Technical method

Experience with a three-hour electron microscopy biopsy service

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In the last decade there has been a significant increase in the use of electron microscopy in experimental and diagnostic pathology. This development is most clearly exemplified in the numerous ultrastructural investigations of human and animal renal disease. Ultrastructural evaluation and fluorescent microscopy are now considered to be of more than mere academic interest, and if this trend continues it is probable that in the field of kidney biopsy interpretation they will become almost mandatory. Information assisting in diagnosis obtained by means of electron microscopic analyses, in addition to permitting improved management of patients, has also provided valuable insights concerning the pathogenesis of a number of disease states involving the kidney. The next decade will doubtless witness further development of the field of submicroscopic pathology.

The requirement for rapid assessment of biopsy material has led to a number of attempts to shorten the processing techniques generally employed in transmission electron microscopy (Estes and Apicella, 1969; Bencosme and Tsutsumi, 1970; Hayat and Giaquinta, 1970). These rapid processing schedules have, however, not as yet gained universal acceptance. The reasons for this may be partly in the conservatism of electron microscopists, who have been reluctant to alter radically existing methods, which have been shown to give excellent structural preservation. Pathologists, however, have been prepared to accept somewhat different degrees of structural preservation in order to obtain the bonus of a rapid diagnostic test. Inconsistency in the results has been noted and this is undoubtedly a contributing factor in determining the widespread acceptance of rapid electron microscopic processing techniques. Johannessen (1973) has recently reported that the rapid methods have only given satisfactory results with normal and near normal kidney specimens and were unsatisfactory for severely scarred specimens. The reasons for the inconsistent results obtained in some laboratories are not known, but in many instances variations in the epoxide content of the embedding media, which are known to alter the cutting characteristics of the specimens, would seem to be an important factor.

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We have been using a rapid method that was adapted from the previously published reports, over a period of a year and have experienced no problems of reproducibility. The method has now been tested in other laboratories in Canada, the United Kingdom, and the USA with equal success. Interest expressed in the technique by numerous pathologists has stimulated us to make this report, in the hope that others will make use of the facility for rapid ultrastructural analysis that is now available.

Methods

The schedule was initially designed for a kidney biopsy service in which the ultrastructural results were available three hours after receiving the sample. As a result the electron microscopic analyses were on hand at the same time as the fluorescent microscopy and usually well in advance of the conventional histopathology slides. The degrees of preservation of ultrastructure and the ease with which 1 mm square grey sections were obtained by ultramicrotomy were the major criteria, by means of which the success or failure of the technique was assessed.

The details of the method are as follows:

1 Fix small pieces of tissue for 15 minutes, ie, not more than 1 mm^3 or strips with cross sections of not more than 0·5 mm^2. The primary fixative was Karnovsky's glutaraldehyde-paraformaldehyde fixative made up to half strength at pH 7·4 (Clark and Bretton, 1971).

2 Rinse in 0·1M cacodylate buffer pH 7·4; three changes over three minutes.

3 Postfixed in 2% s-collidine-buffered osmium tetroxide for 10 minutes.

4 Repeat step 2.

5 Block stained/fixed in saturated uranyl acetate pH 3·3 for 10 minutes.

6 Dehydrate in 70% ethanol: two changes over three minutes.

7 Dehydrate in 85% ethanol; two changes over three minutes.

8 Dehydrate in 95% ethanol; two changes over three minutes.

9 Dehydrate in absolute ethanol; three changes over eight minutes.

10 Dehydrate in absolute acetone; three changes over eight minutes.

11 Infiltrate with absolute acetone; Epon mixture (1:1) for 10 minutes.

In steps 1-11 the solutions were agitated by means of a rotator or with a magnetic stirrer/stirrer bar.

12 The specimens were removed from the infiltrate and blotted before transfer to Epon mixture and then rotated for 10 minutes. All of the steps 1-12 were at room temperature.

