# OBSERVATIONS ON A ONE-DIP NUCLEAR STAINING PROCEDURE FOR THE DIRECT MICROSCOPIC EXAMINATION OF MILK, MILK PRODUCTS AND OTHER FOODS

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**Editorial Note: The following description and comments relative to a new staining procedure is given to acquaint others with it and to encourage interested individuals to test it and report their findings.**

In the present report, a description is given of a chloroform-methyl-alcohol-Azure A (CAA) nuclear staining procedure for the direct microscopic examination of milk. The new method appears to have important advantages over the polychrome methylene blue (PMB) procedure (*1*). A description of the two-dip PMB staining method was given in a previous report (*2*). Although preparation of the stain was not difficult, it required approximately two hours to prepare the staining solution and defatting agent.

In the preparation of the defatting-fixing solution, a combination of two parts chloroform and one part ethyl alcohol containing a small amount of gelatin was originally specified. Subsequently, methyl alcohol, anhydrous, was substituted for ethyl alcohol, since the former gave more satisfactory results. This modification has been described by Mantel (*3*) in a comparison of six staining procedures.

## METHYLENE BLUE VERSUS POLYCHROME DYES

The composition of methylene blue is theoretically tetra-methyl thionin (*4*). The dye is so easily oxidized that it is difficult to obtain in a very pure form. Normal oxidation of the dye results in the formation of compounds of lower methylation, such as trimethyl thionin and dimethyl thionin, known respectively as Azure B and Azure A. Since ordinary methylene blue usually contains small quantities of the Azure dyes, it can be considered as a weakly polychromed dye.

The PMB staining solution contains a high proportion of Azure A and B, but the exact composition has not been established. It is a well known fact that Azure A and B have a greater selective staining action than methylene blue. Azure A is generally regarded as more valuable than B. MacNeal (*5*) has reported that Azure A is the most important nuclear staining constituent of polychrome methylene blue.

Scott and French (*6*) have indicated that the important nuclear staining characteristics of ordinary methylene blue dye are due to the normally small amounts of Azure A and B present and that an extremely pure methylene blue is not as satisfactory. These conclusions have been confirmed by Haynes (*7*). Ageing an aqueous solution of methylene blue dye or the addition of a small amount of sodium hydroxide, as in the Loeffler stain formula, tends to increase the Azures present and therefore enhances staining intensity and selectivity.

The relatively inefficient differential staining characteristics of an aqueous methylene blue solution when used for staining bacteria in dried milk films can be largely overcome by using 95% ethyl alcohol as a dye solvent and increasing the dye content. The acid and water free stain (AWF) reported by Levine (*8, 9*) is based upon this principle of increased dye adsorption (*10*) with a suitable dye solvent.

In view of the fact that methylene blue usually contains small amounts and the polychrome stains larger but variable amounts of the lower homologs of methylene blue, depending upon methods of preparation, they do not appear as desirable for wide-scale use as a pure stain with a definite composition. A pure stain can be standardized to give consistent and uniform results if the technique of preparation and staining is carefully carried out.

Since the Azures A, B, and C represent the important staining elements in methylene blue, they were investigated for possible use as a nuclear stain for milk films. After a series of comparative tests Azure A was selected on the basis of superior differential staining qualities.

## ONE-DIP STAINS

One-dip staining procedures for milk films have been reported previously but have not proved to be entirely satisfactory. The difficulties associated with the development of a one-dip stain can be attributed mainly to the unpredictable qualities of the dye solvent and the combined defatting agent in their mutual effects upon the dried milk films. Undesirable effects, such as coagulation and distortion of the stained films, may occur in some cases when acids and tetrachlorethane are used as a part of the dye solvent.

To obtain consistently good results, the defatting-fixing-dye solvent solution should be compatible with the whole milk or other product subject to examination. Although the dried milk film is no longer in a liquid condition, it nevertheless contains a small percentage of moisture. A suitable reagent for this pur-

pose should be compatible with the milk solids, defat the film completely and fix the film firmly to the slide. For convenience the combined dye solvent can be referred to as a dye solvent reagent.

### DYE SOLVENT REAGENT

The chloroform-alcohol reagent used as a defatting agent in the PMB procedure has proven to be an efficient solvent for this purpose. Since the gelatin incorporated as a fixing agent was undesirable for a dye solvent, a small amount of triethanolamine has been substituted.

A dye solvent reagent of this character offers the advantages of a lower interfacial tension resulting in decreased absorption of stain by the milk solids and increased adsorption by bacteria. The reagent is compatible with milk solids, defats completely and is an excellent fixative.

The dye solvent reagent contains 2.7% water and is free from acids. The exact amount and percentage of triethanolamine should be used. An increase in the percentage of water will cause a darker maximum staining effect on the milk solids.

### PREPARATION OF DYE SOLVENT REAGENT AND 0.1% AZURE A STAINING SOLUTION

Measure 650 ml. of chloroform reagent, 320 ml. methyl alcohol, anhydrous, and 30 ml. of 10% triethanolamine, U. S. P., purified, into a liter bottle. Mix by inverting bottle a few times.

