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# Celestine Blue B as a Nuclear Stain in Vaginal Exfoliative Cytology[1]

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*Abstract.* This paper deals with spectrophotometric and chromatographic analyses of the dye and a study of two staining solutions with a variety of counterstains, using material fixed with ether/alcohol. These observations indicate that the optimal combination of solutions are 0.5 gm celestine blue B and 40 ml glacial acetic acid heated to 60° C with stirring, to which when completely dissolved 100 ml water and 140 ml 4% ferric alum are slowly added. This should be incorporated with the Papanicolau procedure with the sole omissions of hematoxylin, the acid bath, and the lithium carbonate bath. The result is a very acceptable slide with all the advantages of the Papanicolau technique while employing a nuclear stain that is not hematoxylin, but which has high nuclear specificity. Slides have remained light-fast for at least one year.

The original technique for exfoliative cytopathologic investigation of the female genital epithelia, as initiated and taught by Papanicolau, stands unchallenged in its precision and overall utility in the detection of cancer. Through the years numerous modifications have been proposed chiefly in the interest of simplicity and time conservation, for the Papanicolau technique is time consuming and requires expert interpretation. Some of the important innovations are completely new staining methods: von Bertalanffy's rapid screening techniques with acridine orange staining and fluorescent miscroscopy, and very recently the Masin's use of the oxazine dye, cresyl violet. The purpose of this study is to determine the feasibility of using the remarkable nuclear-staining abilities of another oxazine dye, celestine blue B, in exfoliative cytology.

Celestine blue B was studied extensively by Gray et al. (1956) as a nuclear stain for amphibian, avian, and mammalian tissue, the nuclear specificity being controlled by pH. Again in 1958, Gray et al. devised a method of obtaining simultaneous nuclear and cytoplasmic staining with celestine blue B and croceine scarlet. The present principal investigator, together with Gray (1958), published an application of the staining procedure to frozen section staining. Roeser (1959) sharply contradicted the findings of Pickle and Gray (1957) concerning the nuclear specificity of this method of compounding celestine blue B, as applied to the staining of nuclei-like bodies in bacteria and al-

[1] This work was initiated during the senior authors incumbency in a Tobacco Industry Student Research Fellowship.

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gae. Such contradictions seem to be a good part of the history of celestine blue B.

## Materials And Methods

The dye used in this study was purchased from National Aniline Division, Allied Chemical and Dye Corporation, C.I. #51050, lots 16070 and 16873.

Vaginal smears of the cervical face, posterior fornix, and occasionally endocervix were obtained from the obstetric and gynecologic clinics of our institution by Dr. Hugh Grover in cooperation with the chief of these services, Dr. Elizabeth Burrows. Four slides were routinely taken: two for regular pathological diagnosis using the Papanicolau technique, and two for research use. Slides were immediately fixed overnight in Papanicolau's fixative (half ether and half 95% alcohol). A total of 400 slides was used. Slides were stained with celestine blue B compounded by the method of Gray and by that of Roeser, because of the controversy his paper introduced.

*Gray's celestine blue B* (hereafter referred to as CBB-G) (Gray, 1956):

Add 0.5 ml concentrated $H_2SO_4$ to 1 gm of celestine blue B, dissolving the resultant granular mass in 100 ml of 2.5% ferric alum containing 14 ml of glycerol at 50° C. Cool to room temperature and adjust to pH 0.8 with concentrated $H_2SO_4$, preferably with a pH meter and a magnetic stirrer. Lacking these, one may add approximately 1.3-1.5 ml concentrated $H_2SO_4$, testing nuclear specificity by staining a slide and adding small amounts of acid.

Staining procedure:

1. After ether-alcohol fixative, rehydrate through 70% and 50% alcohol to water.
2. Place in stain 1 minute.
3. Rinse in running tap water 2 minutes.
4. Dehydrate, clear in xylol, and mount in balsam or other mountant.

*Celestine blue B* modified from Roeser (1959) (hereafter referred to as CBB-R):

To 0.5 gm celestine blue B, add 40 ml glacial acetic acid. Heat this gently while dissolving the dye, preferably under a hood because of the fumes from the acid; the temperature should not exceed 60° C. When completely dissolved, add 100 ml distilled water slowly with stirring; then add 140 ml 4% ferric alum, with stirring. Store in screw-cap bottle. This preparation seems to be the best of several modifications of the solutions recommended by Roeser. It remains stable for at least six months, perhaps longer.

Staining procedure:

1. After ether-alcohol fixative, rehydrate through 70% and 50% alcohol to water.
2. Place in stain 4 minutes.
3. Rinse in running tap water for 2 minutes.
4. Dehydrate, clear, and mount.

