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Fluorescent Staining of Elastic Tissue with Rhodamine B and Related Xanthene Dyes*

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Summary. Frozen sections, cut at 8 μ from various fresh tissues, were dried for 5 minutes over sulfuric acid, and then stained for 4 minutes in a 0.1% aqueous solution of Rhodamine B (Chroma) at pH 8.0. After soaking twice in butanol for 2 minutes each, the sections were kept on a warmer at 60° C for 2 minutes and then mounted in oil of cedar. Under ultraviolet microscopy, elastic fibers selectively stained yellow-orange against a pale blue background. No autofluorescence of elastic fibers was observed in guinea pig, rat, hamster or rabbit tissue in contrast to the autofluorescence of elastic fibers typically seen in human tissue.

Similar fluorescent staining of the elastic tissue could be achieved by using a number of related xanthene dyes including pyronine, eosin B, acridine orange, pholoxin, phloxin B. More distantly related auranine O and thiazol yellow G were likewise used with success as fluorochromes for elastic fibers.

Introduction

Although Lansing (1951) and Pearse (1968) have listed numberous conventional stains for elastic tissue, the literature contains only scattered references to fluorescent staining of elastic tissue. This paucity of reports may reflect the fact that in man elastic tissue is autofluorescent (Vassar and Colling, 1959; Montes and Duran, 1963; Jackson, Puchtler and Sweat, 1968). The nature of such autofluorescence remains obscure, although Ayer (1964) in his review cites four fluorescent compounds which have been isolated from elastic tissue, and Pearse (1968) considers that the autofluorescence could be due to an extrinsic lipid. The problem becomes increasingly complex when one finds that elastic tissue in many species shows no autofluorescence. This could indicate that elastic tissue varies in chemical composition from species to species (Partridge, 1962) or that the fluorescence in man is due to extraneous adsorbed compounds.

Selective fluorescent staining of elastic tissue has been described in four reports. Initially Jarret, Bligh and Hardy (1956) induced marked selective fluorescence of human elastic fibers in frozen sections with acridine orange (1/15,000 aqueous, 5 minutes at 3° C). Vassar and Colling (1959) stated that the autofluorescence of human elastic fibers could be appreciably enhanced by staining with an alcoholic solution of acriflavine. They stained sections from routine formaldehyde fixed tissue. Tappero and Baima-Bollone (1965) stained elastic

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tissue in formaldehyde-fixed lung by a 10-minute incubation in 0.01% brilliant dianyl green (aqueous, pH 9). More recently Roudier and Degeyne (1967) were able to stain elastic tissue in rats with a number of fluorescent stains. They used both frozen sections and regular formaldehyde-fixed tissue. The following dyes proved useful: acridine orange, eosin Y, thioflavine, acridine red, clayton yellow, acriflavine, thiazine red, geranine G, lissamine yellow. Acriflavine (aqueous 1/5000, 5 minutes) followed by differentiation in ammonia solution was recommended as the best technique.

In view of the increasing popularity of fluorescent microscopy, the present study was undertaken to develop a simple useful fluorescent stain for elastic tissue in animals.

Methods and Results

One hundred and fifty-six different dyes were made up in a 1% aqueous solution or suspension and injected intradermally in guinea pigs. After one hour and fifteen minutes skin biopsies were taken, frozen sections prepared and viewed under regular light and fluorescence microscopy (high pressure mercury lamp, HBO, 200 W UG 1 filter, eyepiece filter, Leitz UV absorbing). Only the fluorescent dyes produced in vivo staining of elastic tissue fibers. None of the dyes produced staining of elastic tissue as viewed with visible, whereas seven of the 56 fluorchromes studied led to selective yellowish-orange fluorescence of elastic fibers. These dyes were Auramine O (National Aniline Division Allied Chemical & Dye Corp), Clayton yellow (Harleco), Eosin bluish (National Aniline Division Allied Chemical & Dye Corp.), Phloxin (Harleco), Phloxin B (Harleco), Pyronin B (Chroma-Gesellschaft Roboz Surgical Instrument Co.), Rhodamine B (Chroma-Gesellschaft Roboz Surgical Instrument Co.). Each of these dyes was tested for its staining properties in vitro, using both frozen and paraffin sections. The following procedure proved to be the most satisfactory of all that were tried.

