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for
NEPHROPATHOLOGY

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Techniques for Nephropathology

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PREFACE

It is our aim in this monograph to present techniques for the routine examination of renal tissue that are reliable and provide the maximum information for diagnosis. The methods described have provided consistently good results and to a varying degree represent a compilation of those previously advocated. Personal preference,* experience, and the quality of technical expertise usually determine which techniques are employed in an individual laboratory. Our methods were assembled after an extensive process of trial and error in applying different recommended techniques over recent years.

Accurate diagnosis of the renal biopsy remains the cornerstone of management of renal diseases and that diagnosis is often reached only after immunohistochemical and ultrastructural data have supplemented the light microscopic findings. Thus the techniques outlined cover, in the main, these three aspects of renal biopsy examination.

Morphological techniques, however, have not provided much of the basic information of the renal, especially glomerular, reaction to injury. In vitro glomerular culture promises much in this regard when applied to human tissue and the experimental situation. Thus we have included a chapter on this and related techniques by Associate Professor Robert Atkins and Associate Professor Eric Glasgow and their colleagues who commenced working in this field at Peince Henry's Hospital, Melbourne in 1972.

* Discussion in Strauss, J., Ed., *Pediatric Nephrology Vol. 4: Renal Failure — Current Concepts in Diagnosis and Management*, Garland ST PM Press, New York, 1978, 255.

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David E. Allen
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Dr. Allen has written or co-authored a number of articles on staining methods for electron microscopy and currently has research interests in the areas of electron microscopy and immunohistochemistry.

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At The Royal Melbourne Hospital, Dr. Dowling has pursued this interest in both the diagnostic and technical aspects of nephropathology.

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Chapter 1

COLLECTION AND INITIAL TREATMENT OF TISSUE

David E. Allen and John P. Dowling

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I. INTRODUCTION

Renal tissue is generally acquired by one of three methods, namely closed renal biopsy, open surgical biopsy, or at post-mortem. The biopsy procedures have been very adequately described elsewhere and will not be considered further. Open surgical biopsies if taken as a surgical wedge excision tend to provide mostly cortical tissue, and should deeper medullary tissue be required, a needle biopsy would appear to be more appropriate.

The amount of renal tissue provided for diagnostic purposes is critical. The diagnosis of diffuse renal diseases may require only a small amount of cortex and/or medulla to establish a diagnosis. Focal lesions, if minor, often require a very ample specimen for diagnosis. Thus, while one needle core containing cortex and medulla is often sufficient to provide specimens for light and electron microscopy and immunofluorescent examination, the acquisition of two adequate needle cores is preferable. In our experience the latter has not been associated with increased patient morbidity.

To ensure prompt and appropriate division of the tissue, a technician must attend the biopsy procedure. The use of a dissecting microscope can greatly assist both the clinician and the technician in the performance of a biopsy. Doubt often exists during a closed needle biopsy procedure as to whether renal tissue has been taken. The technician can more accurately determine what type of tissue (kidney, muscle, connective tissue, or fat) has been produced. With experience, naked-eye examination of the biopsy may suffice.

It is crucial that the technician identifies renal cortical and/or medullary tissue in the biopsy cores. Examination of the specimen should be carried out under moist conditions with the biopsy resting on saline-soaked cotton gauze in a Petri dish. It is also important to examine and divide the biopsy as quickly as possible to avoid delayed-fixation artifact for electron microscopy. The saline-soaked gauze will prevent the rapid dehydration that occurs with small-tissue biopsies. A strong indicent light used for examination of the specimen under the dissecting microscope will also dehydrate the specimen and should be avoided.

