MILES

Histo-Logic®

Technical Bulletin for Histotechnology • Scientific Editor, Lee G. Luna, D. Lit. (Hon.) H.T. (ASCP) • Managing Editor, Brent Riley
Vol. XXII, No. 1, January/February 1992

In Situ Hybridization Detection of Type I Collagen mRNA in Neonate Rat Bones: Effects of Decalcification

Janice R. Herring, Thomas G. Plant, Nancy G. Mayne, and Daniel C. Williams

Lilly Research Laboratories
Eli Lilly & Company
Indianapolis, IN 46285

Abstract

We have examined the effects of decalcifying agents upon type I collagen mRNA detection levels and localization in long bone metaphyses of neonate rats by in situ hybridization at the light microscope level. Femurs and tibiae from 1- to 2-day-old rats were fixed in paraformaldehyde for 24 hours, decalcified in either EDTA, formic acid/sodium citrate, or formic acid/paraformaldehyde, then embedded in paraffin, sectioned, and hybridized using a type I collagen 35S antisense riboprobe. The localization and intensity of the signal in the EDTA-treated tissues were similar to the signal observed in calcified control tissue. In either of the formic acid-decalcified preparations there appeared to be a significant reduction in the number of autoradiographic grains as compared with similar areas of the calcified control sections. These findings indicate in situ hybridization can be performed on paraffin-embedded, decalcified tissues, and EDTA appears to be a better decalcifying agent than formic acid/sodium citrate or formic acid/paraformaldehyde solutions.

Introduction

A variety of experimental techniques have been utilized to evaluate bone cell function in normal metabolism and in bone diseases. The power of histological and histomorphometric approaches to understanding bone cell biology lies in their ability to define the response of specific cell types, within their normal tissue architecture, to a particular treatment or stages in bone development. Cytochemical and immunocytochemical procedures have enhanced histological approaches by allowing the localization of specific cellular activity or proteins at the tissue or cellular level. In situ hybridization is a relatively new, powerful technique extending these capabilities to the histological localization of specific DNA and RNA sequences.

Though histological evaluation of calcified tissues is becoming more common, it still remains a technically difficult and labor-intensive process. Relatively early in our attempts to develop the technique of mRNA-RNA in situ hybridization in bone tissue, we determined that the procedure for in situ localization of mRNA was itself a sufficient technical challenge, without including the problems inherent in sectioning calcified tissue.

(continued on page 282)

IN THIS ISSUE

Therefore, we decided to evaluate the effects of tissue decalcification on the in situ localization of mRNA. The model we chose to use for this study was the localization of type I collagen mRNA in bones from neonate rats.

Materials and Methods

Preparation of Tissue Sections

Femurs and tibiae from 1-day-old rats (Sprague-Dawley strain, Charles River Breeding Laboratories, Portage, Mich) were surgically removed and placed in ice-cold 4% paraformaldehyde fixative in phosphate-buffered saline (PBS) at pH 7.2 during dissection (30 - 60 minutes), then fixed in fresh fixative for 24 hours at 4°C on a Lab-Line Orbit Shaker at 150 rpm. The tissues were then rinsed in two changes of PBS (4°C) for 4 hours, placed in 70% ethanol, and stored at 4°C until processed. The samples were divided into four equal groups; one group remained calcified while the rest were decalcified for 24 hours at 4°C in one of the following solutions: 1) 10% EDTA in PBS, pH 7.0; 2) 2.5% formic acid/10% sodium citrate, pH 4.2; or 3) 5.0% formic acid/1% paraformaldehyde in PBS, pH 7.2. Decalcified samples were washed in six changes (1 hour each) of distilled water (4°C). All samples were then dehydrated with increasing concentrations of ethanol (70%, 95%, 100%, 100%), cleared in xylene, and embedded in Paraplast® X-tra. Five-micrometer-thick sections were cut on a Sorvall JB-4 microtome, floated on a 48°C flotation bath containing 1% gelatin, and transferred to acid-cleaned slides. Two sections were placed on each slide (one for hybridization and one as a non-hybridized control), dried vertically, then placed on a 50°C slide warmer for 5 minutes. To increase adherence of the sections to the slides, they were placed in a staining rack and baked at 60°C overnight. Sections were deparaffinized 5 minutes in each of two changes of xylene, 100% ethanol, and 95% ethanol, then air-dried (30 - 60 minutes).

To prevent contamination with nucleases, all procedures were carried out wearing gloves. Reagents (RNase/DNase free) were made up in disposable containers using water that had been treated with diethyl pyrocarbonate (Depc) and autoclaved.[1] Nonautoclavable equipment was detergent-cleaned and rinsed with Depc-treated water.

Preparation of Riboprobe

The probe used in this study was a 54 base riboprobe for rat alpha 2(I) collagen that hybridizes to the nucleotides corresponding to amino acids 1151-1169 in the C-terminal coding region. Riboprobe plasmids were constructed by cloning double-stranded synthetic oligonucleotides into the multiple cloning site polylinker of plasmid pT7/T3-19 (Bethesda Research Laboratories, Gaithersburg, Md). In this plasmid, the multiple cloning site polylinker separates facing T3 and T7 polymerase specific promoter sequences.

The oligonucleotides were designed with appropriate restriction sites for directional cloning in this vector. Use of these riboprobe plasmids and the appropriate RNA polymerase produces either antisense probe or the sense strand (same sequence as alpha 2(I) mRNA) of RNA. Antisense or sense probes of high specific activity were produced in a 20-µL reaction volume containing 0.5 µg linearized probe vector, 125 µCi 35S-UTP (1320 Ci/mmol, NEN Research Products, Boston, Mass), 40 mM Tris-HCl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine-(HCl)3, 10 mM dithiothreitol (DTT), 0.05 mM each of GTP, ATP, CTP, 50 units RNase inhibitor and 30 units T7 polymerase. The reaction proceeded at 37°C for 1 hour. One unit of DNase (RNase free) was added (to destroy the template), and the reaction continued for 15 minutes. The volume was increased to 50 µL with water and 1 µL of the total was removed for counting. Unincorporated nucleotides were removed by centrifugation through a G-25 fine Sephadex column. The effluent was collected and 1 µL counted. The incorporated label was determined and the specific activity calculated (usually 1-5 × 108 cpm/µg). All reagents and glassware were RNase free.

In Situ Hybridization

Sections were pretreated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 (10 minutes). The slides were rinsed twice (10 minutes each) in 2X SSC (standard saline citrate; 1X SSC = 0.15 M NaCl, 0.015 M trisodium citrate), and dehydrated sequentially in an ethanol series (70%, 95%, 95%, 100%, 100% for 1 minute each), and air-dried (1 hour). The hybridization mixture contained 0.7 µg/mL 35S-labeled probe, 10 mM DTT, 1 mg/mL bovine serum albumin, 2X SSC, 50% deionized formamide, 10% dextran sulfate, 2 mg/mL baker's yeast tRNA, and 1 mg/mL sonicated salmon sperm DNA. The mixture was heated in a 75°C water bath for 3 minutes, cooled to 50°C, and maintained at this temperature on a slide warmer until ready to use. The slides were placed flat on a metal slide tray on the warmer and 20 µL of the mixture placed on the sections to be hybridized. A piece of Parafilm® (10 × 15 mm) was placed on each section to distribute the mixture evenly. Sections were hybridized in a moist chamber (Lipshaw Corp., Detroit, Mich) overnight (20 hours) at 50°C.

