An Adaptation of Methacrylate Embedding for Routine Histopathologic Use

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HEMATOLOGISTS have traditionally evaluated hematopoiesis in smears of aspirated marrow and have been aware of some of the errors inherent in this technic, most, if not all of which are corrected by examination of sections.[1] The errors include inability to obtain comparable material for serial studies, lack of correlation between the ratio of cells in smears and in the marrow, failure to demonstrate tissue organization, misleading information about the number and abnormalities of reticular cells, variable dilution of the marrow by peripheral blood making it impossible to determine accurately total cellularity and numbers of cells in a lineage, inability to evaluate fibrosis and poor demonstration of granulomas and lymphomas. Many of these deficiencies were recognized as early as 1934.[2] Similar errors have been encountered in smears of liver and spleen.[3] Nevertheless, hematologists have continued to utilize smears and not sections to study hematopoietic tissues for two reasons. First, the hematologist has been unable to identify cell types in sections of paraffin-embedded tissue, the only material available to him. Second, most hematologists never having had the opportunity to examine sections of plastic embedded tissue, are unfamiliar with the potential advantages of this technic over smears for study of hematopoietic tissues.

The advantages of paraffin are: 1) technical and professional personnel are universally taught this method; 2) availability of equipment, including specialized automated instruments for processing of tissue; 3) the artifacts inherent to the paraffin technic are familiar and accepted. Disadvantages are: 1) difficulty of processing tissues more than 4.0 cm. in length; 2) inability to cut sections less than 2 μ and frequently less than 4 μ in thickness; (usually sections are thicker than indicated by the microtome setting); 3) the necessity to decalcify very hard tissue so long that the staining quality is impaired; 4) the difficulty of preparing sections from a mixture of hard and soft tissues, for example, embryos; and, most important, 5) the inability to differentiate cell types in hematopoietic tissues.

With the advent of the electron microscope, new plastic embedding materials were developed for ultrathin sections. One micron sections of tissue

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prepared as for electron microscopy and stained by any of a variety of technics, demonstrate superior preservation of cytologic detail when examined by light microscopy.[4,5] However, these plastics are unsatisfactory for routine clinical and research work with the light microscope because it has been impossible to cut sections more than 2–3 mm. square.

The purpose of this paper is to describe our modification of Cathey's methacrylate embedding technic[6] which has the following advantages. Cells of hematopoietic tissues may be differentiated from each other as well as in dry smears. The cells are similar in appearance to those illustrated in Bloom and Fawcett's Textbook of Histology.[7] The technic does not require expensive or elaborate apparatus, and is uncomplicated enough to be adapted by any histopathologic laboratory. Tissues may be processed in as short a period of time as 16 hours provided that proper fixation and staining technics are used.[1,8] The technic is applicable to all, and not just to hematopoietic tissues.

Materials and Methods

I. Fixation

The usual histologic fixatives can be used. We have used buffered Zenker-formol, Bouin's, paraformaldehyde, 10 per cent formalin, glutaraldehyde and osmium tetroxide. The length of time of fixation was varied according to the thickness of the tissue. Most tissues examined were fixed in Zenker-formol from one to three hours. Tissues were trimmed to a few tenths of a cm. in thickness to permit better fixation and more rapid dehydration and infiltration of the plastic mixture. The fixative was removed by washing in cold, running tap water for 15 to 45 minutes, depending on the size or thickness of tissue because residual formalin reacts with the monomer causing premature polymerization and thus interfering with infiltration. Tissues may be washed for as long as 48 hours without deleterious effects.

II. Dehydration and Embedding

Tissues were dehydrated through 75 per cent, 95 per cent, and absolute ethanol for at least 20 to 40 minutes, depending upon the thickness of the specimens. Final dehydration was accomplished for 10 minutes in anhydrous acetone (solution #1). Tissues were stored in 75 per cent alcohol if immediate embedding was impractical. (Tissues stored more than three weeks in alcohol prior to embedding may not stain well with Romanowsky mixtures). Tissues keep indefinitely in the plastic after polymerization; in fact, the cutting quality improves with time.

