One of the simplest procedures to demonstrate mucinous substances (acid mucopolysaccharides) in routine histological preparations involves the use of Alcian dyes. Methods involving these dyes have largely replaced the older, more empirical mucicarmine technique and are faster and less complicated than the colloidal iron method of Hale, which suffers from the possibility of false-positive staining due to the presence of intrinsic iron in tissues.

The usefulness of the Alcian dye methods for diagnostic purposes has been extended by combination with other procedures. Mowry used Alcian blue-periodic-acid-Schiff method to demonstrate the acidic groups and the 1,2 glycols of mucopolysaccharides. Putt and Hukill found Alcian green-Verhoeff's elastic stain useful for the histologic diagnosis of lung carcinomas.

Alcian blue 8GS, introduced by Steedman, is a water soluble, copper phthalocyanine dye, which is closely related to the alcohol soluble Monastral fast blue. Since Alcian blue 8GS is no longer commercially available, it has been replaced by Alcian blue 8GX (C.I. No. 74240), a purer and more soluble dye.

The original reaction for demonstrating mucins was of necessity very short: if prolonged, other tissue elements took up the stain. This was presumably due to both strong and weak acid groups in tissue, the former responsible for the fast reaction. In order to overcome this problem, Vailli oxidized sectioned tissues in chromic acid, which limited the staining reaction to the strong acidic groups of mucopolysaccharides. Mowry and Lison further modified the staining procedure by acidifying the dye solution with acetic acid, which permitted a longer and more precise stain with minimal background coloration due to the fact that the low pH allowed only the utilization of strong acid esters. The slight nuclear or connective tissue staining that sometimes occurs with Alcian dyes can be overlaid and minimized with a counterstain.

The purpose of this paper is to introduce a method of preparing Alcian dye solutions by the substitution of calcium chloride (CaCl₂·2H₂O) in place of acetic acid. This replacement results in a solution with a pH of 2.8 which is well within the limits of acidity of the solutions used by Mowry and Lison. The present selective use of Alcian dyes for mucin in a calcium salt solution was first observed inadvertently while carrying out Attwood's differential

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Received for publication 28 April 1970.
stain for amniotic-fluid embolism, in which mucus is stained green with Alcian green, and epithelial squames red, with phloxine. In our particular preparations, selective affinity of Alcian green for mucus and cartilage was ordinarily poor. Restaining the mucins in Alcian green resulted in an intense uptake of the dye and only could be accounted for by the calcium chloride that was present in the counterstain.

MATERIALS AND METHODS

Routine surgical and autopsy material, including tissues that did or did not contain mucins were fixed in 10% formalin and Helly's fluid, embedded in paraffin in the usual manner and sectioned at 5 microns. Egg albumin adhesive was used sparingly as a mounting medium as it also stains. In order to check background uptake of the dyes, mucin positive and negative sections were stained in various Alcian dye solutions for 30 minutes with and without calcium chloride. These were compared with identical sections stained for the same period of time in a 1% solution of Alcian dyes in 3% acetic acid. Unacidified dye solutions stained background proteins, whereas sections immersed in acidified and calcium chloride solutions showed negligible background staining. The dyes used in the present procedure were purchased from ESBE Laboratory Supplies, 3431 Bathurst St., Toronto 19 Ontario, Canada and Roboz Surgical Instrument Co., Ltd., 810-18th Street, N.W., Washington, D.C. 20006.

PREPARATION OF MATERIALS

Celestin blue:

5 gm. of ferric ammonium sulfate (iron alum) was dissolved in 100 ml. of distilled water overnight at room temperature. It was then brought to a boil and 0.5 gm. of Celestin blue added and boiled carefully for 3 minutes. When cool it was filtered and 14 ml. of glycerin was added. The solution keeps well at room temperature and should be filtered before use.

Mayer's haemalum:

| Component                        | Amount |
|----------------------------------|--------|
| Hematoxylin                      | 1 gm.  |
| Sodium iodate                    | 0.2 gm.|
| Potassium alum (aluminum and potassium sulfate) | 50 gm. |
| Distilled water                  | 1000 ml.|
| Chloral hydrate                  | 50 gm. |
| Citric acid                      | 1 gm.  |

The hematoxylin, sodium iodate and potassium alum were dissolved in distilled water overnight. Chloral hydrate and citric acid were then added and the solution boiled for 5 minutes. When cool the dye mixture is ready for use and is stable when stored.

Alcian green 2GX solution:

| Component                           | Amount     |
|-------------------------------------|------------|
| Alcian green                         | 1 gm.      |
| Calcium chloride (CaCl₂2H₂O)        | 0.5 gm.    |
| Distilled water                     | 100 ml.    |

Calcium chloride was dissolved in the water prior to addition of the dye. A crystal of thymol was added as a preservative. The solution was filtered before use and was stable when stored.
**Martius yellow counterstain:** (C.I. No. 10315)

| Ingredient                                      | Amount   |
|-------------------------------------------------|----------|
| Martius yellow                                  | 0.5 gm.  |
| Distilled water                                 | 20 ml.   |
| Phosphomolybdic acid (1 gm.) in absolute alcohol| 80 ml.   |

Ingredients were combined and filtered. Solution is stable at room temperature.

