The combined use of anaerobic culture with added carbon dioxide and the rapid confirmation by plate bile solubility test enabled clearcut results to be obtained even in the mixed flora from respiratory specimens. The method thus saves the time required to obtain pure cultures.

The test is simple to perform, gives rapid results, compares well with the standard identification methods, and is therefore recommended as a routine screening procedure for the diagnostic laboratory.

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Plastic embedding of transbronchial biopsy specimens for light microscopy

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Transbronchial biopsies are increasingly used in the investigation of pulmonary disease, but the interpretation of conventional paraffin sections of such material can be difficult. The specimens are small and consist of several fragments, each 2 mm or less in diameter. Air spaces are often torn, distorted, or collapsed. Furthermore, because of the limitations of paraffin wax as an embedding medium, finer details are obscured by the thickness of the section and shrinkage artefact.

In recent years it has been shown that, if tissue is embedded in synthetic resins, shrinkage artefact is minimised and sections 1 μm or less in thickness are easily obtained (Green, 1970; Burns, 1973; Lee, 1977; Philpotts, 1977). It is thus possible to prepare sections that provide a simple and useful intermediate step between light and electron microscopy. Histological preparations of this type are now used routinely in many centres, particularly in the diagnosis of lymphoreticular and glomerular disease. However, they have not previously been applied to the study of pathological processes in the lung.

This paper deals with two methods for embedding transbronchial biopsy material, which we have been evaluating in our laboratory: the first uses hydroxyethyl methacrylate, and the second an epoxy resin first described by Spurr in 1969. Both these techniques are applicable to larger biopsies or postmortem material with appropriate minor modifications.

Material and methods

It must be emphasised that many of the reagents used in the two techniques described below are toxic, carcinogenic, explosive, or inflammable. They must be handled with extreme care, and a fume cupboard is mandatory. All the materials mentioned below are available from BDH Chemicals Ltd, Poole, or from TAAB Laboratories, Emmer Green, Reading.

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THE 2-HYDROXYETHYL METHACRYLATE TECHNIQUE

Monomer:	2-hydroxyethyl methacrylate stabilised with 1200 ppm hydroxyquinone	400 ml
	2-butoxyethanol	40 ml
	Benzoyl peroxide	7·5 g
Promoter:	Polyethylene glycol 400	8 ml
	N-N-dimethylaniline	1·0 ml
Embedding mixture:	Monomer	10 ml
	Promoter	0·25 ml

Processing
(1) 10% buffered formalin: 24 hours
(2) 70% alcohol: 1 hour
(3) Four changes of 100% alcohol: 1 hour each
(4) Three changes of monomer: ½ hour each
(5) Monomer: overnight.

Embedding
The specially shaped blocks (Fig. 1) consist of a wedge-shaped upper part containing the specimen and a rectangular lower part which is held in the microtome chuck. The lower part is 3·5 × 2·5 cm, and the total thickness is 1 cm. They are cast in silicone rubber moulds which are prepared using a male model of paraffin wax.

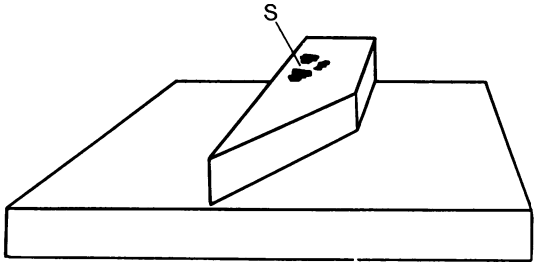

Fig. 1 *The shape of the blocks used in the methacrylate technique. The specimen(S) is embedded towards the rear of the upper part.*

The embedding mixture is made up immediately before use in a universal container, and the specimen is added and gently shaken. The embedding mixture and the specimen are then transferred to the mould, care being taken to position the specimen correctly. Heat is generated during polymerisation, so the mould should be partially immersed in a water bath, taking care not to wet the embedding medium. Sections may be cut within 1 hour, but it is better to leave the blocks overnight.

Section cutting
Sections are cut with a steel knife on a base sledge microtome set at 1 or 2 μm. The blocks are set in the chuck so that the apex of the wedge-shaped upper part leads. Sections are floated out on cold water, collected on albuminised slides, and dried at 60°C.

THE SPURR RESIN TECHNIQUE

Monomer:	Vinylcyclohexane dioxide	39 ml
	Diglycidyl ether of polypropylene glycol	23 ml
	Nonenyl succinic anhydride	104 ml
	Dimethylaminoethanol	2·6 ml

The monomer may be stored in glass bottles at −20°C. No separate promoter is used in this technique.