After staining many slides with these solutions, it was determined that simple nuclear staining did not provide enough information, especially in comparison with the Papanicolau technique. Therefore, a variety of counterstains, both aqueous and alcoholic, were tried. A long-continued attempt to develop simultaneous nuclear and cytoplasmic staining from one solution (suggested for tissues in Gray et al., 1958) resulted in the already well-documented conclusion that vaginal smears stained with aqueous dispersions produced opaque and dull cytoplasmic staining. The best of the aqueous counterstains was Bordeaux red (C.I. 88) (1 gm added to the staining solution and dissolved thoroughly) used as a single staining solution. Alcoholic counterstains proved much more transparent, so it was decided to use the Papanicolau technique with the substitution of celestine blue B (CCB-G and CBB-R) for hematoxylin, omitting the acid and lithium carbonate baths, as the procedure which offers the most advantageous use of this nuclear stain in exfoliative cytology.

Several concentrations of aqueous celestine blue B and various formulations of celestine blue B staining solutions were analyzed by means of paper chromatography and spectrophotometry.

## Results

### *Paper Chromatography*

Descending partition chromatography on paper wicks using butanol: acetic acid: water (40: 10: 50:) as a solvent was carried out on dye dispersions prepared as follows:

1. CBB-G
2. CBB-R
3. 0.5% aqueous solution of celestine blue B.
4. 0.1% aqueous solution of celestine blue B.

CBB-G separated into a homogeneous blue-violet band, a light pink band which became turquoise after twenty-four hours, and a small light blue tail. CBB-R separated into a light blue band, a blue-violet band, and a distant tail of very light blue. The ferric alum appeared in both CBB-G and CBB-R below the blue-violet band, with some streaking of the blue-violet around the ferric alum, which was identified by its yellow color. The aqueous dispersions did not separate but streaked; the streak was blue-violet centrally and its edges were pink. These observations suggest metachromasia; Lendrun (1947) used the metachromatic properties of celestine blue B in the staining of acid mucopolysaccharides. In the present study we have no-

ticed that, whereas nuclei stain a blue color, vaginal mucus accepts a faint violet color; also the nucleoli of HeLa cells take a similar light violet color.

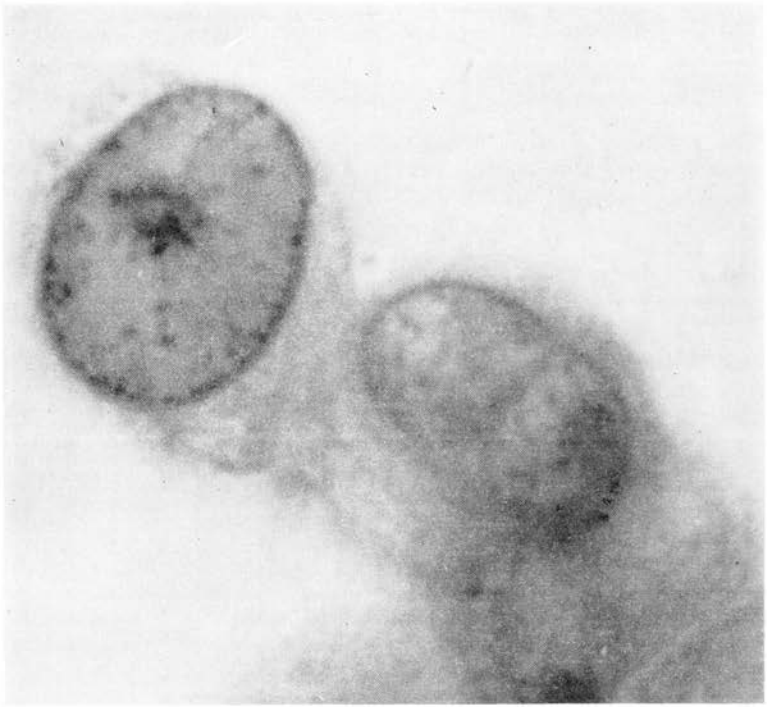


Figure 1. HeLa cells stained with CBB-R. (Obtained from Dr. J. Shannon, University of Nebraska). 2000 X.

*Spectrophotometry*

The transmission curves published by Gray et al. (1956) indicate maximal absorption in the region of 550 millimicrons, using his staining solution at pH 0.4, 0.8, and 1.2. In an attempt to duplicate these results with aqueous dispersions of the dye, it was determined that maximal absorption occurred at exactly 650 millimicrons, regardless of concentration, if the pH were 3.6 or higher. When the pH was 3.3 or lower, maximal absorption took place in the region of 530-550 millimicrons. Furthermore, when identical concentrations were used (0.005% dye in distilled water), this relationship was observed; optical density varies directly with hydrogen ion concentration (Figure 2). These data were obtained with a Beckman spectrophotometer, model DU. Optical densities above 2.0 were obtained with the 0.1 multiplier; they have limited value but illustrate the remarkable change in optical density resulting from pH differences. Gomori (1952) speaks of absorption spectra of dyes with

three maxima: *alpha, beta,* and *gamma.* Using this terminology, the curves assume these relationships:

| Curve | Maximal Absorption (millimicrons) | pH | Designation | Color |
|---|---|---|---|---|
| A,B, | 530 | 2.0 or lower | alpha | red-violet |
| C | 550 | 3.0 | beta | violet |
| D | 550 | 3.3 | beta | blue-violet |
| E,F,G,H,I | 650 | 3.6 or higher | gamma | blue |