To prepare a 0.1% staining solution, dissolve dye in proportion of 0.1 gram Azure A to 100 ml. of the dye solvent reagent. After adding dye to the dye solvent reagent, shake the container occasionally. The dye dissolves within ten minutes and does not require filtration.

### ONE DIP NUCLEAR STAIN FOR MILK

Immerse the dried milk films for one minute in the staining solution. Withdraw the slide and allow to drain for 10 to 15 seconds, until most of the solvent evaporates. Rinse the slide in a container of cold tap water by raising the slide up and down five or six times or optionally by shaking vigorously five or six times in flowing tap water. Dry on end on blotting paper.

### TWO DIP NUCLEAR STAIN FOR HALF AND HALF, WHIPPING CREAM AND FROZEN DESSERTS (1-1)

Immerse the dried films for one to two minutes in the dye solvent reagent. Dry on end for one minute. Immerse the slide for one minute in the staining solution, then drain, rinse and dry as above for milk films.

In dairy products with a high fat content, it is difficult to extract all of the fat with one treatment. In this procedure most of the fat is removed in the first immersion and the subsequent drying shrinks the film to thinner dimensions. A second extraction removes the remaining fat and stains the film.

Similar results can be obtained by immersing the film one minute in the staining solution, drying one minute and immersing a second time for one minute in the staining solution. If the films are immersed initially in the dye solvent reagent, less fat will accumulate in the staining solution.

Dye solvent reagent can be added to the staining solution when necessary to compensate for a moderate amount of evaporation loss. Replace staining solution whenever it becomes unsuitable due to dissolved fat, foreign material, etc.

Bottles containing the dye solvent reagent and staining solution should be kept tightly stoppered at all times when not in use. Containers for both solutions should have lined screw caps.

### USE OF COLOR FILTER

Technicians who devote considerable time to the microscopic examination of milk films may find it highly desirable to use a Corning color filter #3780, Dark Lemon Yellow, 2" x 2", mounted in front of the microscope mirror. The color filter sharply accentuates the outline of bacteria and provides increased color differentiation between the bacteria and the lightly stained background of milk solids. The filter will also reduce the eye strain and fatigue associated with prolonged use of the microscope.

### COMMENTS

The PMB stain previously reported proved to be a considerable improvement over the older procedures but involved a time-consuming method of preparation. Since the composition of polychrome stains cannot be readily determined, it appeared desirable to use Azure A, the most important nuclear staining component of the polychrome stains. Azure A is a pure stain, of known composition and capable of producing uniform results.

Water solutions of methylene blue and polychrome stains will cause over staining of the milk solids if the films are not removed from the staining solution within a specified time interval. Over-staining does not appear possible with the Azure A staining solution. The bacteria and milk solids are stained to maximum intensity in one minute. Dried films of milk have been immersed in the staining solution for 24 hours with no perceptible difference compared to films stained for one minute.

With the new staining procedure, the milk solids stain more lightly than with the PMB procedure and therefore provide a greater contrast of the deeply stained bacteria and leucocytes against the milk solids' background. Very small diplococci and bacilli are clearly visible.

As a means of testing the fixing qualities of the dye solvent reagent, milk films that had been immersed in the staining solution for periods ranging from one minute to twenty-four hours, then drained and dried fifteen seconds, were subsequently shaken vigorously for one minute in a container of flowing tap water. The films remained fixed to the slides and the bacteria were deeply stained. The only ap-

parent difference after this drastic treatment was a lightened color of the milk solids' background.

The preparation of the PMB staining solution and defatting agent necessitates the use of seven ingredients and requires approximately two hours. The new CAA staining solution and the dye solvent reagent can be prepared with four ingredients and requires approximately fifteen minutes.

A small series of direct microscopic counts were made comparing the results obtained with the new CAA procedure and the PMB procedure. The results indicated that the new staining method gives microscopic counts at least equal to or exceeding those obtained by the PMB method.

The new staining method appears to have a useful application for dairy and food products. Excellent results were obtained with frozen desserts, evaporated condensed milk, creamed cottage cheese, and cultured buttermilk when these products were diluted one to one with sterile distilled water. Very good results were obtained with films prepared from ten percent whole milk powder and ten percent skim milk powder. Ice cream mix requires a dilution of one part to two parts distilled water.

The new method also appears to be suitable for staining films prepared from ten percent solutions of dried egg powder and one to one dilutions of frozen eggs. In both instances, the background of egg solids had a uniformly stained appearance with no evidence of coagulation caused by the dye solvent.

For experimental purposes, films may be prepared from various kinds of liquid, semi-liquid, and emulsified food products to determine the presence of bacteria, molds, etc. Variable time intervals may be required to obtain maximum staining and penetration of films with different ingredients. In many cases dilution to the proper film thickness will be necessary. Additional experience and testing will indicate whether the staining procedure will be suitable for such purposes.

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