

## Technical methods

### A new procedure for the identification of *Pneumocystis carinii* cysts in tissue sections and smears<sup>1</sup>

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Pneumonia associated with *Pneumocystis carinii*, once thought a rarity, has been reported with increasing frequency during the past decade. Failure for the earlier recognition of this pathogenic agent during post-mortem examination of tissues may partly be attributed to the fact that unless special stains are applied the organism can be altogether missed in histological preparations.

The purpose of this paper is to report a simple technique which has proved reliable in demonstrating the cysts of *P. carinii* in sections of lungs from eight patients. The stain was also successfully applied to touch preparations from the lungs of three of the above cases.

Mowry (1958) described a histochemical technique whereby free hydroxyl groups are sulphated by using a mixture of concentrated sulphuric acid and ether and subsequently revealed by the use of metachromatic dyes. This method was successfully used in demonstrating fungi by Kelly, Morgan, and Saini (1962).

In our earlier attempts, Mowry's technique was found to stain the cysts adequately but the background intensity interfered with the rapid localization of the organisms. A slightly modified technique was therefore devised and found to be satisfactory on several trials.

#### SOLUTIONS

##### 1 SULPHATION REAGENT

Wet diethyl ether.....25 ml.  
Concentrated sulphuric acid..25 ml.

About 35 ml. of reagent grade diethyl ether is vigorously shaken with 5 to 10 ml. of distilled water in a separating funnel and the two layers are allowed to separate until both are clear. The lower aqueous phase is then discarded and a few millilitres of the upper ether phase is run off to wash away any hanging drops of water from the stem of the funnel and 25 ml. of the ether, now saturated with water, is transferred to an Erlenmeyer flask immersed in an ice bath. Then 25 ml. of concentrated sulphuric acid is added slowly with

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constant agitation of the flask. It is essential that the reagent solution is well mixed. All glassware must be dry so that no more than the water saturating the ether is present in the final solution. The sulphuric acid-ether solution is transferred from the flask to a Coplin jar, the cover of which is sealed with grease. As a precautionary measure, the solution is stirred with a glass rod before use to ensure the homogeneous distribution of sulphuric acid in ether as the former may tend to settle at the bottom of the jar.

##### 2 TOLUIDINE BLUE O SOLUTION

Toluidine blue O.....8 mg.  
Distilled water.....15 ml.  
Hydrochloric acid.....0.5 ml.  
Absolute ethanol.....35 ml.

The dye is dissolved first in water then the acid is added. Ethanol is poured in last to make a final concentration of 0.016% toluidine blue O. Proper allowance should be made if the percentage dye content of the brand of toluidine blue O used differs from 73%<sup>3</sup>. For staining smears, 75 mg. of toluidine blue O is used in order to make a final concentration of 0.15%.

##### 3 0.25% AQUEOUS METANIL YELLOW

#### FIXATIVE

Either 10% formalin or Zenker-formol can be used. Sections are cut 5 to 7  $\mu$  thick.

#### STAINING PROCEDURE FOR TISSUE SECTIONS

- 1 Deparaffinize sections in xylene. If fixed in 10% formalin, proceed to step 3.
- 2 If fixed in Zenker-formol bring sections to water, remove mercury precipitates through iodine and hypo solutions, dehydrate and clear in xylene.
- 3 Wash off xylene by several dips in pure ether.
- 4 Place sections in sulphuric acid-ether reagent for five minutes.
- 5 Wash slides in running tap water.
- 6 Stain in 0.016% toluidine blue O solution for three minutes.
- 7 Wash off excess dye in running water.
- 8 Counterstain with 0.25% metanil yellow for two minutes.
- 9 Dehydrate through three changes of isopropyl alcohol, clear in xylene, and mount.

**RESULTS** *Pneumocystis carinii* cysts, mucin, and sometimes cartilage, appear violet or purple. Other tissue elements stain yellow or different shades of light to dark green. The cysts are demonstrated as dark bodies in Figure 1.

<sup>3</sup>In this study toluidine blue O was purchased from National Aniline Division, Allied Chemical and Dye Corporation, 40 Rector Street, New York 6, New York.