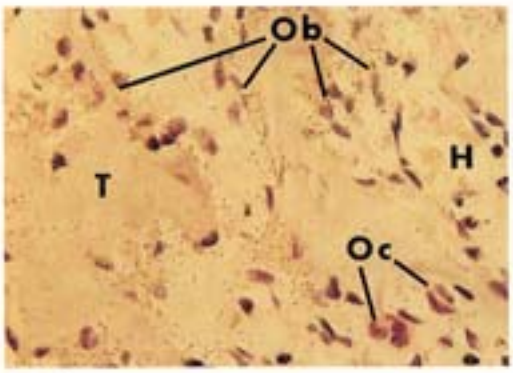

Figure 1A

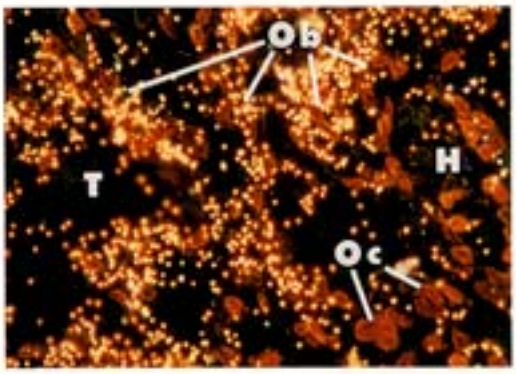

Figure 1B

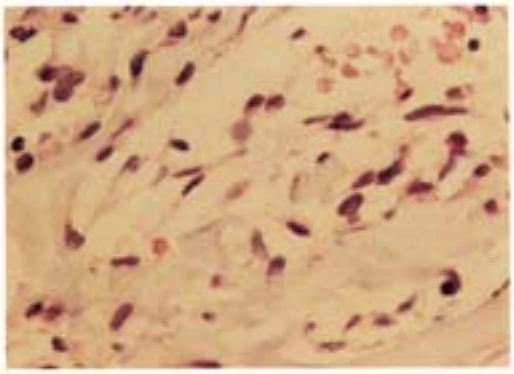

Figure 1C

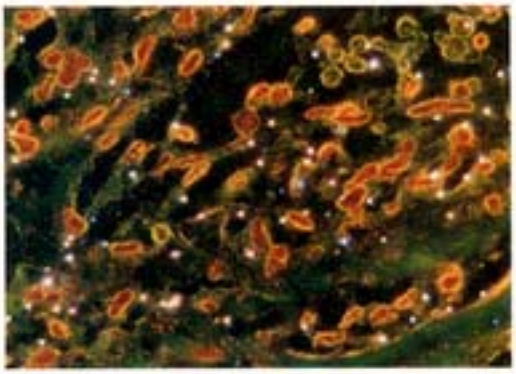

Figure 1D

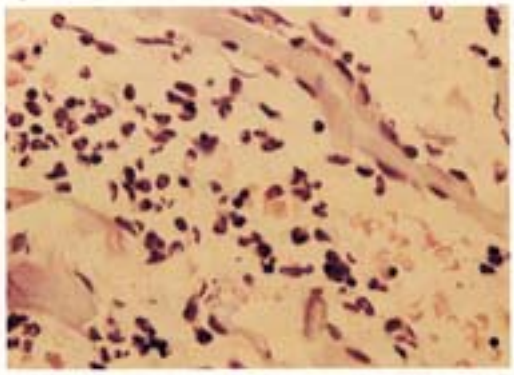

Figure 1E

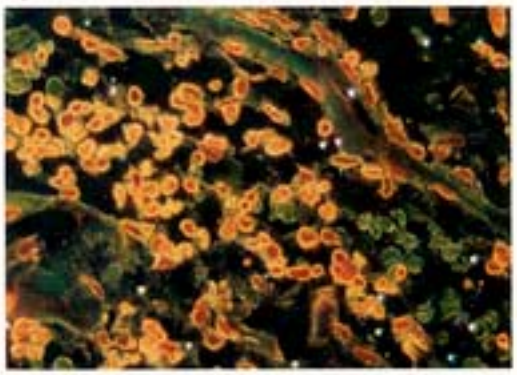

Figure 1F

Localization of type I collagen mRNA with the 35S-labeled antisense probe in metaphyseal region of calcified, 1-day-old rat long bones. Labeled osteoblasts (Ob) can be seen located on the surface of several bone trabeculae (T) in Figs. 1A, 1B. Osteoclasts (Oc) and hematopoietic cells (H) remain unlabeled. The sense probe (Figs. 1C, 1D) and a nonhybridized (Figs. 1E, 1F) control are shown for comparison.

The number of autoradiographic grains in the sense and nonhybridized controls are sparse, and the distribution is random. Sections on the left are bright-field images. Their matching dark-field images appear to the right (Figs. 1B, 1D, 1F) of the bright-field illustrations and are shown for better localization of the autoradiographic grains. Hematoxylin and eosin stain, magnification × 425.