Processing
(1) 10% buffered formalin: 24 hours
(2) 50% alcohol: 15 minutes
(3) 70% alcohol: 15 minutes
(4) 95% alcohol: 15 minutes
(5) Three changes of 100% alcohol: 30 minutes each
(6) Three changes of propylene oxide: 30 minutes each
(7) Equal parts of propylene oxide and resin: 1 to 2 hours
(8) Spurr resin: 1 to 2 hours
(9) Spurr resin: overnight.

In our laboratory the processing is carried out in a Reichert EM tissue processor.

Embedding
The blocks are larger than those used for electron microscopy and measure 1 × 1 × 2 cm. They are cast in specially prepared silicone rubber coffin moulds. The specimen is held in the appropriate position, and resin is carefully run in with a Pasteur pipette. The mould is then heated to 60°C in an oven for 4-24 hours.

Section cutting
A standard microtome with a steel knife will not cut satisfactory sections of material embedded in Spurr resin. Therefore, in our laboratory, we use a glass knife in a Reichert OMU 3 Ultramicrotome set at 1 or 2 μm. The sections are transferred to a drop of water on a microscope slide. The slide is placed on a hotplate at 85°C to expand the section. Excess water is removed, and the slides are left on the hotplate so that the sections adhere securely.

STAINING
Haematoxylin and eosin (H and E), elastic van Gieson (EVG), and periodic acid-silver (PAAg) are

routinely used in each case. Periodic acid-Schiff (PAS) is used when fungal infections or alveolar proteinosis is suspected.

Before staining, Spurr resin must be removed from the sections by a 2-minute application of a solution containing 10 ml 74 OP alcohol, 10 ml propylene oxide, and 10 pellets sodium hydroxide. This is not necessary, or indeed possible, in the case of methacrylate-embedded material.

Haematoxylin and eosin

(1) Methacrylate sections:
Celestine blue: 10 minutes
Harris's haematoxylin: 10 minutes
Spurr sections:
Harris's haematoxylin: 5-10 minutes
(2) Differentiate in 1% acid alcohol
(3) Blue in tap water
(4) 1% eosin in 1% calcium chloride: 10 minutes
(5) Adjust colour balance in water.

Elastic van Gieson

(1) 0·5% potassium permanganate: 5 minutes
(2) Bleach in 1% oxalic acid: 2 minutes
(3) Rinse in 70% alcohol
(4) Methacrylate sections:
Miller's elastic stain: overnight
Spurr sections:
Miller's elastic stain: 4 hours
(5) Rinse in absolute alcohol
(6) Rinse in water
(7) Slidder's van Gieson counterstain: 2-3 minutes

Periodic acid-silver (modified after Gomori (1952))

Stock solution:	3% hexamine	200 ml
	5% silver nitrate	10 ml
	store at 4°C	
Staining solution:	Stock solution	20 ml
	Distilled water	20 ml
	5% borax solution	1·75 ml

(1) 1% periodic acid: 45 minutes
(2) Rinse in distilled water
(3) Staining solution preheated to 60°C: 1-2 hours
This is carried out in a Coplin jar angled at 60° to prevent precipitate falling on the section. Over-staining should be avoided by periodically checking the section
(4) Wash in distilled water
(5) Tone in 0·2% gold chloride
(6) Counterstain with dilute aqueous light green.

Periodic acid-Schiff

(1) 1% periodic acid: 30 minutes
(2) Wash in water
(3) Double strength cold Schiff reagent (Lillie and Fullmer, 1976): 6 hours
(4) Wash in water overnight
(5) Counterstain with Mayer's haematoxylin: 4 minutes.

Clearing and mounting

All sections are cleared in xylol and mounted in DPX.

Results and discussion

Sections from both methacrylate and Spurr-embedded material are vastly superior to those obtained from conventional paraffin blocks (Fig. 2), so that the light microscope can be used to the limit of its resolving power. The preservation of cytological detail is such that macrophages, alveolar lining cells, and endothelial cells may be differentiated with ease (Fig. 3). At lower magnifications, the walls of small air spaces are well defined, and even when the specimen has been partially crushed a useful diagnostic opinion can be given.

Staining reactions are identical with those in paraffin sections. However, with EVG, although the elastic tissue stains in the conventional way, the counterstain is pale. This causes no difficulty when the sections are examined microscopically, but photographs of such material are disappointing. When material has been embedded in Spurr resin and stained with haematoxylin and eosin, the elastic tissue is brilliantly eosinophilic. The PAS reaction is identical with that seen in paraffin sections, except that intracellar PAS-positive particles are much more sharply defined.

