MODIFICATION OF FLUORESCENT-ANTIBODY PROCEDURES EMPLOYING CRYSTALLINE TETRAMETHYLRHODAMINE ISOTHIOCYANATE

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Fluorescent antibody applications with fluors having a color other than fluorescein have been suggested by several investigators employing dyes fluorescing in the red-orange range of the spectrum (Chadwick, McEntegart, and Nairn, Lancet **1**:412, 1958; Borek and Silverstein, Arch. Biochem. Biophys. **87**:293, 1960; Hiramoto, Engel, and Pressman, Proc. Soc. Exptl. Biol. Med. **97**:611, 1958; Scott, Immunology **3**:226, 1960; Loh and Riggs, J. Exptl. Med. **114**:149, 1961).

The availability of a chromatographically pure red isomer of tetramethylrhodamine isothiocyanate (TMRITC; Felton and McMillion, Anal. Biochem. **2**:178, 1961) has stimulated the present study. Certain aspects of previously published labeling and staining procedures were re-evaluated to determine optimal conditions for employing this compound.

The globulin fraction of horse antirabies serum was labeled at various dye-to-protein ratios ranging from 1:10 (0.10 mg dye per mg protein) to 1:80 (0.0125 mg dye per mg protein). The globulin solutions were buffered with 0.5 M carbonate-bicarbonate to pH 9.5. TMRITC was added as a dry powder. Conjugation proceeded overnight in the cold, and was followed by dialysis against repeated changes of 0.01 M phosphate-buffered saline (pH 7.4) until free rhodamine no longer appeared in the dialyzate. Conjugates were then absorbed as necessary on animal-tissue powder and tested.

Rabid mouse brain impression smears were stained directly with several dilutions of the conjugates for time intervals from 30 min to 8 hr. After washing in phosphate-buffered saline, the slides were mounted and examined using a Reichert monocular microscope with ultraviolet-transmitting filter 8079 and ultraviolet-excluding eyepiece filter 8082. Staining was subjectively graded from minimal 1+ fluorescence to maximal 4+ fluorescence. Representative results obtained with antirabies conjugate labeled at 1:40, diluted 1:5, are summarized in Table 1.

TABLE 1. *Relationship of staining time to staining intensity*

Staining time	Staining intensity	
	Specific	Nonspecific
30 min	±–1+	0
2 hr	2–3+	0
4 hr	4+	0
7 hr	4+	1+

TABLE 2. *Relationship of staining intensity to conjugation at various dye-protein ratios*

Dye-protein ratio	Staining intensity	
	Specific	Nonspecific
1:10	?	2–3+
1:20	4+	±–1
1:40	4+	0
1:80	1+	0

Another series of results, using several conjugates for a 4-hr staining period, is summarized in Table 2.

Antihuman globulin (from goat) was labeled in the same manner described above. These conjugates were evaluated using the indirect staining technique, the fluorescent treponemal antibody test (Deacon, Freeman, and Harris, Proc. Soc. Exptl. Biol. Med. **103**:827, 1960). The recommended staining time for this test when employing fluorescein-labeled conjugates is 30 min. When evaluating the rhodamine-labeled conjugates, staining time was increased to 40 min, 1, 1.5, 2, and 4 hr. Minimal brightness resulted after staining periods of 40 min, 1, and 1.5 hr. After 2 and 4 hr, intensity increased to the maximal 4+. Conjugates labeled at dye-to-protein ratios of 1:20 or 1:40 provided the most satisfactory staining. The antigen in this test system is relatively free from background tissue, therefore nonspecific staining was not a problem.

Optimal results were obtained with conjugates

labeled with TMRITC at a dye-to-protein ratio of 1:40 in a staining period of 4 hr in two different fluorescent-antibody systems. The optimal labeling ratio and staining time for rhodamine-labeled antibody may vary with other immunological systems.

NEW PRESS FOR DISRUPTION OF MICROORGANISMS

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Among a wide variety of methods we have tested for the preparation of cell-free extracts from microorganisms we have had best results, particularly with yeasts, using the Hughes Press (Hughes, Brit. J. Exptl. Pathol. **32**:97, 1951). This apparatus, however, suffers from two major disadvantages: (i) the stainless steel blocks are often difficult to separate after use, and (ii) recovery of the crushed material is tedious and entails considerable loss. The press described here overcomes these disadvantages. The blocks are easily separated at any time during the process, and the crushed, frozen, cell mass is collected almost quantitatively in a plastic centrifuge tube which can readily be removed.

Construction. The cylinder block, bottom plate, and receiving tube block are machined from a cylindrical bar of 303M stainless steel as described in Fig. 1. In addition to the centrally drilled hole, 2 cm in diameter, four equally spaced holes are drilled in one end of the cylinder block and tapped to receive the bolts which attach the bottom plate. The bottom plate has a 1-mm hole drilled centrally, holes for the passage of the four attaching bolts, and two holes drilled partially through to receive aligning pins that are inserted in the top of the receiving-tube block. The receiving-tube block shown has been drilled to accept a 12-ml plastic centrifuge tube. The dotted lines on either side of this hole (Fig. 1) and crossing the bottom of the block represent narrow grooves which allow passage of air around the tube when the press is in operation. The piston is machined from 440C stainless steel, hardened and ground to a diameter 0.05 mm less than the diameter of the cylinder. A shallow mark is cut near the top of the piston to indicate its bottom position in the cylinder. In earlier attempts, pistons made of the same material as the cylinder block were found to be unsuitable because of compression and binding to the cylinder wall under pressure. In an effort to avoid this binding and to assist in aligning the piston in the cylinder, these pistons were tapered slightly from the mid-point toward the top (exaggerated in the diagram) and the top of the piston was rounded. Hardened stainless-steel pistons were subsequently made in this way, although neither modification appears to be necessary, since straight, hardened, stainless-steel pistons with flat tops have been used successfully. While this manuscript was in preparation, details of a similar apparatus were reported (Edebo, Acta Pathol. Microbiol. Scand. **52**:300, 1961). The press described here, however, appears to have certain advantages in simplicity of construction and operation.

Operation. The cylinder block (with bottom plate attached), receiving-tube block and piston are packed in crushed dry ice for a period of at least 20 to 30 min before use. The receiving tube is inserted, the two blocks are assembled, and the suspension of cells to be crushed is run into the cylinder and allowed to freeze. The piston is inserted, and pressure up to about 8,000 psi is applied with a hydraulic laboratory press. When the plunger is fully depressed, the apparatus is removed from the hydraulic press, the cylinder block is lifted off, and the receiving tube removed. Depression of the piston often occurs in short "jerks" and requires from 2 to 15 min. The only difficulty thus far encountered is that with samples of more than 3 to 5 ml occasionally the material freezes around the top of the receiving tube. As more material is extruded through the hole in the bottom plate, the frozen block is forced toward the bottom of the receiving tube, creating sufficient air pressure to break the tube. This has been avoided by using a receiving-tube block that will accept a tube of larger diameter.