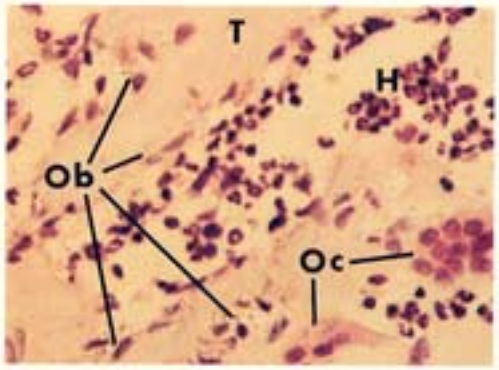

Figure 2A

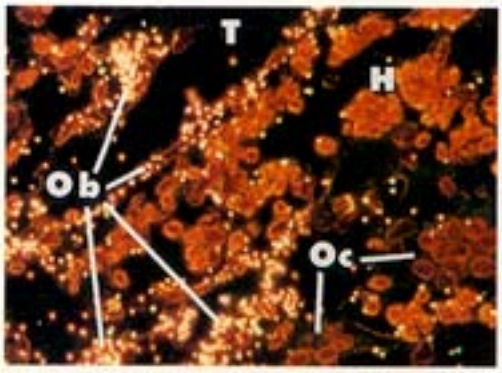

Figure 2B

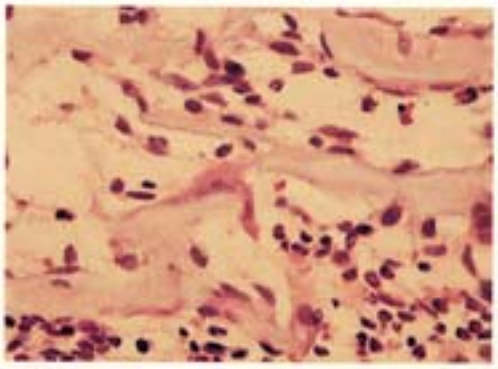

Figure 2C

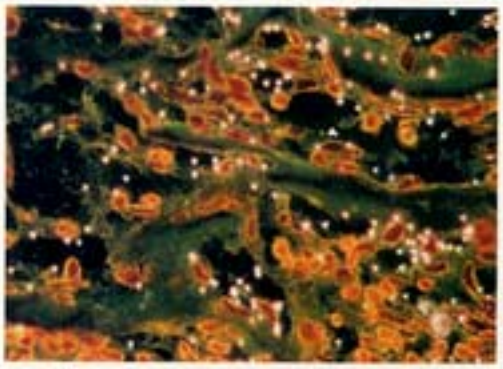

Figure 2D

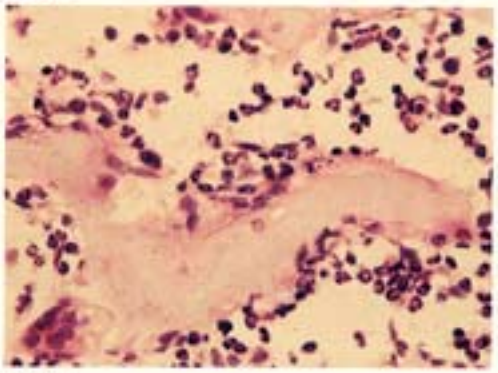

Figure 2E

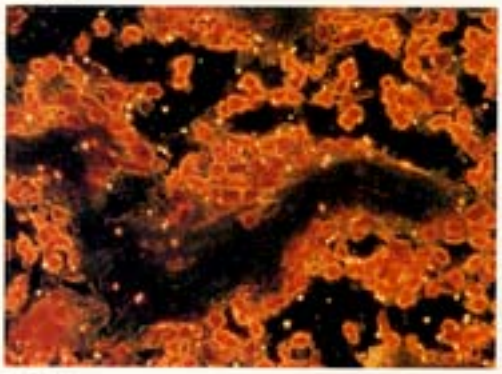

Figure 2F

Metaphyseal region of decalcified, 1-day-old rat long bones demonstrating the localization of type I collagen mRNA with 35S-labeled antisense probe. The localization and intensity of the radiographic grains in the EDTA-treated section (Figs. 2A, 2B) appear to be similar to that of those in the calcified control section (Fig. 1A). As in the calcified section (Fig. 1A), osteoblasts (Ob) on the trabecular bone surface (T) are labeled with the osteoclasts (Oc) and hematopoietic cells (H) remaining unlabeled.

In comparison to the calcified and EDTA-decalcified sections, the signal intensity in the formic acid/sodium citrate (Figs. 2C, 2D) and formic acid/paradormaldehyde (Figs. 2E, 2F) decalcified sections is significantly reduced. The few grains that are present appear only as random, nonspecific labeling throughout the section. To the left are the bright-field images (Figs. 2A, 2C, 2E) with their matching dark-field images (Figs. 2B, 2D, 2F) to the right. Hematoxylin and eosin stain, magnification × 425.

After hybridization, the slides were submerged in 2X SSC, 10mM DTT for 15 minutes at 50°C to remove the Parafilm.® Sections were rinsed four times (15 minutes each) in 2X SSC, 10mM DTT at 42°C, twice in 2X SSC at 23°C (15 minutes each), then treated with RNase A (20 µg/mL)/RNase T1 (25 units/mL) in 2X SSC for 20 minutes at 37°C to remove nonspecific bound probe. Following three rinses (10 minutes each) in 0.1X SSC, 10 mM DTT at 42°C, and 3 changes of 2X SSC (5 minutes each) at 23°C, the slides were dehydrated in 70%, two changes of 95%, and two changes of 100% ethanols with 0.3 M ammonium acetate for 1 minute each and air-dried (1 hour). Controls used to examine the specificity of hybridization included hybridization with a sense probe (same sequence as alpha 2[I] mRNA) and hybridization carried out with no probe present in the hybridization mixture. Reagents (RNase/DNase free) were obtained from Sigma Chemical Co., St. Louis, Mo, and Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Detection of probe

Autoradiography was performed, in total darkness, by dipping slides into a Kodak NTB-2® autoradiography emulsion melted at 45°C and diluted 1:1 (v/v) with 0.6 M ammonium acetate. Slides were dried vertically for 1 hour at room temperature, in a light-tight container, then transferred to another light-tight box containing DriRite® dessicant for 4-7 days at 4°C. The exposed slides were processed at 15°C in Kodak D-19® developer for 2.5 minutes, rinsed in distilled water, fixed for 5 minutes in Kodak® fixer, and rinsed again in distilled water for 15 minutes. Slides were either stained with Gill's hematoxylin No. 3 (Sigma Chemical Co., St. Louis, Mo) and Eosin Y/Phloxine B, or 1% Toluidine Blue, then dehydrated with increasing concentrations of ethanol and xylene, and mounted in Permount.®

Results

The localization of mRNA within the tissue appears to be similar in both calcified and EDTA-decalcified preparations (Figs. 1A and 2A). The highest level of autoradiographic grains (indicating the localization of alpha 2[I] collagen mRNA) are observed associated with osteoblasts on the bone surface in both EDTA-decalcified as well as calcified control sections. The intensity of the signal in the EDTA-treated tissues is similar to the signal observed in the calcified control tissue. In the formic acid/sodium citrate-decalcified section (Fig. 2C), there appears to be a considerable (4- to 6-fold) reduction in the number of autoradiographic grains in comparison with similar areas of the calcified control section (Fig. 1A). In the formic acid/paraformaldehyde preparation (Fig. 2E), very few grains are present and these appear only as random, nonspecific labeling throughout the section. The "sense" and nonhybridized probe controls are essentially the same for all groups (controls in the calcified preparations are shown as examples — Figs. 1C and 1E). The level of autoradiographic grains in the nonhybridized control is relatively low and the grains are distributed in a random manner. As expected, grain levels in the "sense" probe control are slightly higher than the nonhybridized control, though considerably less than in the antisense probe preparations. Grain distribution in the "sense" probe control is random.

Discussion

In situ hybridization is used to localize specific mRNA in histological sections. Detailed protocols dealing with the practical aspects of in situ hybridization procedures have recently appeared in the literature.[2,3,4] Despite significant interest, in situ hybridization has been used infrequently to examine mRNA expression in the cells of calcified tissues.[5,6,7]

The development of in situ hybridization protocols for the study of calcified tissues will be facilitated by an understanding of how procedures unique to these tissues, such as decalcification, affect the results of in situ hybridization. McDonald and Tuan[8] evaluated the effect of fixation and decalcification regimens on the detection of collagen transcripts in chick tissue by cDNA-RNA hybridization. They utilized a biotinylated probe with a streptavidin-alkaline phosphatase detection system and settled on a protocol that included fixation in a modified Carnoy's solution, followed by decalcification of sections in a two-step procedure involving both hydrochloric acid and EDTA. Sandberg et al.[9] have recently reported the successful use of a formalin-fixed, EDTA-decalcified preparation of fracture callus to investigate the localization of collagen mRNA by DNA-RNA hybridization. Utilizing the procedures described in the present study, we have localized riboprobes for IGF-I and IGF-II in neonate long bones, and riboprobes for collagen type I and collagen type II in long bones from adult rats.[10] In addition to specific procedural parameters (e.g., EDTA-decalcification), critical attention to "clean" technique, accurate titering of probe intensity (35 S label levels), and controlled emulsion exposure are important to obtain sufficient spatial resolution for cellular localization and quantitation of mRNA in histological preparations. We have found that optimum conditions must be adjusted for each probe.