The most interesting result of our investigation is the clarity with which alveolar capillaries are demonstrated by the PAAg stain. These minute vessels are indistinct in paraffin-embedded material but are strikingly obvious in plastic sections. Furthermore, in cases of cryptogenic fibrosing alveolitis, they undergo aneurysmal dilatation and often appear to lie each side of the thickened alveolar interstitium (Fig. 4). This dilatation, which may be missed in paraffin sections, occurs in parts of the lung not affected by honeycomb change. Its significance is not yet apparent, but it is highly characteristic and a useful diagnostic feature in biopsy material.

When the two methods are compared, methacrylate produces superior results. With the Spurr technique the maximum size of sections obtainable is 0·5 × 0·5 cm, so that larger biopsies cannot be accommodated. This is due to the small travel of the ultramicrotome and the limited width of the glass knife. On the other hand, with methacrylate, although the width of the blocks is restricted due to the hardness of the material, their length can be as

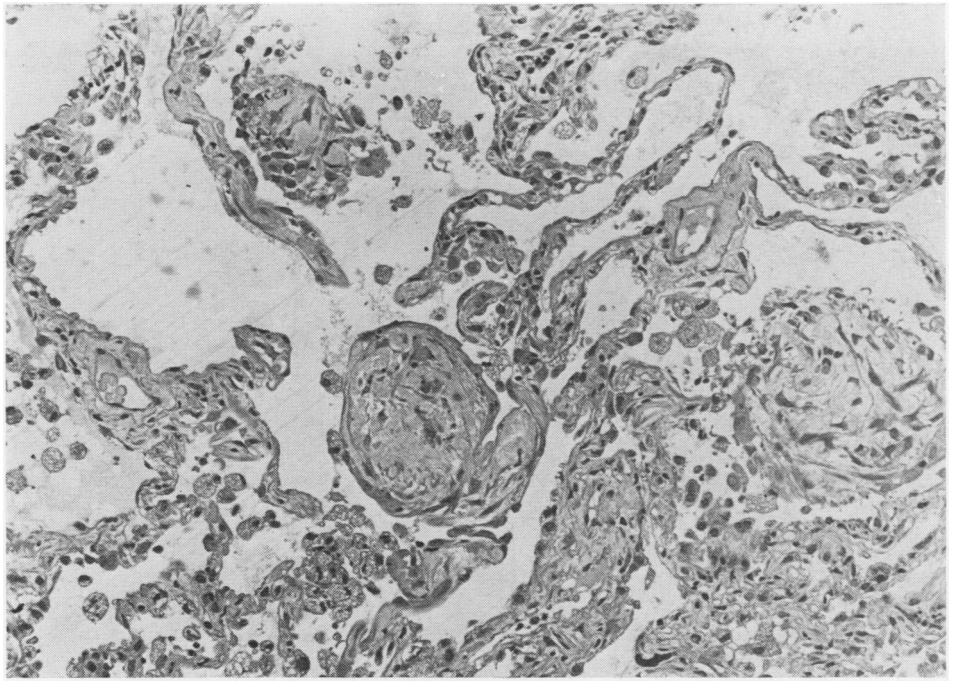

Fig. 2 *A transbronchial biopsy from a case of organising pneumonia. Alveoli are partly collapsed but easily recognisable. The embedding medium has taken up the stain to a slight extent and appears as diagonal background streaks in the upper left side of the picture. 2μm methacrylate section. Haematoxylin and eosin × 112.*

