Improved processing technique for renal biopsies for light microscopy

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To obtain the maximum value from the small amount of tissue available by renal biopsy, histopathological preparations of the highest quality are necessary. By simple modification of conventional methods of fixation and processing we have developed a scheme whereby thin sections (1-2μ) of excellent histological quality are available for microscopy within four and a half hours of taking the renal biopsy. Although the total processing time is very short, this scheme was not developed in an attempt to speed up diagnosis. Rather it was because conventional fixation, processing, and staining methods do not produce renal biopsy sections of a sufficiently high quality. However, there is no doubt that the short preparation time is of great value because it allows early diagnosis and treatment.

Fixation

Formaldehyde forms the basis of nearly all histological fixatives and 10% neutral or buffered formol saline is commonly employed for renal biopsies. Helly's fluid has also been recommended for renal biopsies with a fixation time of three hours followed by overnight washing (Muehrcke and Pirani, 1962).

CORROSIVE FORMOL

Corrosive formol (Lendrum, 1941), however, appears superior to both these methods of fixation in several different respects.

Advantages

1 Fixation is rapid, 30 to 45 minutes being adequate for renal biopsies.

2 Excellent histological detail with particularly good nuclear preservation is achieved.

3 Mercury potentiates many of the special staining techniques which are applied to renal biopsies. For example, much brighter and more contrasting tones are achieved with the PAS techniques after mercury fixation and stains for fibrin such as the picro Mallory and MSB methods (Lendrum, Fraser, Slidders, and Henderson, 1962) are more specific. The mercury does not distort the tissues or interfere with sectioning, so it is not necessary to wash the biopsy for long periods or pretreat it with iodine before processing. Mercury may be removed from sections before staining by the iodine/hypo sequence.

Disadvantages

Mercuric chloride is expensive, very poisonous, and corrodes metals. Also, as pointed out by Jones (1957), mercury interferes with the periodic acid silver methenamine technique which is widely employed in staining renal biopsies. These may be overcome as follows.

1 Because of its low solubility (5-7%) (Lillie, 1965) large amounts of mercuric chloride are not required.

2 Formalin is also poisonous but this has never been considered a reason for abandoning it as a routine fixative. It is the practice in this laboratory to colour the corrosive formol pink with a little acid fuchsin in order to facilitate identification (Lendrum, 1966).

3 Containers of corrosive formol should have a plastic or bakelite cap. No significant deterioration in microtome knives has been experienced after cutting material fixed in corrosive formol. (As suggested by Lendrum (1941), corrosive formol is used routinely for all necropsy histology.)

4 There is no doubt about the inhibition of silver staining but it may be satisfactorily overcome by leaving deparaffinized sections in 2% ammonia in 96% alcohol overnight and then carrying out the periodic acid silver methenamine technique.

Preparation of corrosive formol

Take saturated aqueous mercuric chloride 9 parts and concentrated formalin (40% formaldehyde) 1 part. Mercuric chloride ($HgCl_2$) is only slightly soluble in water 5-7% depending on temperature. Although formalin-mercury mixtures are fairly stable, they tend to become cloudy with the formation of precipitates after a few days. The mercuric chloride ($HgCl_2$) may be reduced to mercurous chloride or calomel (HgCl) or even metallic mercury (Lillie, 1965).

Small amounts of corrosive formol for renal biopsies are dispensed as and when they are required.

Fixation time with corrosive formol

Thirty to 45 minutes is an ample time for fixation. If the biopsy is received late in the afternoon, or at any time when it is inconvenient to begin immediate processing, it should be transferred directly to 70% alcohol. There it may remain without harm until processing can be carried out. Increased fixation times do not improve the histological details and prolonged fixation makes the biopsy brittle and difficult to section. If, for any reason the biopsy has to be sent by air and processing and embedding cannot be carried out, after 45 minutes' fixation in corrosive formol the biopsy should be transferred to ordinary 10% formol saline for transit. This is because 70% alcohol is inflammable.

Processing

The times given have been developed whilst processing over 600 renal biopsies over a period of several years.