13 The specimens were removed from 12, blotted and embedded in flat rubber embedding moulds in a new Epon mixture.

14 The resin was polymerized at 100°C for one hour.

15 Blocks were cooled rapidly at −20°C for five minutes.

16 Blocks were sectioned with either glass or diamond knives and observed in the electron microscope (Philips EM 300), with or without lead citrate counterstaining.

The Epon embedding mixture was prepared in two batches during the schedule. The first batch was utilized for steps 11 and 12, whilst the second batch was used for the embedding. Any residual embedding medium was mixed with an equal volume of absolute acetone and kept in airtight bottles at −20°C for subsequent processing schedules. The various components of the Epon mixture were mixed thoroughly using a motor-driven teflon helix. The mixture was compounded either by weighing or dispensing in volume by means of disposable plastic syringes. The following ratio of epoxy resin to anhydrides proved to be suitable (see Hayat and Giaquinta, 1970):

Epon 812....13 ml (16 g)

Dodecenyl succinic anhydride (DDSA)....8 ml (8 g)

Nadic methyl anhydride (NMA)....7·5 ml (9·2 g)

Polymerization was accelerated by the addition of 0·42 ml (0·3 g) of DMP-30.

The ratio of anhydride chemical equivalent to epoxy chemical equivalent in the above mixture was 0·76. Cutting grey sections is known to be facilitated if the ratio is lowered below 0·7. However, a higher ratio is necessary when curing occurs at temperatures in excess of 95°C (Coulter, 1967). The epoxide equivalent (WPE) of the Epon 812 that we have used varied from 150 to 160 and the proportions of the various components in the embedding mixture were adjusted to retain the 0·76 A:E ratio by application of the formulae and tables previously published (Coulter, 1967; Burke and Geiselman, 1971). The WPE of the Epon 812 in the formula stated here was 156.

Results

Specimens embedded by this method sectioned easily. Large surface area sections were obtained thereby facilitating orientation in terms of histological structure. This proved to be important in kidney biopsy samples where the glomeruli are of particular interest. The thin sections often required no lead counterstaining and were remarkably stable in the electron beam. Excellent low magnification micrographs were obtained from such material (fig 1).

The elevated temperature used in the curing schedule produced no discernible distortion of fine structure when compared with control specimens processed by conventional methods requiring approximately 36 hours for completion. Similarly, shrinkage in the specimens was minimal and equivalent to that observed in control preparations.

In addition to its application to kidney samples the method has now been utilized for a wide range of pathology specimens. Of particular interest have been the results with dermatological biopsy specimens. There have often in the past been problems in attaining satisfactory infiltration and embedding in keratinized tissues, yet the rapid method permitted thin sections to be cut with ease from a range of skin biopsies (fig 2).

Comment

An unexpected bonus derived from rapid processing was the high standard of structural preservation obtained. At the outset we had expected that ultrastructural preservation might be inferior as a result of the vigorous processing. Undoubtedly, the reduction in the time of exposure to solvents brings about a proportionate reduction in extraction of material from the specimens. This feature has been noted in both animal and plant specimens processed by rapid schedules (Hayat and Giaquinta, 1970; Bain and Gove, 1971). Provided that the initial aldehyde fixation is rapid and penetrates well, such labile cytoplasmic structures as microtubules and cytoplasmic filaments are well preserved. There is an overall increase in cytoplasmic density compared with conventional preparations.