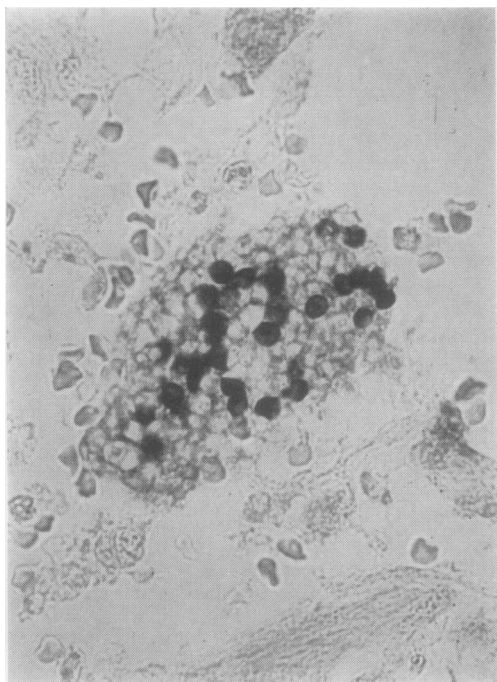


FIG. 1. *P. carinii* cysts appear as dark round bodies in the midst of the honeycomb alveolar exudate. Stain as described in text.  $\times 800$ .

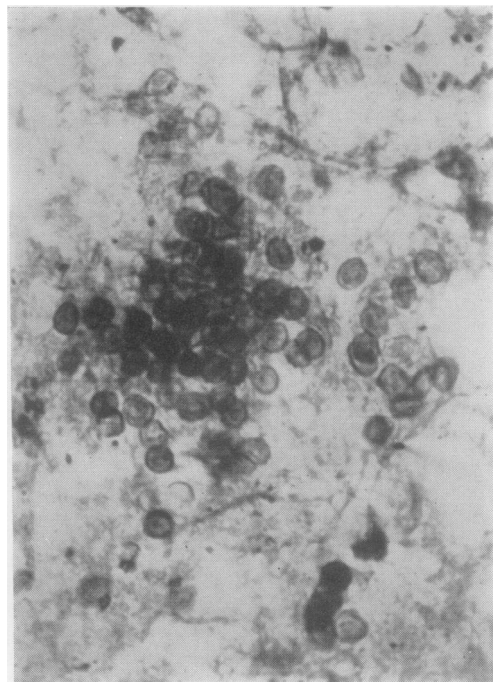


FIG. 2. Cysts from a touch preparation of lung tissue at necropsy. The organisms are clumped together and an internal structure can be seen within some cysts.  $\times 800$ .

#### STAINING PROCEDURE FOR SMEARS

- 1 Air-dry the smears.
- 2 Place slides in sulphuric acid-ether reagent for five minutes.
- 3 Wash slides in running tap water.
- 4 Stain in 0.15% toluidine blue O solution for three minutes.
- 5 Dehydrate through three changes of isopropyl alcohol, clear in xylene, and mount.

**RESULTS** *P. carinii* cysts are purple; tissue fragments and debris stain blue. The organisms are seen clumped as dark bodies in Figure 2.

#### COMMENTS

By using ether saturated at room temperature with water, a decrease in the intensity of background staining was noted, thus accentuating the contrast. In addition, the cysts appeared to be better preserved.

The honeycombed material in the alveolar spaces of the lungs in pneumocystic pneumonia tends to stain

occasionally blue or dark green but this does not interfere with the identification of the dark purple cysts.

Staining and differentiation have furthermore been incorporated into a single step by making up the staining solution in acid and alcohol, thus eliminating possible variations in the demonstration of the cysts. In dehydrating the sections before clearing and mounting, ethanol has been found to decrease the staining intensity of both the background and organisms. For this reason isopropanol has been substituted.

This stain is simple and rapid and has consistently given satisfactory results. It should be of value in detecting *P. carinii* in the sputum and thus help in establishing a definite diagnosis in suspected cases during life.

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#### REFERENCES

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