**PROCEDURE**

1. Deparaffinize and hydrate sections.
2. Remove mercury crystals if tissue is fixed in Helly's solution.
3. Stain in Celestine blue for 5 minutes.
4. Wash in tap water for 2-3 minutes.
5. Stain in Mayer's haemalum for 5 minutes.
6. Wash well in tap water.
7. Differentiate nuclei in acid alcohol for a few seconds.
   - (1 ml. of hydrochloric acid in 99 ml. of 70% alcohol).
8. Wash in running tap water for 5 minutes.
9. Stain in Alcian green 2GX solution for 5 to 10 minutes.
10. Rinse in distilled water.
11. Counterstain in Martius yellow for 15-30 seconds.
12. Rinse in tap water.
13. Dehydrate in 95% alcohol, 2 changes; absolute alcohol, 2 changes.
14. Clear in xylene. Mount in Permount.

**RESULTS**

The following staining results were obtained: Nuclei, blue-black; connective tissue mucopolysaccharides, mucus and ground substance of cartilage, green; erythrocytes, muscle and fibrous tissue, yellow. Mast cell granules and cryptococcus are also demonstrated.

**DISCUSSION**

The present study indicates a simple, effective repeatable method for staining mucins with Alcian dyes. In this regard the staining method was selective, since the exact staining reaction is unknown and will require further investigation. The present Alcian modification shows good agreement with that produced with acidified Alcian dyes.

The present use of Alcian green 2GX is in agreement with the works of Pearse" and Humason" because it reacts rapidly and at low concentrations. However, other Alcian dyes may be used and include Alcian green 3BX, Alcian yellow GXS, and Alcian blue 8GX. Alcian greens, subjected to thin layer chromatography, are revealed to be mixtures of the blue and yellow dyes.

In the present work the sequential stain of nuclei by Celestine blue and Mayer's haemalum as devised by Lendrum and McFarlane" prevents nuclei
from being decolorized by the subsequent acid-containing solutions. Their method can be replaced by that of Slidders which is a simple iron hematoxylin solution that can replace with advantage the older double staining method.

SUMMARY

A method of preparing Alcian dye solution in calcium chloride is presented. The method has proven advantageous in the routine staining of tissue mucins.

ACKNOWLEDGMENT

I wish to thank Dr. Levin Waters for his interest and suggestions and Dr. Russell Barnett for reviewing the manuscript.

REFERENCES

1. Hale, C. W.: Histochemical demonstration of acid mucopolysaccharides in animal tissues. Nature, 1946, 157, 802.
2. Barka, T. and Anderson, P. J.: Histochemistry, Theory, Practice, and Bibliography. Hoeber Medical Division. New York, Harper and Row, 1963.
3. Mowry, R. W.: Revised directions for the colloidal iron stain, the use of Alcian blue 8GX and their combination with the Periodic Acid Schiff reaction. Anns. N.Y. Acad. Sci., 1963, 106, 402-423.
4. Putt, F. A. and Hukill, P. B.: Alcian green. A routine stain for mucins. Arch. Path., 1962, 74, 169-170.
5. Steedman, H. P.: Alcian blue 8GS: A new stain for mucin. Quart. J. Micro. Sci., 1950, 91, 477-499.
6. Vailli, M.: Osservazioni sull'uso del' Alcian blue 8GS nello studio die mucopolisaccaridi. Boll. Soc. Ital. Biol. Sperm., 1951, 27, 597-599.
7. Mowry, R. W.: Alcian blue technique for histochemical study of acidic carbohydrates. J. Histochem. Cytochem., 1956, 4, 407-413.
8. Lison, L.: Alcian blue 8G with Chlorantine Fast Red 5B. A technic for selective staining of mucopolysaccharides. Stain Tech., 1954, 29, 131-138.
9. Attwood, H. D.: The histological diagnosis of amniotic fluid embolism. J. Path. Bact., 1958, 76, 211-215.
10. Pearse, A. G. E.: Histochemistry. Theoretical and Applied, 2nd Ed. Boston, Little, Brown and Company, 1960.
11. Humason, G. L.: Animal Tissue Techniques, 2nd Ed. San Francisco, W. H. Freedman and Company, 1967.
12. Lendrum, A. C. and McFarlane, D.: A controllable modification of Mallory's trichrome staining method. J. Path. Bact., 1940, 50, 381-384.
13. Slidders, W.: A stable iron-hematoxylin solution for staining the chromatin of cell nuclei. J. Micro., 1969, 90, pt. 1, 61-65.