Subsequent to its trials for biopsy material, the method has been tested with research specimens as diverse as tissue culture cells (fig 3), bacteria, fungi, pellets of cell organelles, and a range of animal and human tumours. In all instances the

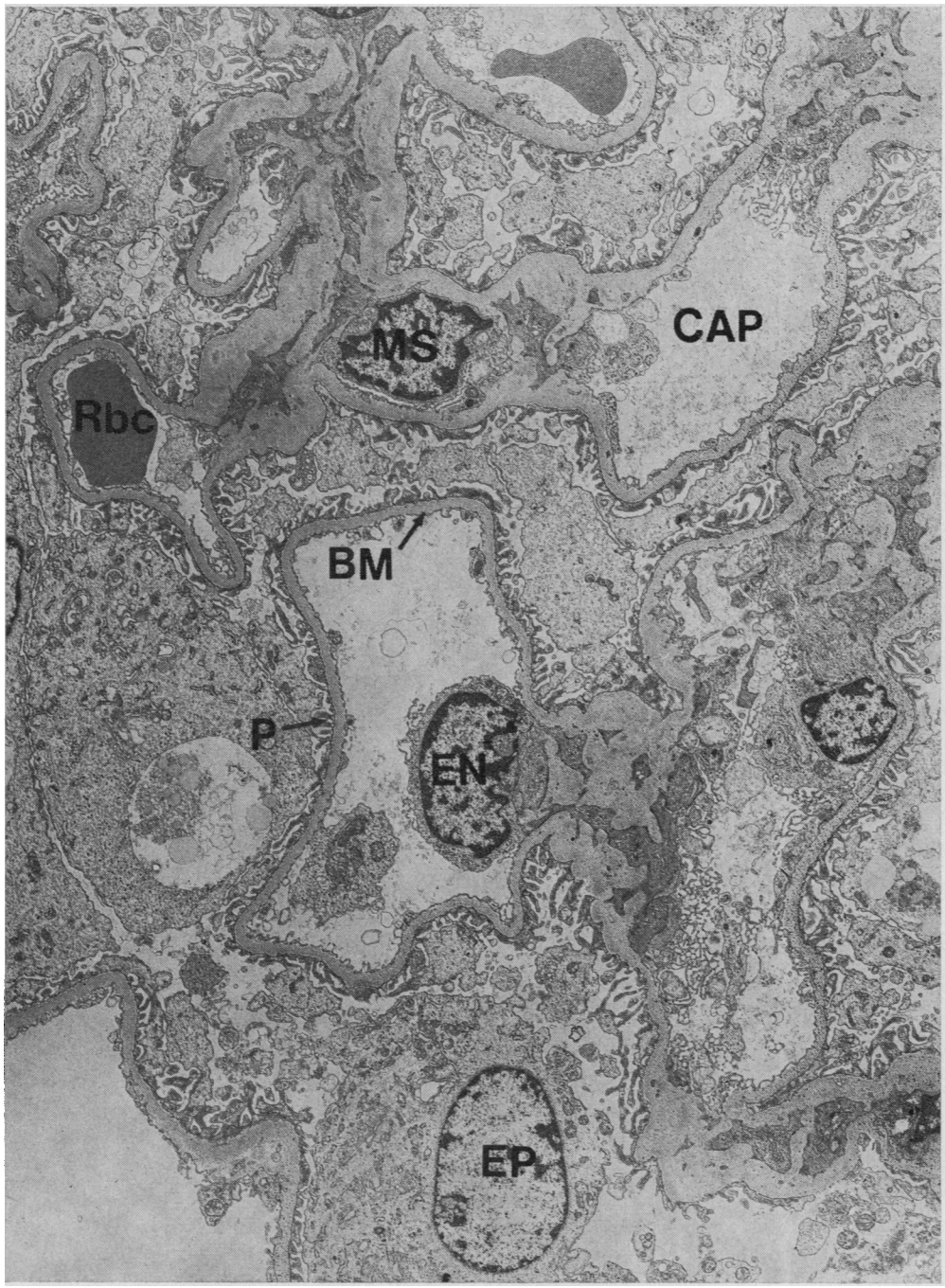

Fig 1 *Kidney biopsy specimen showing normal distribution of pedicels (P), podocytes (EP), and basement membrane (BM). The fenestrated endothelium is evident and endothelial cell nuclei (EN) are prominent. Erythrocytes (Rbc) are present in two of the capillary lumina. Mesangial cells (MS) lie between the capillary loops; × 4125.*