Impregnation and Embedding

The special paraffin wax is prepared as follows:

Bioloid paraffin wax (melting point 53°-55°C)	1,000 g
Beeswax (white)	100 g
Colophonium	20 g

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The beeswax and colophonium are first melted together and then added to the molten Bioloid wax. After thoroughly stirring, the wax mixture is cast in cardboard boxes lined with waxed paper, and small quantities are melted and used as required. It is essential to have a hard compact embedding medium in order to obtain thin (1-2μ) sections. Even with the highest quality microtome and the sharpest knife it is impossible to cut very thin sections with histological paraffin wax. The formula noted above allows thin sections to be cut without lengthy impregnation techniques on the standard rotary microtomes available in any well equipped histopathology laboratory. The beeswax produces the required degree of hardness and both the beeswax and colophonium impart a cohesive quality to the wax.

Section Cutting

In cutting thin sections it is essential to cool the paraffin block and the microtome knife. This may be most simply achieved by placing both in the deep freeze compartment of a standard household refrigerator. This method, whilst not necessitating any special equipment, is rather inconvenient. In a recent paper Wagener and Musil (1967) described a simple cooling device which may be easily attached to the microtome knife. This piece of equipment is inexpensive and is easily manufactured by any competent surgical mechanic and it is extremely satisfactory. The blocks, however, are cooled in the deep freeze compartment of a standard refrigerator for 30 minutes just before sectioning.

Staining Techniques

Although most of the standard staining methods are applicable to renal biopsies, it is necessary to mention some of the problems which may be created by mercury fixation and the use of very thin sections.

HAEMATOXYLIN AND EOSIN

Any good staining sequence may be used. Lillie-Mayer's haematoxylin (Lillie, 1965) is very satisfactory. Also 1-2% eosin yellowish (water and alcohol soluble) in 2% calcium chloride is used in this laboratory (Conn and Holmes, 1928). The calcium chloride fixes the eosin and prevents it being washed out in water and the more dilute grades of alcohol during dehydration. The best differentiation of eosin is obtained by overstaining and then differentiating by washing in running water, remembering that differentiation will continue in the dilute grades of alcohol used in dehydration.

When thin sections are employed the standard staining times all have to be increased. For 2μ sections the conventional staining times should be doubled, and with 1μ sections increased three or four times.

PERIODIC ACID SCHIFF

Coleman's variant of de Tomasi's Schiff reagent has been found to be the most satisfactory (Coleman, 1939). Coleman's reagent employs twice the amount of potassium metabisulphite and half the amount of N hydrochloric acid. Over several years no difficulty has been experienced in consistently duplicating the manufacture of Schiff's reagent, provided the same technique is strictly adhered to. However, all brands of basic fuchsin do not produce a reagent that stains the renal basement membranes of thin sections brightly. The most satisfactory Schiff's reagent has been manufactured from a basic fuchsin (BDH batch number 2778900). To produce the best results it may be necessary to experiment with different brands of basic fuchsin before blaming indifferent staining on other facets of treatment. The practice of Pearse (1961) is followed of not using a sulphite rinse after Schiff's reagent but washing in running water for five to 10 minutes.

TECHNIQUE OF THE PERIODIC SCHIFF REACTION FOR RENAL BIOPSIES

1 Take sections to distilled water via the iodine and hypo sequence to remove mercury pigment.
2 Oxidise for 10 minutes in 0·5% aqueous periodic acid.
3 Wash in running water for five minutes.
4 Flood slide on the staining rack with Schiff's reagent for 15 to 20 minutes.
5 Wash in running water for five to 10 minutes.
6 Counterstain nuclei with haematoxylin for five to 15 minutes (staining time depends on the thinness of the sections).
7 Differentiate briefly in 0·5% acid alcohol, blue, and wash in running water.
8 Dehydrate in Cellosolve, followed by counterstaining in a saturated solution of Lissamine Flavine FFS (ICI) in Cellosolve for 10 to 20 seconds (Lendrum *et al*, 1962).
9 Rinse off excess dye with Cellosolve and immediately clear in xylol.
10 Mount in a synthetic mounting medium.