Infiltration of the plastic was initiated by placing the dehydrated tissue into solution #2 for two 20 minute changes and then into a mixture of equal parts of solutions #2 and #4 for 60 minutes. Next, the tissue was placed in solution #4 for at least one hour. The tissue may be infiltrated in this solution for up to 48 hours after which it will slowly polymerize at room temperature.

Infiltration rates of plastic mixtures #2 or #4 may be increased by one or two methods. Tissues in the plastic solutions may be placed in a desiccator and exposed to a negative pressure of 500–600 mm. (water aspirator or laboratory vacuum), or the specimens may be placed in vials and rotated at ⅓ rpm. This is accomplished by means of a disc with holes for the vials which is inclined 30° from the vertical.

III. Polymerization

"Peel-away" plastic embedding molds, about 2 cm. deep, containing a 1 to 3 mm. layer of previously polymerized solution #4, are used for embedding. This layer prevents the tissue from settling onto the bottom of the mold. The tissue in solution #4 was placed in the mold, oriented, and the mold filled completely with solution #4.

Polymerization of the plastic was initiated under long wave ultraviolet light for 4 hours and final curing completed at 56° for 4 hours. The ultraviolet light was mounted horizontally on 2 blocks in the oven so that the molds are 5–6 cm. below the bulb. It is convenient to arrange times at which these two steps are performed to conform with the routine of the laboratory. For example, we polymerize with ultraviolet light from 10:00 p.m. to 2:00 a.m., and polymerize with heat from 2:00 a.m. to 6:00 a.m. by attaching automatic timers to the ultraviolet light and to the oven heating unit. Tissues may be cut as soon as cool, usually 20 minutes after removal from the oven. Blocks may be more rapidly cooled in ice water.

These times may be altered according to the size of the tissue and the need for rapid processing. For example, needle biopsies of marrow, spleen, kidney or liver have been prepared for sectioning within 8 hours after removal from the fixative by decreasing the time of polymerization to three hours. On the other hand, large orthopedic specimens require longer dehydration and infiltration. Two days may be needed for infiltration and 16 hours for polymerization of the plastic.

We have not adapted the processing of tissues for automation.

IV. Sectioning

The block was held in a vise and moistened with 50 per cent alcohol while excess plastic was trimmed with a small coping saw. The block was then inserted directly into the microtome chuck. Alternatively it may be cemented to a support with a fast setting epoxy resin prior to placing it in the chuck.[9] After sectioning, the block may be removed from the support by hitting it with a hammer. These plastic embedded specimens may be sectioned with either a sliding or rotary microtome while the block and the knife are moistened with 50 per cent alcohol. We have sectioned pieces of tissue as large as 5 × 3 cm. Sections were transferred by a spatula moistened with 50 per cent alcohol to a slide coated with a thin layer of albumin. The section was flattened by covering with a drop or two of 95 per cent ethanol and then placed on a heating platform at about 45 C. until dry.

The dried section was flooded with clove oil for about 10–20 minutes or until cleared. The clove oil was removed by passing the slide through three changes of 95 per cent alcohol. Thereafter, the sections were handled in the same manner as those from which paraffin or nitrocellulose has been removed.

V. Solutions and Reagents

Solution 1

Acetone (Baker Reagent Grade) over anhydrous Na_2SO_4

Solution 2

Methyl Methacrylate Monomer

Solution 3

Methyl Methacrylate	27.0 cc.
Polyethylene Glycol Distearate #1540	6.0 Gm.
Dibutylphthalate	4.0 cc.

Heat to 56° to dissolve distearate and cool to room T° as soon as distearate is dissolved.

Solution 4

Solution 3	90.0 cc.
Benzoyl Peroxide	0.6 Gm.
Wetting Agent (nonyl phenoxy polyethylene ethylene (oxy) ethanol 100 percent reactive, non-ionic) (IGEPOL 630)	5 drops
Plexiglas	30.0 Gm.

Plexiglas added gradually with constant stirring, such as with a magnetic stirrer. Store in refrigerator to prevent polymerization. Bring to room temperature before using. Stable at room T° for 48 hours.