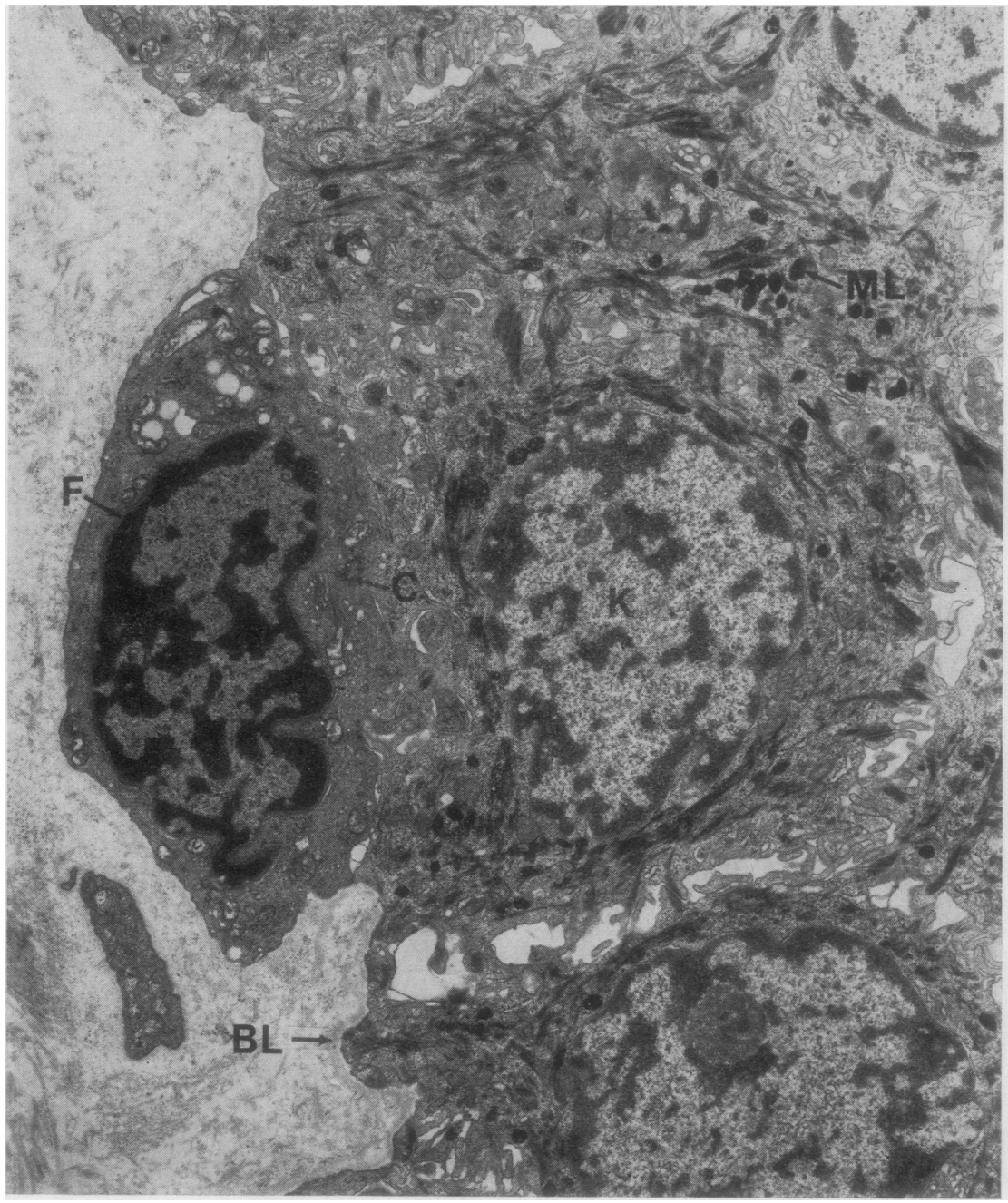

Fig 2 *Dermo-epidermal junction from a biopsy specimen of pemphigus vulgaris. A melanocyte with a prominent centriole (C) and a distinctive nuclear fibrous lamina (F) lies adjacent to the basal lamina (BL). Numerous melanin granules (ML) are scattered throughout the cytoplasm of the adjacent keratinocytes (K); × 14 500.*