Glomeruli, particularly those that are congested, are just visible to the naked eye (Figure 1). The dissecting microscope aids identification of those that are not congested by showing them as small rounded discs that are seen particularly over the lateral margins of the biopsy. The cortical aspect of the biopsy often readily contrasts with the medulla which possesses parallel arrays of congested peritubular venules. However, the renal parenchyma obtained may not be congested in some instances making distinction

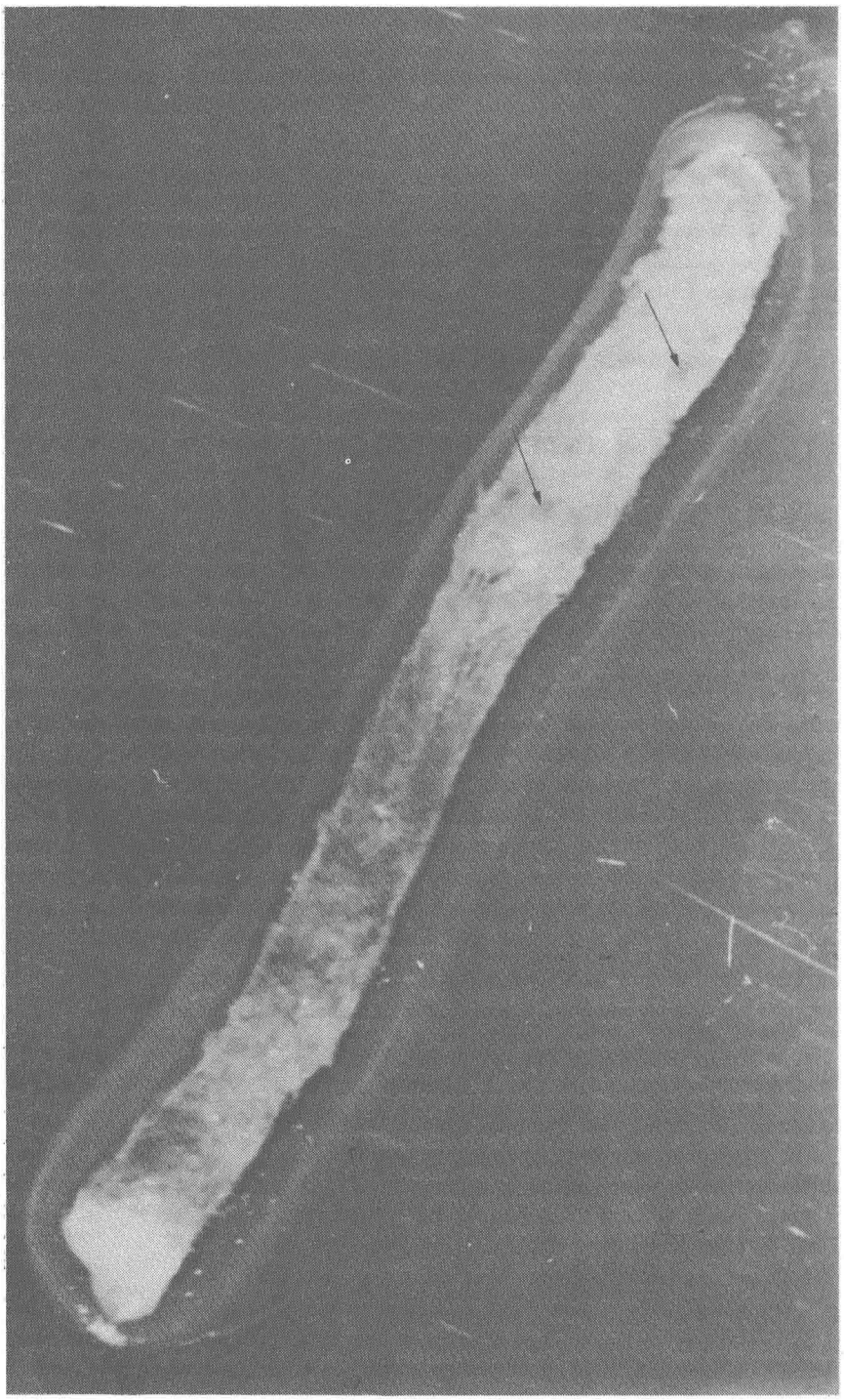

FIGURE 1. Renal needle core biopsy Glomeruli (arrowed) are just visible in the renal cortical tissue. (Original magnification × 10.)

between cortex and medulla very difficult; the presence of white fibrous capsular tissue may distinguish the cortical end of the biopsy in this circumstance.