VI. Sources

Methyl Methacrylate (Monomer BP 100–101 C.)
Dibutylphthalate (BP 172–174 C./5mm.)
Benzoyl Peroxide (MP 105–106 C. [dec])
Matheson, Coleman & Bell
Division of the Matheson Co., Inc.
Norwood (Cincinnati) Ohio, or Rutherford, N. J.
Polyethylene Glycol Distearate #1540 (no substitutes)
Ruger Chemical Co., Inc.
Irvington on Hudson
New York, N. Y.
Wetting agent (nonyl phenoxy polyethylene ethylene (oxy) ethanol
100 percent reactive, non ionic) (IGEPOL 630)
Chemical Sales Company
1125 - 7th Street
Denver, Colorado
Plexiglas Molding Powder A–1 (colorless pellets)
Rohm and Haas Company
2 Washington Square
Philadelphia, Pa. 19105
"Peel-a-way" Embedding Molds
Lipshaw Mfg. Company
7440 Central Avenue
Detroit 10, Michigan

Results and Discussion

Our experience with this technic is extensive; since November 1966, all tissues from patients on the Hematology Service, surgical specimens from the Orthopedic Service, tissues from the fetal opossum including entire "embryos," frog kidney tumors, and rat neural tissues have been processed. Renal biopsies are now processed only by this technic. A total of about 4,000 blocks and 16,000 slides have been prepared. All but the very largest pieces of tissue were sectioned at 2 to 4 μ using ordinary, commercial steel microtome knives. The largest pieces have been sectioned at 4 μ to 6 μ. With extra care, we have been able to section pieces of tissue the size of a renal or hepatic needle biopsy at 0.5 micron. The tissue sections can be stained with most routine histologic stains since the plastic is readily removed when the sections are passed through xylene for several minutes during the staining procedure. Most of the tissue sections were stained with hematoxylin eosin-azure II; other stains used were Malloryazan, Gomori's silver impregnation, periodic acid Schiff, hematoxylin and eosin, muco-carmine, Best's carmine and azure B. The best results for hematopoietic tissues were achieved by fixation in Zenker-formol for 1 to 3 hours followed by staining with hematoxylin eosin-azure II or other Romanowsky mixtures.[7,8] Excellent slides have also been prepared from tissues previously embedded in paraffin by dissolving the paraffin and replacing it with methacrylate.

Figure 1 is a 6 μ thick section from the center (where infiltration would be the poorest) of a 4 x 3 cm. section, through an entire femoral head of an adult. We are now able to process tissue this size in 3 days following decalcification. This contrasts to a recently published technic using nitrocellulose in which