In summary, we have evaluated three histological decalcification procedures for effects on the detection and localization of a 35S-labeled, 54 base riboprobe for rat alpha 2(I) collagen mRNA in sections of neonate rat bone. EDTA-decalcification appears to have little effect on either the level of detection (number of autoradiographic grains) or the distribution of label in the tissue sections compared with calcified sections. Decalcification procedures employing formic/citrate or formic/formaldehyde solutions have distinct deleterious effects on the detection level and distribution of the riboprobe. The ability to use decalcified tissue preparations, in combination with careful control of the hybridization procedure, should facilitate the application of in situ hybridization to the study of bone metabolism and development.

References

1. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning*. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989:74.
2. Denny P, Hamid Q, Krause JE, Polak JM, Legon S. Oligo-riboprobes: tools for in situ hybridization. *Histochemistry*. 1988;89:481-483.
3. Moench TR. In situ hybridization. *Mol Cell Probes*. 1987;1:195-205.
4. Simmons DM, Arriza JL, Swanson LW. A complete protocol for in situ hybridization of messenger RNAs in brain and other tissues with radiolabeled single-stranded RNA probes. *Journal of Histotechnology*. 1989;12 (No. 3): 169-181.
5. Metsaranta M, Young MF, Sandberg M, Termine J, Vuorio E. Localization of osteonectin expression in human fetal tissue by in situ hybridization. *Calcif Tissue Int*. 1989;45:146-152.
6. Otsuru O, Leboy PS, McDonald SA, Tuan RS, Shapiro IM. Developmental expression of genes in chick growth plate cartilage detected by in situ hybridization. *Calcif Tissue Int*. 1989;45:182-192.
7. Sandberg M, Vuorio E. Localization of types I, II, and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. *J Cell Biol*. 1987;104:1077-1084.
8. McDonald SA, Tuan RS. Expression of collagen type transcripts in chick embryonic bone detected by in situ cDNA-mRNA hybridization. *Dev Biol*. 1989;133:221-234.
9. Sandberg M, Hanna A, Maltimaki P, Aho H, Vuorio E. In situ localization of collagen production by chondrocytes and osteoblasts in fracture callus. *J Bone Joint Surg*. 1989;71-A:69-77.
10. Herring JR, Plant TG, Mayne NG, Williams DC. Effects of decalcification on the histological detection of type I collagen mRNA in neonate rat bones by in situ hybridization. *J Bone Miner Res*. 1989;4(suppl 1):352. Abstract.

Yes! I used to do a lot of histology. But, now all I do is cell blocks!

Quinnipiac College Prepares Students for a Career in Veterinary Technology

Brent Riley
Managing Editor

Several schools across the country have developed veterinary technology programs. These programs are designed to train students in the proper maintenance, care, and use of laboratory animals. Histotechnology is also a major element in the educational process. One of the more prominent programs in the United States is at Quinnipiac College in Hamden, Connecticut.

"We have a 4-year program for a bachelor of science degree in veterinary technology," explained Layne Ochman, Director of the Veterinary Technology Program at Quinnipiac. "We originally referred to our program as Laboratory Animal Technology," Ochman continued, "but we decided the word *laboratory* was a bit too confining in some respects. We found that people were not just doing research on animals, but many were going into veterinary clinical services. So we changed the name of our program to Animal Technology." More recently the name was changed again to Veterinary Technology based on a recommendation from the American Veterinary Medical Association (AVMA).

The Quinnipiac program, which was started in 1971, has about 50 students. Many of the students go on to graduate school or veterinary school. "The curriculum is set up so they have a very good basic core of science courses so they're prepared to go on," Ochman said. The curriculum includes a full semester, four-credit course in histology. In addition, students must work in the histology laboratory during on-the-job training at one of the nearby clinical sites.

"Job opportunities for my students are numerous," Ochman revealed. "There are probably 10 openings to every student." At recent meetings of the AVMA and the American Association of Veterinary Colleges, members have discussed the dramatically increasing need for qualified veterinary technology professionals. These organizations are developing strategies to curb the

nationwide drop in total applications at veterinary technology schools.

"Things have changed a bit this past year," Ochman claimed, "and we're hoping that this is a true indication because we've had a 300% increase in the number of applications."

Most of Quinnipiac's graduates go into some type of biomedical position either at a pharmaceutical company, a toxicology company, or a university where animal research is underway. A few go into veterinary hospitals, but higher salaries in the pharmaceutical industry draw a majority of trained technologists.

Quinnipiac College, which is located a few miles north of New Haven, has about 4,000 students. The College offers a number of degrees in allied health and sciences, as well as business and liberal arts. Their veterinary technology program was the first fully accredited 4-year program. It was provisionally accredited in 1980 by the AVMA. (At the time, the program was called Animal Laboratory Technology.) Full accreditation was granted in 1985.

"It takes time to put together a full 4-year curriculum," Ochman explained. "It is necessary not only to have a working facility that meets certain standards, but we also need to set up clinical sites. It takes a lot of money, space, and equipment to maintain our academic standards as well as our animal health and care standards."

Students spend about 12 hours each week at one of two clinical sites. One of the clinical sites is at New Haven Central Hospital for Veterinary Medicine. The other is at the Section of Comparative Medicine at Yale University. There are no more than five students at a clinical site at any time. This allows students to get individual attention as they work in the various departments at each site.

"When our students enter the job market, they have the necessary skills because they've had actual experience," Ochman said. "They're really very good at what they do." Applicants are carefully interviewed before they are accepted into the Quinnipiac program. The primary purpose of the interview is to assess the prospective student's interest in and understanding of the Veterinary Technology Program. "Some students have mixed feelings when they come in," Ochman explained. "We want them to be absolutely sure that this is what they want."

The recent controversy surrounding the use of animals in research has not had a significant impact on the program, according to Ochman. "The animals in this facility are used specifically for teaching," she explained. "We don't do any research on them." Still, the college must follow the regulations of the animal welfare act. In addition, the curriculum includes a study of the ethical issues involved in animal care.

"The students in this program are probably more compassionate than anyone when it comes to animals," Ochman said. "They are very concerned that if animals are to be used in research, that the three Rs of reduction, refinement, and replacement always be kept in mind. We follow the laws and maintain a high ethical, moral consideration of what's going on.

"We encourage our students to discuss ethical issues, and they have some heated discussions at times. Although we would all like to see animal research replaced by alternative methods, it simply is not feasible at this point. We try to look at it in terms of all the good that has resulted. The medical advances that are possible through animal research have helped millions of people."