If only one needle core comprising both cortex and medulla is available, it is dissected usually in one of two ways. From each end 1 mm^3 is taken and fixed for electron microscopy as soon as possible, ensuring that the small segments consist of renal parenchyma and not capsular or calyceal epithelial tissue. A further small section (1 to 2 mm in length) is taken from the cortical parenchyma for immunofluorescence. The remaining tissue consists of a variable amount of cortex and some medulla and is then fixed for light microscopy. Alternatively, the same sections may be taken for electron microscopy and the core is then divided longitudinally and their respective halves of the biopsies used for light microscopy and immunofluorescent studies, respectively. The division of the biopsy may be carried out within the gauze-containing Petri dish. Some workers section the needle core in a small longitudinal trough which has been fashioned from beeswax.

The single needle core obviously limits the amount of tissue available for each technique for examination. One further disadvantage seen more frequently with biopsies taken from shrunken kidneys is that the outer cortical piece selected for electron microscopy often contains glomeruli in advanced stages of sclerosis and is noncontributory. If sufficient time is available a second small section from the inner cortex will often overcome this problem. With two adequate needle cores containing cortex and medulla, one biopsy may be used in its entirety for light microscopy while the second may be divided for immunofluorescent and electron microscopic examination. Additional tissue may be available for glomerular culture. The piece selected for immunofluorescence may be dealt with in a number of ways. Rapid attachment to a microtome chuck with polyethylene glycol mounting medium (e.g., Miles OCT compound — see list of suppliers in Appendix) followed by immersion in liquid nitrogen is often employed. If available, a slurry of isopentane and nitrogen gives somewhat better morphological preservation. This is prepared by cautiously adding liquid nitrogen to about 50 mℓ of isopentane contained in a widemouthed insulated vessel. Care should be taken to avoid splashing the skin with slurry during mixing. Nitrogen is added, with stirring, until the isopentane begins to solidify. The slurry then has a temperature of $-160°C$. A less satisfactory alternative is to freeze the tissue by placing it in contact with a metal vessel or block chilled with a mixture of dry ice (solid carbon dioxide) dissolved in ethanol.

If the specimen must be transported or stored before sectioning it may be wrapped in aluminium foil and frozen by one of the above methods. The frozen biopsy is then placed in a (cold) tube and stored or transported in liquid nitrogen or dry ice. The latter is the more convenient and safer transport medium. If these materials are not available, it is essential that the tissue be stored at or below $-70°C$ as deterioration will occur at higher temperatures.

An alternative ammonium sulfate-ethylmaleimide transport medium was developed by Michel et al.[1] The authors claim that this medium will preserve the activity of tissue-fixed immunoglobulins for several days without the need for freezing. We have not tested this medium with renal biopsies, but the method appears promising and details are given below.

Because of autolysis, post-mortem renal tissue may present problems, particularly for electron microscopic and immunofluorescent studies. However, sections of high technical quality may be obtained for electron microscopy and basement membrane structures are sufficiently durable to provide adequate ultrastructural detail, often many hours after death. Cytoplasmic detail is often grossly distorted with poor preservation of subcellular organelles. It has recently been shown that protein deposits of immunoglobulin, com-

plement components, and fibrin maintain their antigenicity for up to 15 hr post-mortem.[2] Thus useful information may be obtained from immunofluorescent examination of tissues obtained at autopsy.

II. AMMONIUM SULFATE-ETHYLMALEIMIDE TRANSPORT MEDIUM[1]

Tissue for immunohistochemical studies may be placed in this medium for several days before further processing. This permits transport of the tissue through the post etc. After removal from this medium the tissue is washed three times in the buffer (without the ammonium sulfate — 10 min each wash) and then frozen by the usual method.