Frozen sections of various tissues were cut at 8 micra, using Cryoform (International Equipment Company, Needham Heights, Massachusetts) for blocking. After 5 minutes exposure to the ambient air over concentrated sulfuric acid in a Coplin jar, the slides were covered for 4 minutes with a 0.1% aqueous solution (pH 8) of Rhodamine B (Chroma) or a 0.02% solution of Rhodamine B (Allied Chemical). This was followed by two periods of dehydration with butanol, each for 2 minutes. After an additional 2 minutes on a slide warmer at 60° C, the sections were mounted in oil of cedar for viewing under ultraviolet light. The elastic tissue appeared as fluorescent yellow-orange fibrils (Fig. 1). There was no fading over a period of weeks.

The animals studied were guinea pigs, rats, hamsters, and rabbits, and the tissues employed included skin, lung, aorta, nerve and muscle. Control slides were stained with 1% solution of acid orcein (synthetic for 4 minutes) with differentiation in 95% ethyl alcohol, followed by dehydration in absolute ethyl alcohol, clearing in xylene and mounting in oil of cedar. Human skin was also used. In this instance the elastic tissue was also stained with Rhodamine B with a

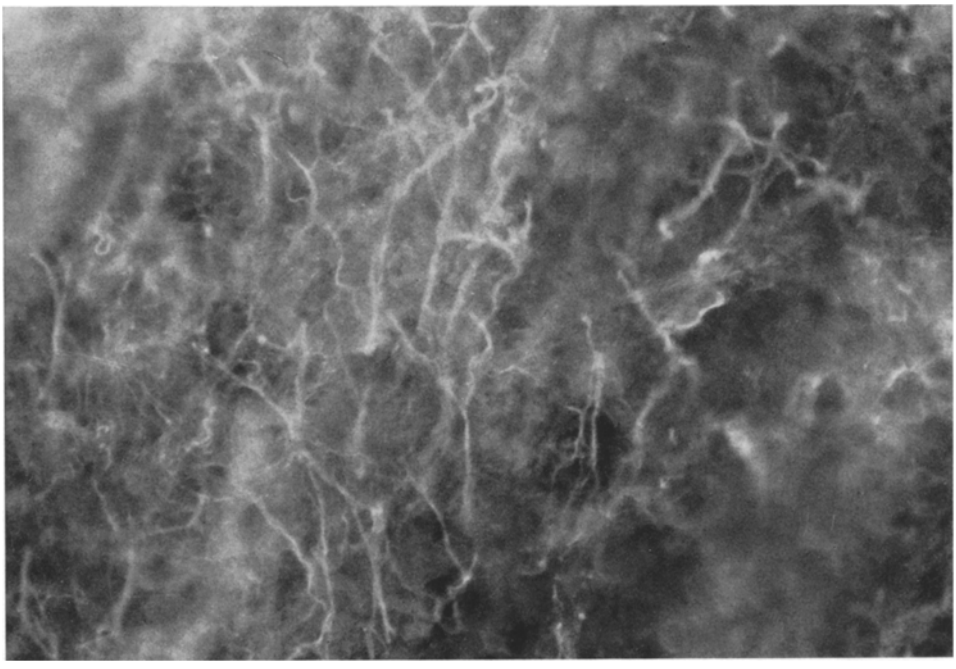

Fig. 1. Fluorescent elastic fibers in skin of guinea pig. Staining was achieved by immersion of frozen sections in 0.02% Rhodamine B (Allied Chemical) for 4 minutes. Control slides show no autofluorescence of these fibers

consequent accentuation of its normal autofluorescence. By contrast, the elastic fibers of animals showed no autofluorescence.

Varying the pH of the Rhodamine staining solution from 2.0 through 10.0 produced no remarkable differences, although pH 8 was chosen as probably optimal. Using Rhodamine B (Allied Chemical) produced a more intense stain so that appropriate dilutions had to be made. Variations in the dye concentration and staining time produced predictable results.