The program was started at Quinnipiac College in 1971. Ochman has been the director since 1984. She is a 1977 graduate of the program and was certified by the American Association of Laboratory Animals in 1980.

NSH Symposium/Convention Breaks More Records

Brent Riley
Managing Editor

To borrow a line from a recent television commercial, "It just doesn't get any better than this." But when "this" is referring to the NSH Symposium/Convention, it most certainly does get better—year after year. The recently concluded 1991 symposium/convention will be remembered—at least for the next year—as the biggest and best NSH meeting ever.

"*Fantastic* is the only word to describe it!" exclaimed Phyllis Boris, cochairman with Kerry Crabb of the NSH Convention Committee. "Our expectations were more than met."

Marilyn Gamble, NSH president, agreed. "I don't know how they do it," Gamble said, "but each symposium/convention seems to surpass the preceding one."

The 17th Annual Symposium/Convention of the National Society for Histotechnology opened on September 28 and ended on October 4. The Orlando, Florida, meeting drew more than 1,100 histology professionals from around the world. In fact, the meeting resulted in a record number of participants and exhibitors, including some from Italy, Australia, France, the United Kingdom, the Netherlands, Switzerland, Sweden, Norway, Canada, Alaska, and Hawaii.

"The society is gaining a reputation as being the place to present papers, not just nationally but internationally," Gamble said. "We're known as a very dominant educational force in the field. In fact, I don't think it would be boasting to say that this is the educational experience of a lifetime for Histotechnologists."

"It was the biggest meeting we've ever had," Boris stated. "We've been increasing our attendance by a little more than 100 every year since 1986. And if that trend continues, we'll be very big by the mid-nineties. We're also looking to expand our international representation to other countries and continents. It's going to be a big challenge for the next few years but, fortunately, the number of people helping has increased in proportion to the work load."

The entire block of hotel rooms reserved for the meeting was sold out by June. According to hotel officials, this was the first time a block of rooms was sold out that far in advance. More than 200 of the registrants were from the state of Florida, the highest attendance ever from a single state. Boris explained that the Florida state licensor requires Histotechnologists to have 24 CEUs within a 2-year period. Because all of the workshops provide CEUs, Floridians took advantage of an excellent opportunity to earn their credits.

Three hundred sixty-five people attended the meeting for the first time. "That's a very significant increase," Gamble said, "and statistics show that a very large percentage of people who are attending for the first time will come back."

While first-timers account for the impressive growth of the symposium/convention, returnees make a strong statement about its value. More than three quarters of those who attended had experienced an NSH symposium/convention before.

The Miles-sponsored first-timers party also drew a record crowd of about 250 people. The banquet is held on the first night of the symposium/convention to give first-time attendees an opportunity to meet with NSH officers and committee chairpersons. "It's become very successful," Boris said. "People look forward to it because it breaks the ice and makes first-timers feel more comfortable throughout the rest of the week."

The symposium/convention lived up to its theme, "Excellence Through Enlightenment." "The workshops were excellent, and there were certainly enlightened people afterward," Boris recalled. Seventy-three workshops kept the participants busy during the first 4 days of the symposium/convention. Then for the following 2½ days they were occupied with scientific sessions.

All the limited-attendance workshops were filled to capacity. The others also drew large crowds. "We always try to expand our variety of workshops," Gamble said. Basic, intermediate, and advanced workshops covered a wide range of technical topics, including microscopic anatomy, basic chemistry, staining techniques, human cytogenetic analysis, immunocytochemistry, fluorescence microscopy, immunohistochemistry, oncogenes, in situ hybridization, ploidy analysis, microwave techniques, specimen photography, marine histology, and image cytometry.

Other topics of interest to Histotechnologists were also covered in workshops. Participants could learn about

safety in the laboratory, hazardous chemicals, CAP workload recording and quality assurance monitoring, and the use of computers in the laboratory. They could also learn how to prepare for the HT and HTL registry exams, how to start a school of histotechnology, how to write procedure manuals, how to write and submit a research paper for publication, and how to write goals, objectives, and lesson plans. Self-improvement workshops were also presented covering topics such as "How to Market Yourself," "The Empowerment Process," "Presentation Skills," and "Dealing with Difficult People." In addition, Dr. J. B. McCormick repeated his popular workshop, "Study and Preparation of Antique Prepared Microslides."

The scientific sessions, which included both clinical and V.I.R. (Veterinary, Industrial, Research), were highlighted by the Professor C.F.A. Culling Memorial Lecture. The lecture was presented by Robert W. Mowry, M.D., of the University of Alabama. Mowry discussed the "Evolution and Present Status of Methods Utilizing Alcian Blue 8gx (and related dyes) for the Detection of Complex Carbohydrates Containing High Concentrations of Acidic Groups Whether Mainly Sulfates or Mainly Carboxyles or Both."

The Culling Lecture was followed by the Swiss Society Exchange Lectures sponsored by Miles Inc. Brigette Greiner of Sandoz AZ in Pratteln discussed "In Situ Hybridization" and Esther Busser of the Institute of Pathology in Winterthur discussed "Dyes."

Separate panel discussions were conducted for clinical and V.I.R. concerns. Three-hour sessions included panels of experts answering questions that were submitted by participants throughout the week, as well as during the discussions.

A record number of entries was also presented in the poster sessions and technical exhibits. In addition, more than 100 exhibits were prepared by technical and scientific companies to show their latest products.

The symposium/convention was held at the Stouffer Resort in Orlando. "We had a beautiful property this year," Boris said. "The hotel said they'd never had such a fun group."

Reception for first-time attendees

Dr. Mowry, presenter of the Culling Lecture

International guests were entertained by Marilyn Gamble and Leonard Noble

Attendees learning about new products and instrumentation

Land Shark and monk at Cast Party

Four out of 101 isn't bad...

As always, the social functions attracted large crowds. On Tuesday night, Miles Inc. sponsored a "Cast Party" where everyone dressed as their favorite movie character. One group, made up of Histotechnologists from the same area, came as the infamous Chicago Black Sox team from *Field of Dreams*. Another group came as 101 dalmatians. Individuals were dressed as Dick Tracy, Blondie, Mae West, Tweety Bird and Sylvester, Indiana Jones, Cher, Cleopatra, Beetlejuice, and the Teenage Mutant Ninja Turtles. There were also two Scarlett O'Haras, but only one Rhett Butler.

"Social events are a very important part of the meeting because that's a time when you can meet your colleagues in an informal, entertaining setting," Boris continued. "That's when people really get to know each other and do the networking that is so valuable to our profession."

The main social event was the Thursday night Awards Banquet, which was preceded by a cocktail reception sponsored by Miles Inc. An estimated 700 people witnessed the presentation of the most prestigious awards in the profession. More than 17 awards and scholarships were presented at the banquet.

"All in all, it was very successful," Boris said. "There was excellent local help. Everything was beautifully done." Carolyn Floyd was the local coordinator for the symposium/convention. "And, of course, none of this could have been accomplished without the help of Roberta Mosedale and Sylvia Palmer at the national office," Boris continued. "They're fantastic."