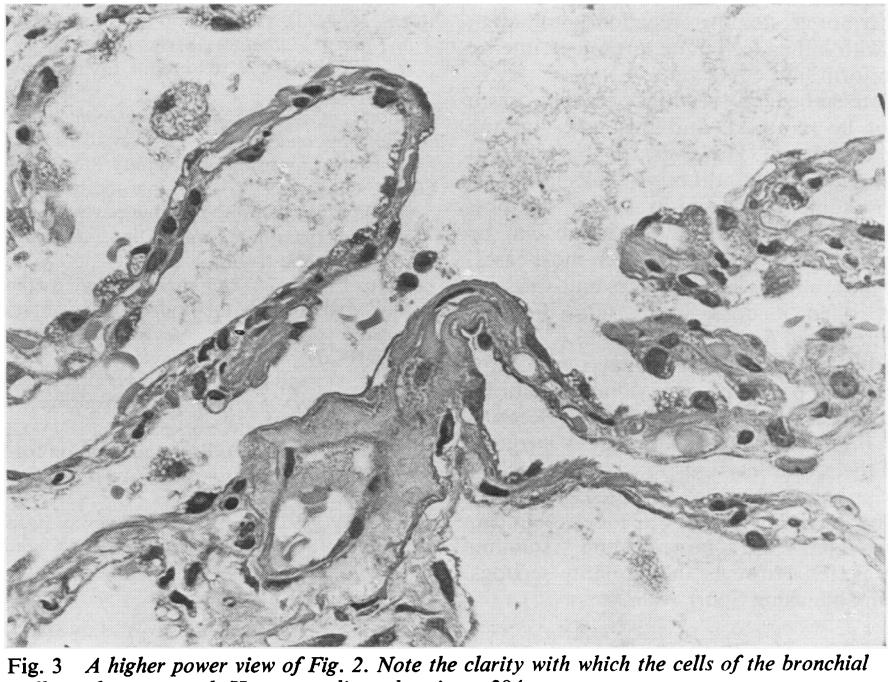

Fig. 3 *A higher power view of Fig. 2. Note the clarity with which the cells of the bronchial wall are demonstrated. Haematoxylin and eosin × 284.*

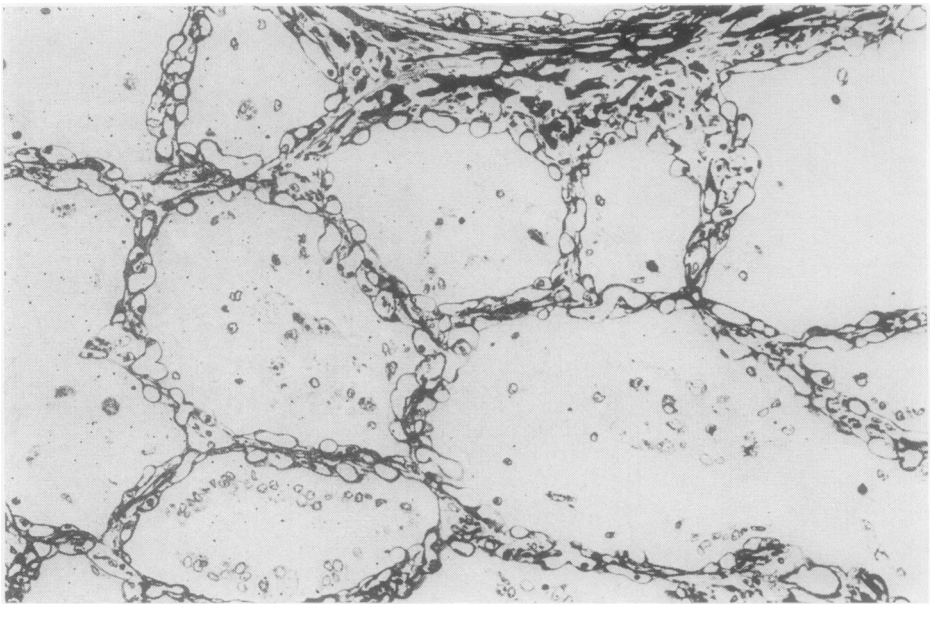

Fig. 4 *A transbronchial biopsy from a case of fibrosing alveolitis. There is no honeycomb change, but the characteristic aneurysmal dilatation of the alveolar capillaries is well shown. Periodic acid-silver* × *112.*

much as 2 centimetres. An added advantage is that a standard base sledge microtome with a steel knife is used. Furthermore, staining reactions, although satisfactory with either technique, are more intense and more uniform in methacrylate.

Methacrylate-embedded sections contain resin which cannot be removed, and this takes up the stain to a certain extent. However, this background staining is extremely faint, although a precipitate of silver salts is sometimes seen in PAAg sections. With Spurr resin the embedding medium can be completely removed from the sections in most cases, but it sometimes remains in air spaces and vascular lumens, and when it does so it stains heavily, obscuring histological details.

In conclusion, it may be said that plastic embedding techniques open up a new dimension in the investigation and diagnosis of lung disease. Sections are of such high quality that we would strongly recommend their routine use for transbronchial biopsies. In our hands, the more satisfactory method is that using methacrylate. The tissue blocks that can be accommodated are larger, and staining is marginally better. However, high-quality sections are also obtainable using Spurr resin.

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