Buffer	2.5 mℓ of 1 *M* potassium citrate buffer pH 7.0 5 mℓ of 0.1 *M* magnesium sulfate 5 mℓ of 0.1 *M* N-ethyl maleimide 87.5 mℓ distilled water Adjust pH to 7.0 with 1 *M* potassium hydroxide
Working solution	To 100 mℓ of buffer add 55 g of ammonium sulfate

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Chapter 2

FIXATIVES AND FIXATION

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I. INTRODUCTION

Correct fixation of the renal biopsy is of great importance as improper preservation presents a severe handicap to the pathologist. Further, the effects of poor fixation cannot be rectified by subsequent processing steps, so particular care should be taken over this initial stage. Many factors influence fixation including delays before the tissue is placed

in the fixative fluid, its penetration rate, the time of fixation, and the subsequent storage conditions. These factors are important for both optical and electron microscopy though the superior resolving power of the latter instrument necessarily places greater demands on the quality of fixation. It is convenient to deal separately with fixation techniques for optical microscopy in this section; methods for electron microscopy are covered later. However, the following points apply to both disciplines.

Tissues should be fixed as promptly as possible after excision, even a few minutes delay will produce observable ultrastructural changes. The tissue blocks should be of such a thickness that the fixing fluid readily penetrates throughout the block in a short period of time. This time will vary with the fixative and also inversely with temperature. While low temperatures retard fixation, they also slow autolytic changes so that improved fixation may follow longer immersion in a fixative at a low temperature. Note though that freezing of tissue blocks near 0°C must be avoided as considerable ice-crystal artifact will be produced. In the case of multicomponent fixatives the penetration rates of the various components will differ and if the specimen blocks are large, deeper layers of the tissue may show adverse effects due to reaction with only parts of the fixation mixture. The volume of fixative employed should be 15 to 20 times that of the tissue to be fixed and the container used should be of such dimensions that the tissue is not bent or folded. After the appropriate time interval the fixed tissue should be rinsed in a suitable washing medium and processed as soon as possible.

II. OPTICAL MICROSCOPY

A. Routine Methods

Many workers use formalin solutions as fixatives for renal biopsies. Usually these are prepared from commercial formalin (37 to 40% formaldehyde) diluted with either saline or (preferably) with a buffer (pH approximately 7) until the formaldehyde concentration is of the order of 4%. These fixatives are readily available and they allow the majority of staining reactions to be carried out successfully. However, formaldehyde is a rather "soft" fixative for renal tissue and often results in defects such as fraying of the brush border of the epithelium. Better results are obtained with Duboscq-Brasil solution.

In this ethanol-acetic acid-formaldehyde-picric acid mixture,[1] the picric acid acts as a "hard" fixative, overcoming the disadvantages of formaldehyde used alone. The solution penetrates well and fixes small biopsies rapidly. Staining methods of the Mallory and Masson type are enhanced by this fixation method. We have used this fixative successfully for a number of years and the complete formula and method are given below. An advantage that Duboscq-Brasil solution shares with buffered formaldehyde is that sufficient tissue antigenicity is retained to permit demonstration of some antigens with immunoperoxidase methods following paraffin embedding.

If material originally preserved with formalin is to be used, we have found it advantageous to apply Duboscq-Brasil solution as a post-fixative. The post-fixation is carried out by treating the tissue with DuboscqBrasil solution for several hours prior to processing. Subsequent staining is considerably enhanced by this treatment.

A disadvantage of Duboscq-Brasil solution is that it may cause red cells to be lysed. If this is considered undesirable, it may be overcome by a short pre-fixation of 10 min in Corrosive Formol. However, subsequent removal of mercury precipitates will also be required (see below).

Meadows and Schoemaker[2] have recommended Corrosive Formol as a superior fixative for renal biopsies since fixation is very rapid (30 to 45 min) and excellent histological detail, particularly of nuclei, is achieved. The mercury content of this fixative also potentiates some of the special staining procedures applied to renal biopsies. However,

mercuric chloride is expensive, poisonous, and corrosive and its use results in the deposition of reduction products within the tissue. These must be removed with iodine and thiosulfate prior to staining. Even after this treatment, tissues fixed in mercury containing fluids are resistant to staining with the silver methenamine procedure. This difficulty can be overcome by treating deparaffinized sections with ammonia in alcohol overnight,[3] but silver staining times are still long and this may lead to excessive nonspecific silver deposition. Also, the silver impregnation tends to fade with time. Mercury-containing fixatives are also unsuitable for histochemical and immunohistochemical purposes. Full details of a Corrosive Formol fixative are given at the end of this section. Muehrcke and Pirani[4] recommended Helly's fluid as a fixative for renal biopsies. However, this mercury-containing fixative would seem to have no advantage over the above method.