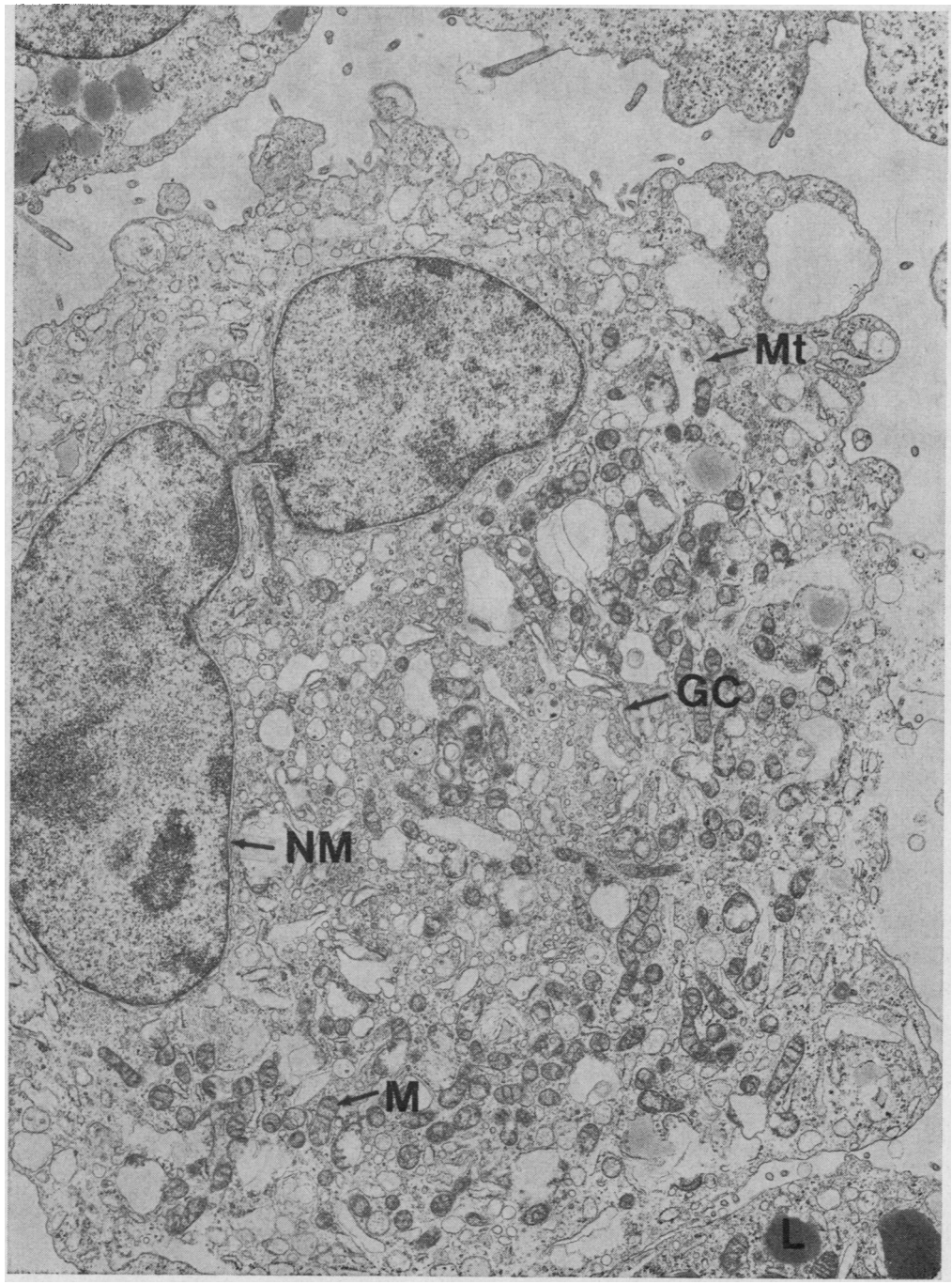

Fig 3 *Amelanotic malignant melanoma cells maintained in culture for six months. Numerous mitochondria (M) with dense matrices are present in the cytoplasm. An extensive Golgi complex (GC) is evident, as are numerous microtubules (Mt). The two membranes of the nuclear envelope (NM) are clearly visible and the pernuclear cisterna is uniform around the nucleus. Many of the cultured cells contain lipid droplets (L);* × *16 000.*

results have justified the adoption of the rapid schedule as the standard method for processing in our laboratory. Material under investigation by special techniques such as cytochemistry, autoradiography, peroxidase- and ferritin-labelled antibody tracing, has been shown to be unaffected by the compressed processing schedule. No evidence of artefactual redistribution of label has been observed in any of the above cases.

It seems probable that provided the inherent problems of adequate sampling are borne in mind, there is a great potential for utilizing electron microscopy in diagnostic pathology. Rapid processing schedules such as those outlined here, will fulfil an important function in this development, until such time as alternative methods like cryo-ultramicrotomy (Morgenstern, Neumann, and Werner, 1973) are more readily available.

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Letters to the Editor

Mycoplasma Antibodies in Sarcoidosis