Although the weather was dampened by rain throughout the week, the spirits of those who attended this annual event were not. And if Boris's predictions come true, the symposium/convention will just keep getting better. Next year, it will be held September 12-18 in Monterey, California. "I think that will be a great meeting," Boris said.

The National Society for Histotechnology has declared the 1990s to be the "Decade of Progress." If the national symposium/convention is any indication, there is a lot to look forward to for histotechnology. Because, as good as it is now, it will just keep getting better.

Methods for the Use of Hyaluronidase Amylase and Neuraminidase (Sialidase)

Lee G. Luna
and
Darryl E. Luna
American HistoLabs, Inc.
Gaithersburg, MD 20879

The enzymes cited above have been used extensively for the identification of hyaluronic acid, glycogen, and sialic acid in paraffin-embedded tissue sections. There appears to be some confusion and lack of knowledge in some laboratories regarding the proper utilization of these methods. The information presented herein is intended to diminish some of the confusion and provide up-to-date information and methods for the proper use of these enzymes. The following remarks provide general information, some of which one should remember when making up the enzyme solution.

Human beings have long taken advantage of enzymatic reactions to improve their well-being without understanding the exact nature of the process, as, for example, the process in baking and in the manufacture of cheese and alcoholic beverages. It is only in comparatively recent years that specific enzymes have been isolated and their mode of action studied. The determination of enzyme activity in serum or body tissues or fluids has become an indispensable tool of modern diagnosis. Practical experience has shown that an expert knowledge of enzyme chemistry is not essential in order to benefit from its use. Enzymes are biological catalysts. Like other catalysts, they accelerate chemical reactions or make them possible by lowering the reaction energy requirement. They are protein in nature and can be isolated and sometimes crystallized like other proteins. Like all proteins, enzymes may be denatured and thereby inactivated by physical or chemical means. They are susceptible to heat, pH, and microorganisms.

Many factors influence the rate of enzyme reactions. The most important of these are *pH*, *ionic strength*, *temperature*, *buffer*, and *technical factors*, such as inhibitors from contaminated glassware or vigorous shaking, which may cause denaturation. Most enzyme assays utilize *37°C* as a reaction temperature. This assures a more natural or physiological condition.

There are various simple precautions that can frequently improve accuracy and precision in clinical enzymology:

1. *Glassware contamination* is a frequent cause of error.
2. *Sample handling presents problems:* Since enzymes may be denatured at air-liquid interfaces (due to distortion of the protein molecules), mixing should be done by *gentle inversion* rather than vigorous shaking.
3. *Accurate temperature* control during incubation is essential.

With this general background information on enzymes, the following point needs emphasis as it relates to histotechnology. Enzyme activity is expressed in *units of activity*, not necessarily in grams of enzymes. Therefore, when making a solution utilizing an enzyme, the concentration should not be expressed in terms of weight (grams) but units of activity. This is especially significant for hyaluronidase and amylase since they are commercially available in a wide variety of units of activity.

Provided below are the enzymes and the entity they catalyze:

Hyaluronidase — (Hyaluronate glycanohydrolase) is an enzyme that catalyzes the hydrolysis of *hyaluronic acid*, which is one of the mucosubstances found in abundance in human and animal tissue.

Amylase — is an enzyme that catalyzes the hydrolysis of starch into smaller molecules. Amylase acts in a random manner in catalyzing the hydrolysis of glycogen, which is found in many organs, particularly liver and muscle in human and animal tissue.

Neuraminidase (sialidase) — is an enzyme that catalyzes the hydrolysis of sialic acid, which is found in abundance in salivary glands and is a component of the airway secretion of the lungs.

Hyaluronidase Digestion Method

The following provides information for compounding solutions of hyaluronidase of different TR units. Hyaluronidase can be purchased in different units of activity from 300 to 2,000 units of activity per milligram (300 units/mg - 2,000 units/mg). The activity in terms of

units for removing hyaluronic acid from sections should be 150 - 200 units per milligram. This being the case, one should purchase hyaluronidase in units of activity that are easy to calculate. For example, if the available hyaluronidase is 400 units of activity per milligram, one should weigh out 0.05 g and dissolve it in 100 mL of normal saline or distilled water. The concentration in terms of activity is 200 units per milliliter.

Example:

(400 units/mg) (1000 mg/g) 0.05 g = 20, 000 units

20,000 units/100 mL = 200 units/mL

This general formula can be used to calculate the amount of milligrams or grams necessary for mixing working hyaluronidase solutions with a unit of activity between 150 to 200 units per milligram.

Hyaluronidase from Bovine testes lyophilized powder should be used for removing hyaluronic acid from tissue sections. The use of hyaluronidase containing various agents such as protein or lactose should not be used.

Solutions

0.1M Potassium Phosphate, Monobasic

Potassium phosphate, monobasic ..... 13.61 g
Distilled water ..... 1000.0 mL

0.1M Sodium Phosphate, Dibasic

Sodium phosphate, dibasic ..... 14.20 g
Sodium phosphate, dibasic, 0.1M ..... 1000.0 mL

Buffer

Potassium phosphate, monobasic, 0.1M ..... 94.0 mL
Sodium phosphate, dibasic, 0.1M ..... 6.0 mL

Hyaluronidase

Hyaluronidase ..... 0.05 g
Buffer solution ..... 100.0 mL
Mix just before use.

Remarks

The buffer solution should be used to dissolve hyaluronidase when critical studies on mucosubstances are being performed. Distilled water works quite well when less than critical studies are performed.

Digestion Procedure

Use two slides from the same block, one for digestion with hyaluronidase and one with buffer solution control.

1. Decerate slides in xylene, 2 changes, 2 minutes each. Place in absolute alcohol, 2 changes, 2 minutes each; 95% alcohol, 2 changes, 2 minutes each; then rinse in distilled water.
2. Digest slides in hyaluronidase solution at 37°C for 1 hour. Incubate the duplicate slides in buffer solution at 37°C for 1 hour.
3. Wash both sets of slides in running tap water for 5 minutes.
4. Stain as desired.

Results

Staining attributable to hyaluronic acid, chondroitin 4 sulfate, or chondroitin 6 sulfate is selectively eliminated.

Amylase Digestion Method

The digestion of glycogen is best accomplished with the use of amylase instead of the often-used diastase. This is suggested since numerous problems have surfaced in recent years with the use of diastase to digest glycogen. The most significant problem is related to the fact that for some unknown reason diastase often does not digest all the glycogen from a given section. Amylase, on the other hand, removes all the glycogen very well and therefore should become the enzyme of choice for digesting glycogen from tissue sections (Figs. 1-3). It should be pointed out that glycogen is also removed completely from sections when saliva is used. It may not be difficult to understand why amylase and saliva work so much better than diastase if one considers the following fact: A-amylase is an enzyme that catalyzes the hydrolysis of starch into smaller molecules. The a-amylases are found in animals and include salivary amylase and pancreatic amylase.

On the other hand, diastase is a white, amorphous, soluble enzyme produced during the germination of seeds, and contained in malt. Diastase of malt converts starch into maltose and then into dextrose, which in turn should dissolve in water.