This grouping of data clearly shows that the solutions represented by all the *gamma* curves, with a pH of 3.6 or higher, are blue, whereas all the solutions represented by *beta* and *alpha* curves, with pH values of 3.3 or lower, are colored red-

FIG. 2

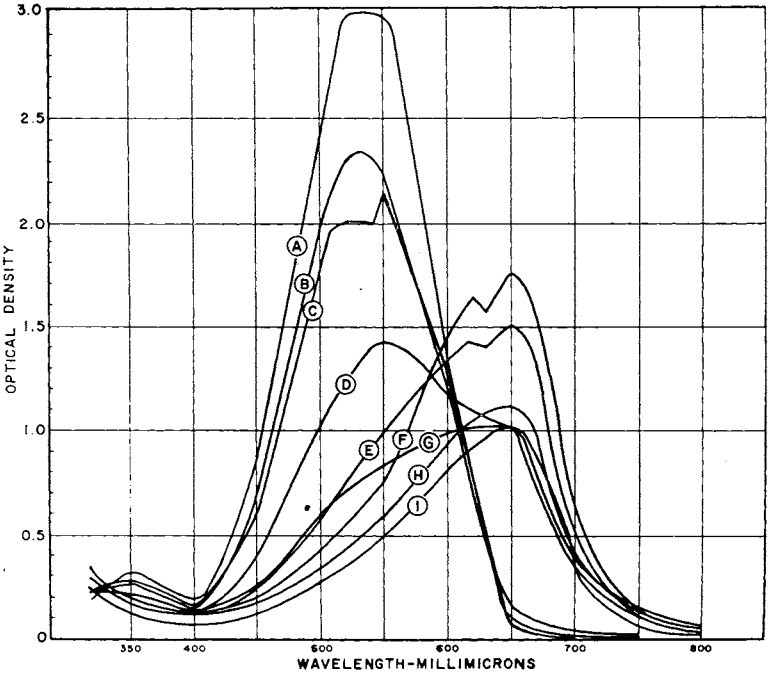



Figure 2. Spectral absorption of aqueous solutions of celestine blue B: stains.

| Curve | Concentration (gm/100 ml) | pH |
|---|---|---|
| A | 0.005 | 0.8 |
| B | 0.005 | 2.0 |
| C | 0.005 | 3.0 |
| D | 0.005 | 3.3 |
| E | 0.008 | 3.8 |
| F | 0.01 | 4.4 |
| G | 0.005 | 3.6 |
| H | 0.005 | 4.5 |
| I | 0.004 | 4.1 |

violet to blue-violet. In the actual staining of exfoliated cells, all staining solutions tested colored nuclei blue, whereas mucus and metachromatic granules were colored violet. The staining solutions all have a blue transmission. It would seem then that these solutions, having pH values ranging from 0.8 (CBB-G) to 1.6 (CBB-R), owe their nuclear staining abilities to colloid formation as suggested by Gray et al. (1956). These data further suggest a molecular alteration related to oxidation and free radical formation in acid media, the free radical being stabi lized by resonance of the odd electron in the ion.