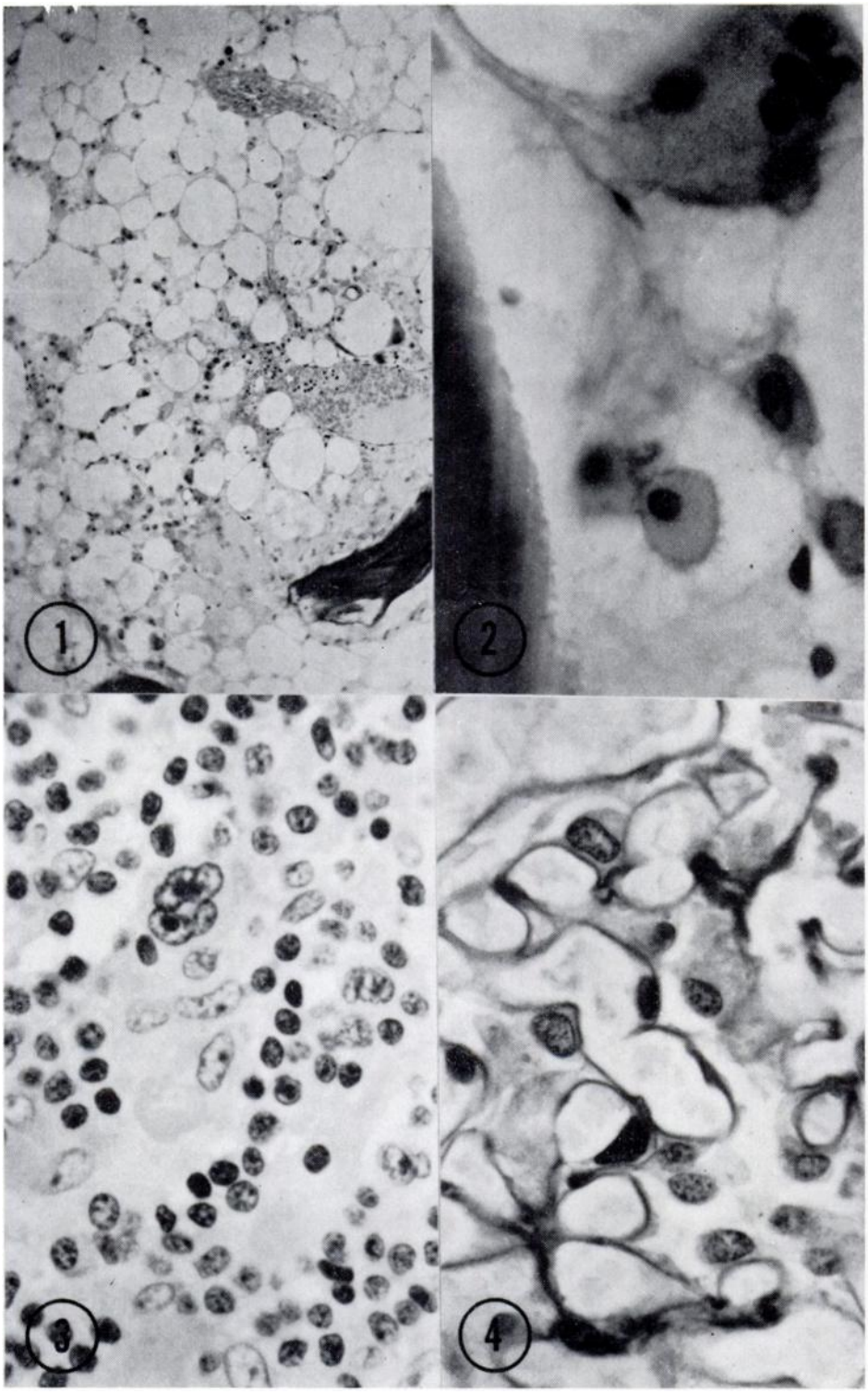

Fig. 1.—Center of a 4 x 3 cm. section of an adult femoral head, 200×.

Fig. 2.—Same block as Figure 1,900×.

Fig. 3.—3.0 x 1.5 cm. section from a lymph node of a patient with Hodgkin's disease, 900×.

Fig. 4.—Glomerulus from a renal needle biopsy, 1200×.