Personal experience in our laboratory strongly suggests that commercially available a-amylase and the amylase contained in one's saliva work equally well for dissolving (removing) glycogen from tissue sections. The practice of

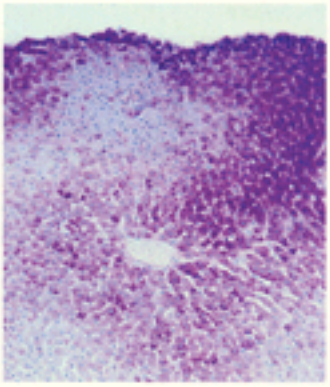

Figure 1: This is a section of liver stained with a periodic acid Schiff reaction (PAS). The section was not treated with an enzyme (sialidase or amylase). Note the heavy presence of glycogen in the upper right quadrant.

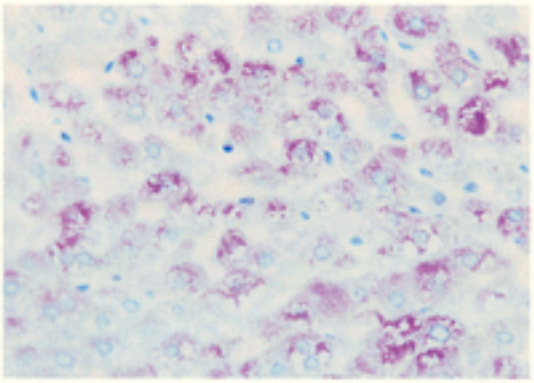

Figure 2: This photograph was taken from the upper right quadrant of the slide seen on Figure 1. The section was decerated in the usual manner, treated with *diastase*, and stained with PAS reaction. Notice the presence of glycogen in some of the cells.

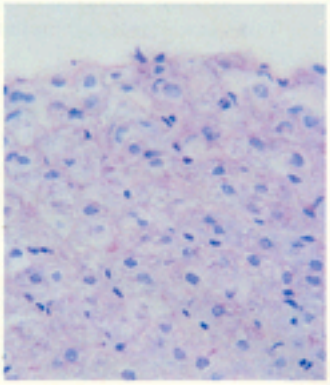

Figure 3: This photograph was taken from a section that contained heavy deposits of glycogen similar to Figure 1. The reaction was decerated in the usual manner, treated with *amylase*, and stained with the PAS reaction. Notice the complete absence of glycogen.

using saliva to digest glycogen should be used only in emergencies, i.e., when amylase is not available in the laboratory to perform the test. The use of saliva is nonsanitary; sections often contain bacteria from the technician's mouth, and, in general, the practice of expectorating on a slide is not appealing to those in the same environment.

Solutions

Phosphate Buffer pH 6.0

Sodium chloride	8.0	g
Sodium phosphate, dibasic	0.282	g
Sodium phosphate, monobasic	1.97	g
Distilled water	1000.0	mL

Amylase Digestion

Amylase of malt	0.1	g
Phosphate buffer solution	100.0	mL

Mix just before use. Note: Distilled water can also be used to dissolve the amylase powder.

Digestion Procedure

1. Decerate slides in xylene, 2 changes, 2 minutes each. Place in absolute alcohol, 2 changes, 2 minutes each; 95% alcohol, 2 changes, 2 minutes each; then rinse in distilled water.
2. Place slides in amylase digestion solution at 37°C for 30 minutes or room temperature.
3. Wash in running water for 5 minutes.
4. Stain with McManus method for glycoproteins (PAS) or selected method.

Results

Staining attributable to glycogen (glycoproteins) is selectively eliminated.

Neuraminidase (Sialidase) Digestion Method

Solutions

Buffer #1

Sodium acetate	26.2	g
Calcium chloride	8.9	g
Distilled water	200.0	mL

Buffer #2

Sodium acetate	2.6	g
Calcium chloride	0.89	g
Distilled water	200.0	mL

Neuraminidase (Sialidase)

Neuraminidase (sialidase)	1.0	mL
Buffer solution #1	0.1	mL

(continued on page 297)

Digestion Procedure
Use two slides from the same block, one for digestion and one for treatment with buffer solution #2 (control).

1. Decerate slides in xylene, 2 changes, 2 minutes each. Place in absolute alcohol, 2 changes, 2 minutes each; 95% alcohol, 2 changes, 2 minutes each; then rinse in distilled water.
2. To contain enzyme solution, circle sections to be digested with diamond or hydrophobic marking pencil.
3. Allow slides to air-dry for 1 hour.
4. Place slides in humidity chamber or on glass rods close above layer of water in a petri dish. Place sialidase digestion solution on one slide in sufficient quantity to completely cover section. Cover the second slide, to be used as "control," with buffer solution #2. The humidity chamber or petri dishes are then incubated for 3 hours at 37°-40°C.
5. Remove from oven and rinse in distilled water.
6. Stain as desired.

Results
Staining attributable to the sialomucins will be selectively eliminated.

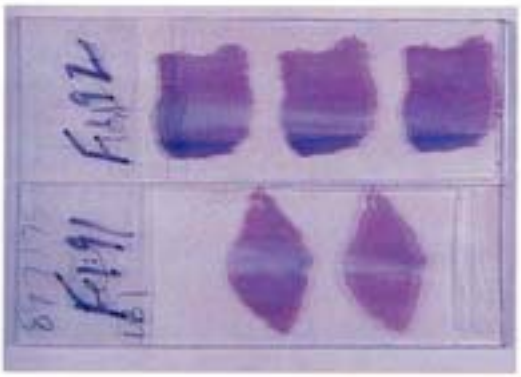

Figure 1. H&E stained section that demonstrates blue bands across the specimen. Notice that the blue non-eosin-staining band is thinner on the right side and gradually increases in size from right to left.

Editor's Note: It would be interesting to know if this is a recurring staining problem in other laboratories using automatic H&E staining systems. Anyone experiencing any problems in staining with automatic stainers is asked to write to the scientific editor (LGL) of *Histo-Logic*. This information could be of extreme importance since I have seen a suspicious trend in questionable quality of hematoxylin- and eosin-stained slides with the use of automatic stainers. A report on this subject will be forthcoming in a future issue of *Histo-Logic*.

Questions in Search of an Answer

■ Figure 1 illustrates a hematoxylin and eosin staining problem for which I would appreciate an answer. The slides were stained with an automatic staining system with Harris' hematoxylin followed by decolorization in 0.25% acid alcohol, with blueing in a weak solution of ammonium hydroxide water.

Juan Bassett
Gaithersburg, MD

■ We have a most unusual problem in our laboratory concerning endoscopy specimens. Whenever we use 10% neutral buffered formalin, or just 10% buffered formalin, the endoscopy specimens appear on the slides as fragmented, crushed, and overstained with hematoxylin.

We have made up the formalin ourselves and we have ordered commercially made formalin and have gotten the same results with both. We have no problem with other tissues. The endoscopy specimens are fine when we use plain 10% formalin without a buffer, but then we have formalin precipitate pigment.

We would be interested in knowing if anyone else has encountered this problem, and, if so, their solution.