Although mercury and chromate fixatives are not recommended for general histological purposes, Helly's fluid has been suggested as the fixative of choice when it is desired to demonstrate juxtaglomerular granules. The method used by Pitcock and Hartroft[5] in their application of Bowie's stain is given below. A possible alternative to the use of Helly's fluid in this method is the Corrosive Formol mixture suggested by Meadows and Schoemaker.

Chambers et al.[6] have suggested that glutaraldehyde be used as a fixative for routine histopathology and reported increased staining intensity with many stains. Agodoa et al.[7] have used this dialdehyde for fixing renal biopsies. It would be convenient to use the same fixative for both optical and electron microscopy, but after testing electron microscopy grade glutaraldehyde from several sources we cannot recommend the method. This dialdehyde penetrates tissue slowly and without long fixation times tissue preservation was poor and staining weak. It is notable that Chambers et al. used glutaraldehyde purified with activated charcoal and employed fixation times of 24 hr or more.

III. FIXATIVES FOR OPTICAL MICROSCOPY

A. Formol Saline Solution

37 to 40% formaldehyde solution (formalin)	100 mℓ
Sodium chloride	8.5 g
Tap or distilled water	900 mℓ

B. Neutral Buffered Formaldehyde Solution

37 to 40% formaldehyde solution	100 mℓ
Water	900 mℓ
Sodium dihydrogen phosphate.1 H_2O	4 g
Disodium hydrogen phosphate.anhydrous	6.5 g

Adequate fixation is achieved in 16 to 24 hr; longer times may make sectioning more difficult. Tissues should remain in the fixative until processing into paraffin or plastic as washing reverses the fixation effect. Solution 2 is preferred as unbuffered formalin mixtures may be acidic and cause deposits to form in the tissues. See also the Section VI on fixatives for electron nicroscopy where an alternative phosphate buffered formalin mixture is described.

C. Duboscq-Brasil Solution

80% ethanol	150 mℓ	
37 to 40% formaldehyde solution	60 mℓ	
Picric acid	1 g	Solution A
Glacial acetic acid	15 mℓ	Solution B

Solution A should be prepared freshly at least once monthly and the acetic acid added immediately before use (14 parts of A to 1 part B). This avoides chemical decomposition and results in better fixation. We cannot recommend storage of the complete fixative as is sometimes suggested.

The minimum fixation time for a renal biopsy core is 1 hr, 4 to 5 hr is optimal, and 18 hr should not be exceeded. After fixation the tissue is washed in several changes of 70% ethanol, preferably overnight. The tissue may remain in this solution until processing is possible. This washing step is particularly important if the tissue is being processed into plastic. *Note:* Solid picric acid is explosive when dry.

D. Corrosive Formol

Saturated mercuric chloride	90 mℓ
37 to 40% formaldehyde solution	10 mℓ

The mixture should be prepared shortly before use as it becomes cloudy on standing. Fixation time is 30 to 45 min. Longer times should be avoided as biopsies become brittle and difficult to section. After fixation, tissues should be washed and stored in 70% ethanol as for Duboscq-Brasil solution. Corrosive formol is poisonous and corrosive and must be used with care; containers must not be of metal or have metal caps. After sectioning, the deparaffinized sections are hydrated via the following iodine-thiosulfate sequence to remove the mercury:

Instead of the usual 70% alcohol step substitute 0.5% iodine in 70% alcohol; rinse in water	5 min
Rinse in 5% sodium thiosulfate; wash in running water	5 min

If both mercurial and nonmercurial fixed material are included in the preparations to be stained; the iodine-thiosulfate procedure is harmless to the latter.