Periodic Acid Silver Methenamine

As already noted, the inhibitory effects of the mercury fixation may be overcome by treating the deparaffinized sections overnight in 2% ammonia in 96% alcohol. Jones' technique (Jones, 1957) is very satisfactory, but in our hands a shorter impregnation time in the silver methenamine solution has produced better results. Accordingly, impregnate in the silver solution for 60 to 90 minutes at 60°C instead of one and a half to three hours at 45 to 50°C as in the original method.

We know of no routine staining techniques which do not work satisfactorily after corrosive formol fixation, and believe that these modifications in technique will amply repay the slight departures from conventional practice (Figs. 1 and 2).

Routine Processing Scheme for Renal Biopsies

FIXATION

Thirty to 45 minutes in corrosive formol. The biopsy should be transferred to 70% alcohol if it is not convenient to process it immediately.

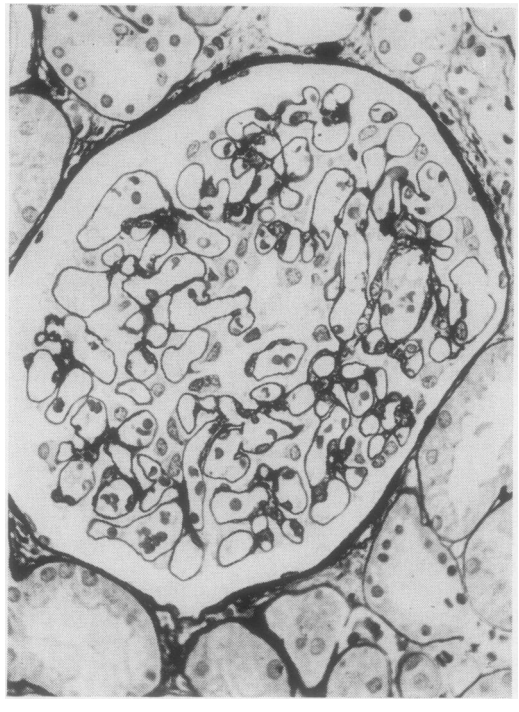

Fig. 1 *Normal glomerulus. Periodic acid-silver methenamine × 360. Even at this relatively low magnification basement membrane endothelial and epithelial cells are clearly visible.*

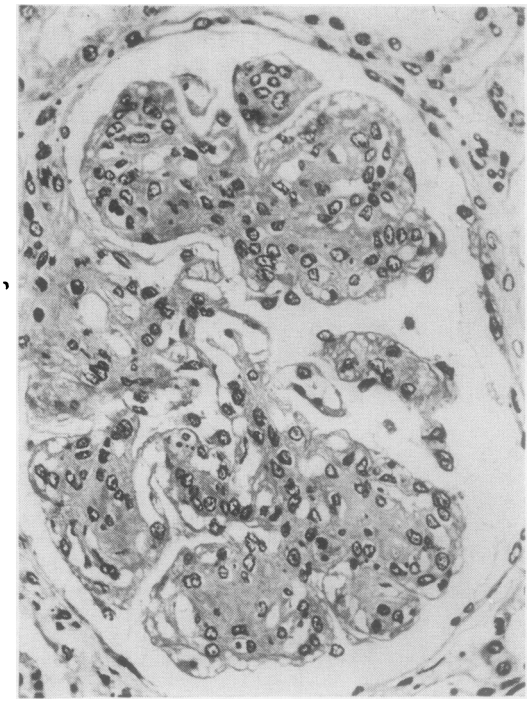

Fig. 2 *Lobular glomerulonephritis. Haematoxylin and eosin × 360.*

DEHYDRATION

Seventy per cent alcohol for seven to 10 minutes.
Ninety-six per cent alcohol for two changes of 10 minutes each.
Absolute alcohol for two changes of 10 minutes each.

CLEARING

Chloroform for two changes of 10 minutes each.

IMPREGNATION

Thirty to 40 minutes in the special paraffin wax.

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