In an earlier study (*J. clin. Path.*, 1972, **25**, 837-842) we reported successful attempts to isolate mycoplasmas from sarcoid tissues. Elevated antibody titres against an isolated mycoplasma were found to be quite common in sarcoidosis. Titres ≧ 16 were encountered in 14% of the patients with sarcoidosis, in 8% of the patients with other diseases, but only in 0·6% of the blood donors.

Most of the sera in that study were collected after Kveim testing the patients. In order to study the effect of Kveim suspension injected intracutaneously, antibody titres against two strains, 215-M and 336-M, both obtained from

Group	*No. of Cases*	*Before Kveim Testing*				*Five Weeks after Testing*
		Antibody Titre ≧ 16				*Rise in Titre* ≧ 2 *Dilutions*
		215-*M*		336-*M*		
		No.	*Highest Titre*	*No.*	*Highest Titre*	
Sarcoidosis						
Subacute sarcoidosis + erythema nodosum	15	1[1]	16	0		16-256[1]
Subacute sarcoidosis − erythema nodosum	27	2[2]	128	2	128	128-1024[2]
Sarcoidosis, duration unknown	28	3	256	2	64	
Chronic sarcoidosis	15	2	16	1	16	
Possible sarcoidosis	17	2	256	1	32	
Total	102	10		6		
Other diseases						
Non-sarcoid erythema nodosum	15	1	128	1	16	
Iritis, uveitis	10[3]	1	1024	0		8-256 (215-M)[3]
Chorioiditis	4	1	256	1[4]	16	16-512[4]
Non-definite panniculitis	6	2	128	1	16	
Tuberculosis	11	2	256	1	32	
Fever of unknown cause	3[5]	0		0		8-32[5]
Collagenoses	8[7]	1	32	1	16	8-64 (215-M)[6]
Cutaneous periarteritis nodosa	1	1	32	0		
Mediastinal cysts	1	1	64	1	16	
Malignancies	6	1	512	1	512	
Primary hypercholesterolaemia	1	1	512	1	64	
Others	24	0		0		
Total	90	12		8		

Table *Antibody titres against two mycoplasma strains (215-M and 336-M) obtained from sarcoid lymph nodes*