*Buffering Capacity*

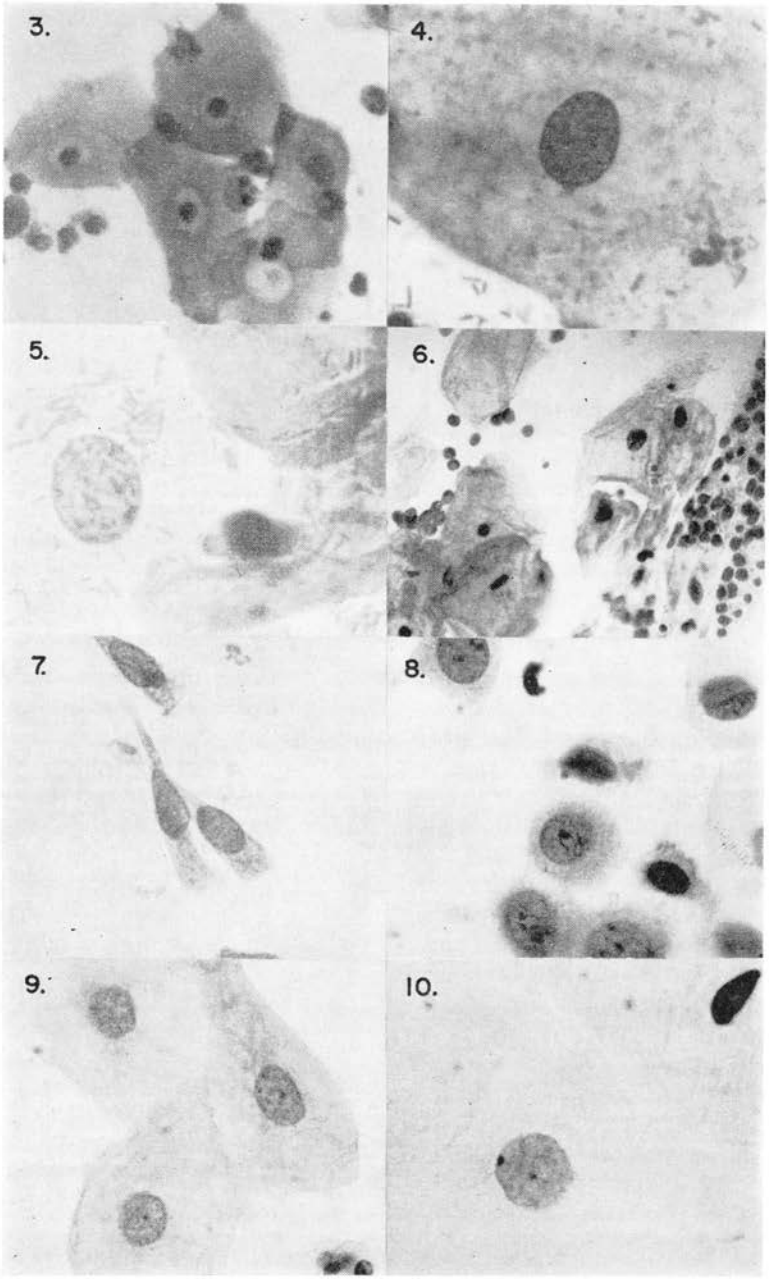
CBB-G has high buffering capacity (Gray et al., 1956), 50 ml of 0.1N NaOH changing the pH of 100 ml CBB-G from 0.8 to 2.0. This information was offered as confirmatory evidence to support the theory that a colloid dispersion is involved and to account for the stability of the preparation.

CBB-R also has considerable buffering capacity. The staining solution has a pH of 1.5; 50 ml 0.1 N NaOH changes the pH to 2.0 and 100 ml changes it to 2.5. This points up the speculation that CBB-G and CBB-R are not too dissimilar at a molecular level.

*Normal Vaginal Epithelium Stained with Celestine Blue B*

The earliest slides were simply stained with CBB-G and CBB-R without counterstains. CBB-G produced excellent nuclear staining but was found also to tint the cytoplasm a faint blue, which slightly interfered with the delicate color reactions of Papanicolau high alcohol counterstains. CBB-R caused minimal cytoplasmic coloring which did not interfere with the counterstains. For this reason, CBB-R was selected as the most compatible nuclear stain for this work. CBB-R is a highly specific nuclear stain. The morphology of the nucleus is clearly shown: the nuclear membrane seems to take a definite coloration, chromatin structures are distinct, the sex chromocenter is clearly visible in vesicular nuclei and frequently in hyperchromatic nuclei, nucleoli stain a darker blue than the nuclear background, leukocytic nuclei stain an intense blue, bacterial cells are stained. The nuclear changes in the life of the vaginal and cervical epithelial cell, from the basal cell with its large vesicular nucleus to the cornified superficial cell with its pyknotic nucleus, and on to karyolysis, can be followed with the same precision as obtained with hematoxylin.

The experiments with aqueous counterstains were interesting but resulted in very opaque cytoplasmic staining, which is quite undesirable in the staining of smears. It was observed, how-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ever, that when perinuclear halos were present aqueous counterstains demonstrated them nicely.

ACKNOWLEDGMENT

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Figure 3. Cornified and precornified superficial cells and leukocytes stained with CBB-G using Bordeaux red as a counterstain. Note perinuclear halos.

Figure 4. Large superficial cell. CBB-G and Bordeaux red.

Figure 5. Naked nucleus surrounded by bacteria. CBB-G and Bordeaux red.

Figure 6. Precornified and cornified superficial cells. CBB-R and Papanicolau counterstains.

Figure 7. Cervical columnar cells. CBB-R and Papanicolau counterstains.

Figure 8. Inner layer basal cells. CBB-R alone.

Figure 9. Outer layer basal cells. CBB-R and Papanicolau counterstains.

Figure 10. Sex chromocenter in nucleus CBB-R alone.