Discussion

Staining sections from fixed tissue was less satisfactory than staining the fresh frozen sections. The best visualization of stained elastic fibers was seen in guinea pig skin, rat lung and rabbit arteries. In all of these the elastic fibers appeared as distinctive, readily viewable structures. In many other tissues the elastric component is so small that staining is less evident (see Table).

The specificity of certain classic stains for elastic tissue is under serious question. (Jackson, Puchtler and Sweat, 1968), but in the present study acid orcein was used as a control stain. The localization and morphology of the fibers that stained with acid orcein, compared with those staining with Rhodamine B, led to the conclusion that Rhodamine B under the conditions employed slectively stains elastic tissue.

The essential molecular structure of the elastic tissue fluorochromes appears to be a diamine derivative of xanthene. Nearly all of the dyes used successfully by others, as well as those used by me, have this diamine-xanthene nucleus or are in close structural relationship (Table). Although the chemistry of elastic tissue is exceedingly complex (Fullmer, 1965; Piez, 1968), one may assume that these representative primary amine xanthene dyes attach to reactive aldehyde groups in the elastin molecule.

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Table. *Fluorchrome stains for elastic tissue*

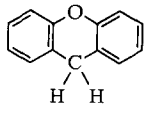
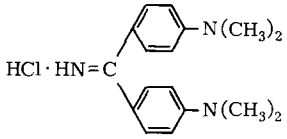
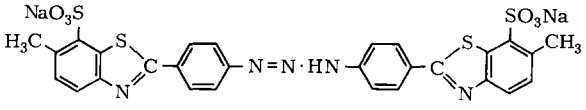
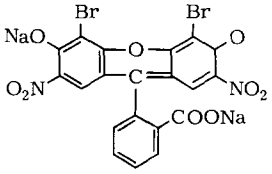
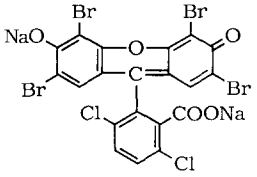
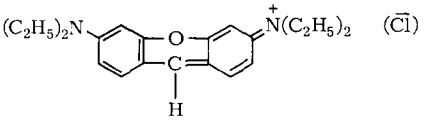
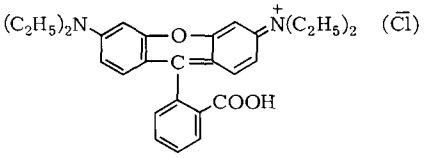
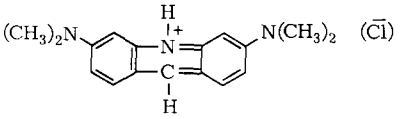
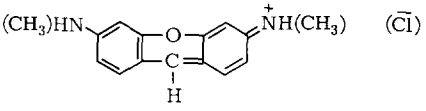
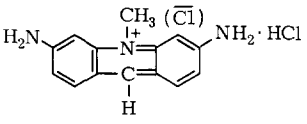
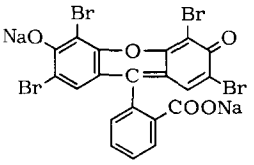
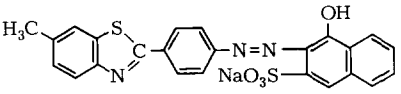
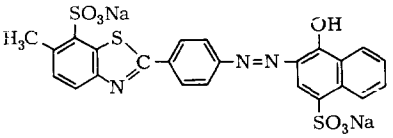
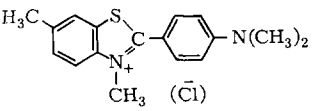
	Formula
Xanthene nucleus	
A. Current study	
Auramine O	
Clayton yellow	
Eosin B	
Phloxine	
Pyronin B	

Table (continued)

	Formula
Rhodamine B	
B. Previous studies	
Acridine orange	
Acridine red	
Acriflavine	
Brilliant dianyl green	Not available
Eosin yellow	
Geramine G	
Lissamine yellow	Not available
Thiazine red	
Thioflavine	

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