A special washing procedure is required if silver impregnation methods are to be used. After hydrating as described above slides are treated with 2% ammonia (concentrated) in 96% alcohol overnight.[3] The silver methenamine procedure will then require 60 to 90 min at 60°C for impregnation.

E. Helly's Fluid for Demonstration of Juxtaglomerular Granules[5]

Distilled water	100 mℓ
Potassium dichromate	2.5 g
Sodium sulfate	1.0 g
Mercuric chloride	5.0 g
Add immediately before use 37 to 40% formaldehyde solution	5.0 mℓ

Thin slices of tissues are fixed for 48 hr and washed in running tap water for 24 hr before embedding in paraffin or plastic.

IV. ELECTRON MICROSCOPY

Fixatives for electron microscopy (EM) require more careful formulation than the mixtures used for optical microscopy in order to preserve ultrastructure and prevent extensive extraction. In general, an EM fixative consists of the fixing agent in a suitable buffer to maintain a contant pH value. Various salts may also be added to balance the ionic concentration and osmolarity of the fixative so that extraction is minimized and tissue components neither swell nor shrink during fixation.

EM fixatives are usually buffered to a pH of 7.4 to 7.6 though for many tissues there is little evidence that the pH is critical within the range of 6.5 to 8.0. It is difficult to quantify the most suitable values for the osmolarity of the fixative since little is known of the internal osmotic pressure of living tissue. A practical procedure is to select a fixative of known effectiveness in similar studies and to make adjustments if swelling or shrinkage of the tissue is observed. The fixatives selected for inclusion here have been shown to be satisfactory in this respect.

Osmium tetroxide is the best of the light microscope fixatives for preserving fine structure and it formed the basis of the earliest fixatives for EM. Sabatini et al.[8] later demonstrated the value of aldehydes as fixatives for EM and in particular the suitability of glutaraldehyde. This dialdehyde gives excellent preservation of a wide range of plant and animal tissues when followed by a second fixation in osmium tetroxide. This double fixation procedure had the advantage that specimens can be stored in buffer for considerable periods of time before further processing. A further advantage is that the fixative may be transported to and used in the biopsy room without undue risk. The toxicity and volatility of osmium tetroxide precludes its use in this manner. The convenience of this double fixation procedure coupled with the good ultrastructural preservation obtained has made it a standard procedure in most laboratories. Formaldehyde, freshly prepared from paraformaldehyde, compares favorably with glutaraldehyde and has the advantage of more rapid penetration into the specimen. Subsequently, mixtures of paraformaldehyde and glutaraldehyde[9] have proved to be superior to either aldehyde used alone for many specimens.

Osmium tetroxide is almost univerasally used as a secondary fixation step following primary aldehyde fixation. With fresh tissue the penetration rate of osmium tetroxide is very low and poor fixation in the center of tissue blocks can occur. The primary aldehyde fixation stabilizes the tissue sufficiently well to overcome these penetration artifacts and also the rate of penetration is increased over that of fresh tissue. A considerable amount of osmium is bound to the tissue following post-fixation. This osmium is bound in the form of "osmium blacks" which are amorphous electron opaque compounds of osmium. These reactions result in blackening of the tissue during post-fixation and also impart some electron opacity to the tissue. This staining effect is not ussually sufficient to enable examination of the tissue without further staining.

A number of buffers have been suggested for the control of pH in fixatives. Phosphate buffers are nontoxic and can produce excellent results. However, they do become slowly contaminated with microorganisms and they are prone to the formation of precipitates during fixation. This is presumably due to the formation of insoluble phosphates and may account for the sectioning difficulties and increased background granularity reported by some workers following the use of phosphate buffers. A number of formulas for the preparation of phosphate buffers are available but, providing they are of the same osmolarity, they give very similar results. Most of the formulas are based on that of Sorensen[22] and details for the preparation of this buffer are given below.

Cacodylate buffers were proposed for EM by Sabatini et al.[8] They are easy to prepare, stable indefinitely, and do not support the growth of microorganisms. In addition, the