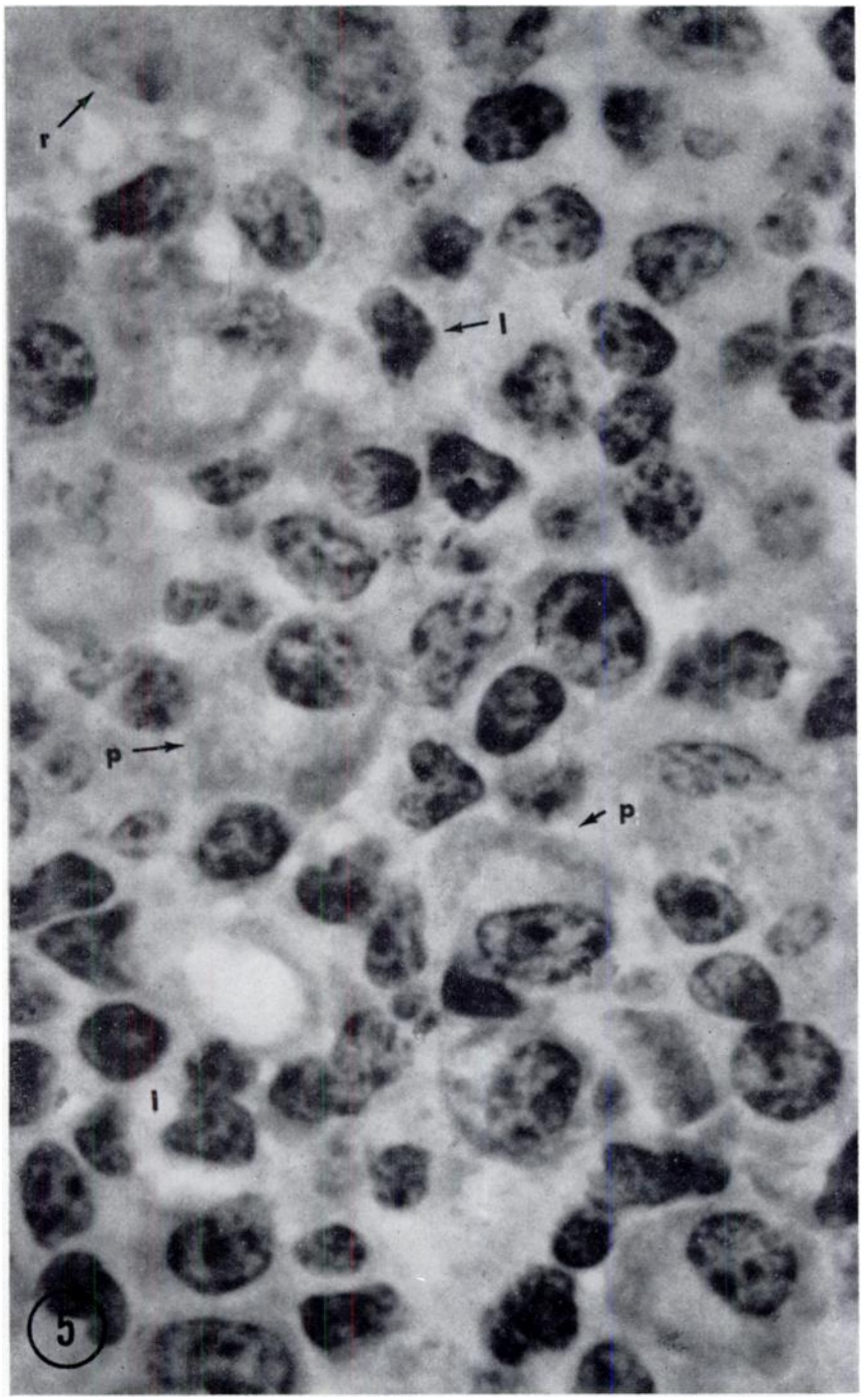

Fig. 5.—Iliac crest marrow obtained by needle aspiration from a patient with Waldenstrom's macroglobulinemia. Lymphocytes (l), reticular cells (r) and immature plasmacytoid lymphocytes (pl), 2400×.

smaller pieces of bone tissue require 3 weeks for processing to obtain 12 μ sections.[10] Figure 2 shows that the delicate fat spaces and tenuous connections between bone trabeculae and myeloid tissue are well preserved.

Figure 3 is from a 3 x 5 cm. section of a node from a patient with Hodgkin's disease and Figure 4 is from a glomerulus from a renal biopsy of a patient with multiple myeloma. Figure 5 is from the marrow of a patient with Waldenstrom's macroglobulinemia and Figure 6 is from the marrow of a patient with chronic granulocytic leukemia with a myleran induced remission. Figure 7 is from a patient with folic acid deficiency in relapse.

Summary

A plastic tissue embedding technic which is rapid and simple enough for routine clinical and research usage has been described. Tissues were routinely processed at 2 μ and 4 μ in a hospital laboratory within 16 hours. The quality of the sections was superior to those obtained with paraffin so that cell types are clearly recognizable. Advantages of this process over paraffin embedding include ease of storage and superior preservation of cytological detail.

SUMMARIO IN INTERLINGUA

Es describite un technica pro le inclusion de tissu in plastico le qual es satis rapide e simplice pro usos routinari in le clinicae pro objectivos de recerca. Specimens de tissu esseva processate a 2 e 4 μ in un laboratorio de hospital intra 16 horas. Le qualitate del sectiones esseva superior a illo obtenite con paraffin de maniera que le differente typos de cellula es clarmente recognoscibile. Le superioritate de iste processo super le inclusion in paraffin es les simplicitate del thesaurisage e le melior preservation de detalios cytologic.