Joyce Schroeder, HT(ASCP)
Henrico Doctor's Hospital
Richmond, VA 23229

Histo-Logic Congratulates Outstanding Histotechnologists

The awards listed below were conferred at the NSH Symposium Banquet, held October 3, 1991, in Orlando, Florida.

Golden Forceps Award
Sponsored by Miles Inc., Diagnostics Division

Lynn Montgomery
Baton Rouge, Louisiana

Histotechnologist of the Year Award
Sponsored by Shandon/Lipshaw, Inc.

Jimmy Stringer
Dallas, Texas

Diamond Cover Award
Sponsored by Miles Inc., Diagnostics Division

Roberta Smith
Durham, North Carolina

Diamond Cover Merit Award
Sponsored by Miles Inc., Diagnostics Division

Winsom Garvey
Toronto, Ontario, Canada

Editor's Award
Sponsored by Miles Inc., Diagnostics Division

Colin Benjamin
Toronto, Ontario, Canada

J. B. McCormick, M.D. Award
Sponsored by Dr. J. B. McCormick

Marilyn Gamble
Hollywood, California

President's Award
Sponsored by the National Society for Histotechnology

Roberta Mosedale
Lanham, Maryland

N.S.H. Convention Scholarship Award
Sponsored by Histology Control Systems, Inc.

Hazel Cannon
Jackson, Mississippi

The Lee G. Luna Foreign Scholarship Award
Sponsored by Surgipath Medical Industries, Inc.

Peggy A. Wenk
Royal Oak, Michigan

Sakura Student Scholarship Award
Sponsored by Sakura Finetek, USA, Inc.

M. Rowley, Jr.
Cobleskill, New York

Shandon/Lipshaw Student Scholarship Award
Sponsored by Shandon/Lipshaw, Inc.

Rebecca L. Webster
Eau Claire, Wisconsin

Shandon/Lipshaw Educational Scholarship Award
Sponsored by Shandon/Lipshaw, Inc.

Jyotsna Patel
Middleburg Heights, Ohio

Dezna C. Sheehan Memorial Scholarship Award
Sponsored by the National Society for Histotechnology

Judi A. Stasko
Ames, Iowa

Miles Educational Scholarship Award
Sponsored by Miles Inc., Diagnostics Division

Rebecca S. Massey
Jackson, Mississippi

Instrumentation Laboratory Educational Scholarship Award
Sponsored by Instrumentation Laboratories, Inc.

Wanda L. Grace
Jackson, Mississippi

Wright-Giemsa Method for Bone Marrow Biopsies

Bonnie Hempelmann
Phelps County Regional Medical Center
Rolla, MO 65401

A microwave modification for a Wright-Giemsa stain was developed in our laboratory and is described herein. In addition to routine use, this method is used for bone marrow biopsies.

Fixation 10% buffered neutral formalin
Smears-absolute methyl alcohol

Process Paraffin sections or air-dried smears

Microtomy Cut sections at 5 micrometers

Solutions

Wright's Stain

Harleco, Wright's stain	2.5	g
Methyl alcohol	1000.0	mL

Filter before use.

Buffer

Sodium phosphate, dibasic	4.5	g
Potassium phosphate, monobasic	5.9	g

Mix together. Add 1 g of this mixture to 1000 mL of distilled water. The pH should be 6.8.

Stock Giemsa

Giemsa powder	1.0	g
Glycerine	66.0	mL
Methyl alcohol	66.0	mL

Working Giemsa

Stock Giemsa solution	100	drops
Distilled water	50.0	mL

Make fresh before use. Discard after use.

Staining Procedure

1. Decerate and hydrate to water.
2. Place in methyl alcohol for 3 minutes.
3. Place in Wright's stain for 1 minute. (Lay slides on staining rack. Add stain to top of slides.)
4. Add an equal amount of buffer to slide containing Wright's stain. Blow gently to mix and until a green sheen is present. Stain for 6 minutes.
5. Rinse in distilled water.
6. Place slide in plastic coplin jar containing working Giemsa stain. Do not cap tightly. Microwave on 100% power for 30 seconds. (Our lab has an Emerson Model AT736A, 500 W microwave.) Do not disturb for 2 minutes after microwaving. Some adjustment in time may be necessary, depending on the wattage of your microwave.
7. Place in 1% glacial acetic acid for 3 quick dips.
8. Rinse in distilled water.
9. Dehydrate, clear, and mount with appropriate media. Use of the microwave saves approximately 45 minutes staining time and produces excellent results.

Kudos

• Please accept our congratulations on *Histo-Logic*'s 20 years of publication. It helped us through many years to make our daily job better. We deeply appreciate Miles' and Lee Luna's effort.

— Fermin E. De Shant, Carlos Alberto Peralta, Osvaldo C. Peralta, Mar del Plata, Argentina

• We want to congratulate *Histo-Logic* for having achieved an important role in helping Histotechnicians and Pathologists, for the last 20 years, with useful articles and helpful information.

Thank you very much and we hope for the best for Mr. Lee Luna and again our congratulations.

— Natalio Guman, Buenos Aires, Argentina

• As chief of the Pathology Department of Hospital Eva Peron, and together with my professional staff (technicians and doctors), we want to send to *Histo-Logic* our congratulations for its first 20 years, being grateful for its helpful information that improved and improves daily our work.

— Sara Kestelboim, Argentina

NSH Introduces Recruitment Video

Brent Riley
Managing Editor

Ask 10 high school seniors what histotechnology is, and you're likely to get 10 very puzzled looks. But a new career video program, produced by the National Society for Histotechnology in conjunction with Miles Inc., is designed to educate career-oriented students about the opportunities in histotechnology.

The video project was spearheaded by Mary Knight and Leonard Noble, with assistance from Marilyn Gamble, NSH president. The main purpose of the 15-minute video is recruitment.

"There are different pathways to becoming an HT," Noble continued, "but they all begin with high school graduation. The ASCP still recognizes high school graduation, plus 2 years in an accredited hospital under the supervision of a qualified Pathologist, to be a viable pathway to a career in histotechnology."

The video targets a younger group—about 18 to 22 years old. The program explains what a Histotechnologist does, featuring Histotechnologists on camera talking about the benefits of their profession. The program closes with information about how to become a Histotechnologist.

The video will be distributed through the national office of the NSH. Copies, which can be borrowed or purchased, will be shown at career-oriented events such as high school career days or allied health fairs. It may also be used to recruit college graduates who might be interested in HTL certification.

The video was shown at the recent NSH Symposium/ Convention in Orlando, Florida. "It was very well received," Noble said.

To receive your own copy of *Histo-Logic*,® or to have someone added to the mailing list, submit home address to: Miles Inc., Diagnostics Division, P.O. Box 70, Elkhart, IN 46515.

The editor wishes to solicit information, questions, and articles relating to histotechnology. Submit these to: Lee G. Luna, *Histo-Logic* Editor, P.O. Box 1263, Lanham, MD 20706. Articles, photographs, etc., will not be returned unless requested in writing when they are submitted.

MILES

Diagnostics Division
Miles Inc.
Elkhart, IN 46515

BULK RATE
U.S. POSTAGE
PAID
Permit No. 499
South Bend, IN

Histo-Logic®