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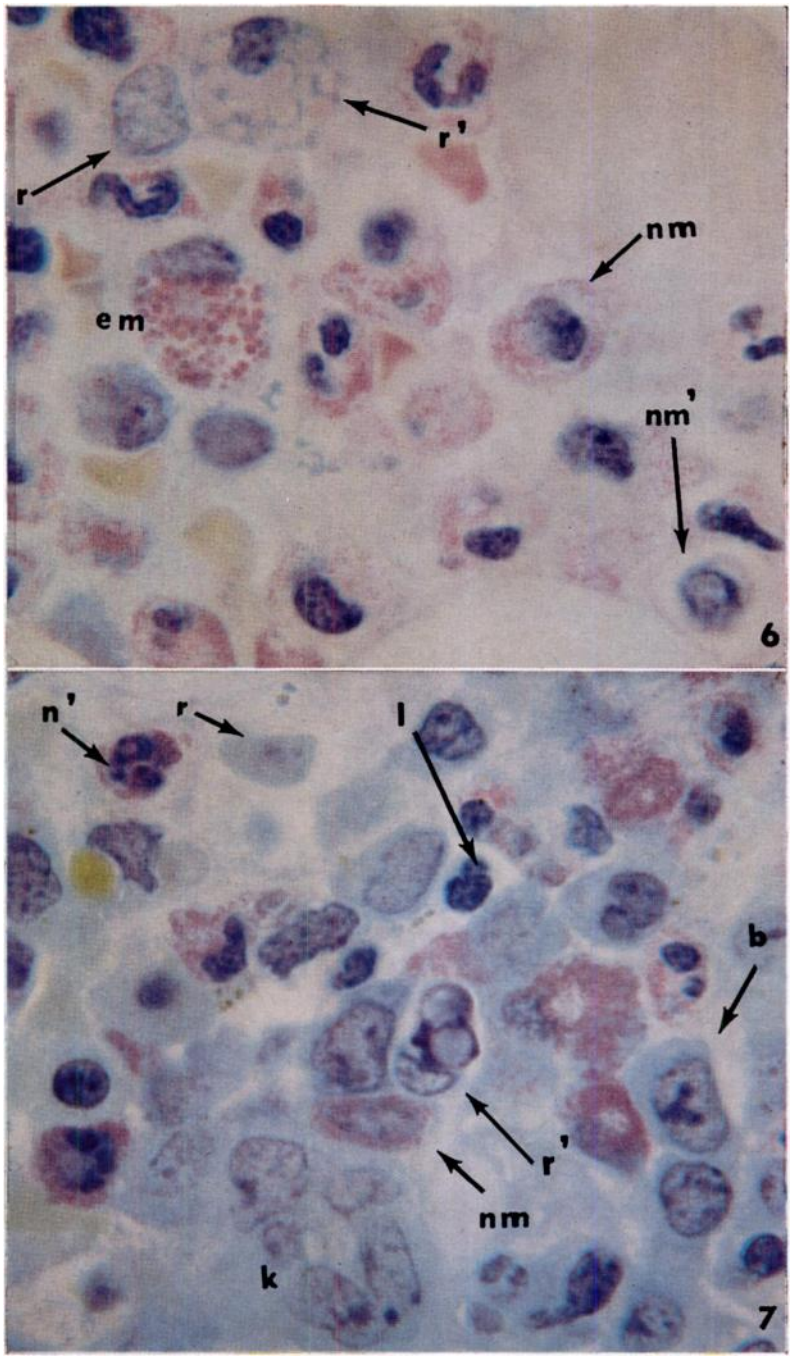

Fig. 6.—Iliac crest marrow obtained by needle aspiration from a patient with chronic granulocytic leukemia in myleran induced remission. Reticular cell (r), reticular cell containing fragments of phagocytized red cells (r'), eosinophil myelocyte (em), neutrophil myelocyte (nm) and neutrophil myelocyte undergoing karyorrhexis (nm'), 1600×.

Fig. 7—Iliac crest marrow obtained by needle aspiration from a patient with cirrhosis and folic acid deficiency. Lymphocite (1), reticular cell with partially digested cellular debris above nucleus (r), reticular cell with two phagocytized nuclei (r'), neutrophil myelocyte (nm), degenerating neutrophil granulocyte (n'), basophil megaloblast (b), and megakaryocyte (k), 1600×.