Differential Staining of Bone and Cartilage *in toto* of Fish

JOSEPH F. MORAN, JR., *University of Notre Dame*

Introduction

The present work was undertaken to fulfill the need of differentially stained specimens for current comparative osteological research work and museum specimens. Several techniques combining the Schultz method (6) for clearing tissues, and an alizarin dye solution for staining calcified elements have been developed since 1920 (Hollister (3), Davis and Gore (2), Stokely (7), and Russell and McCandless (5)). Alizarin dyes stain calcified cartilage and bone, but have no affinity for other cartilage. In 1940 Williams (8) published the summary of a technique using sodium alizarin monosulfonate to stain bone, and toluidine blue to stain cartilageonous elements. The techniques used by the present author are those modified chiefly after Davis and Gore and Williams.

Materials and Methods

Approximately 100 fish (mostly centrarchids and umbrids from 0.8-24 cm long), reagent or U.S.P. grade chemicals, and distilled water were used in the present work.

Hollister cautions against use of tap water in preparing the alizarin solutions since flocculence may occur. A stock solution of 4% potassium hydroxide was prepared from U.S.P. KOH pellets and distilled water. U.S.P. white glycerine was used for final clearing of all specimens. Ninety-five per cent alcohol was used as a stock for all alcoholic solutions. The stock solution of sodium alizarin monosulfonate stain was prepared according to Hollister):

Alizarin saturated solution in	ml
Glacial acetic acid	5
Glycerine (white)	10
Chloral hydrate 1%	60

The stock solution of toluidine blue stain, modified after Williams and Miller (4), was prepared as follows:

Dissolve 2.5 gms. toluidine blue in	ml
Concentrated hydrochloric acid	1.33
95% alcohol	736.30
Distilled water	262.30

The stock toluidine stain contains 0.25 gm. per 100 ml. solution.

Each specimen was given a code number and placed in a correspondingly numbered transparent plastic box. All specimens were treated in these boxes and finished specimens were stored in the same box.

The very small specimens were handled as little as possible and therefore removing the solutions with a large syringe was preferred to changing the specimen to new solutions.

Procedure

The procedure consists of four parts; namely, initial treatment, cartilage staining, initial clearing, and final clearing.

Initial treatment: Fresh material requires fixing and hardening for at least four days. In this technique 95% alcohol proved most effective. Formalin- or alcohol-preserved specimens should be washed for at least 24 hours in running tap water, then placed in 95% alcohol for at least three days to harden. Stokely found that both alcohol-preserved and formalin-preserved specimens gave equally good results when stained for bone alone. This was also observed in specimens differentially stained for cartilage and bone in the present work. After hardening, the specimens are eviscerated and the skin and scales carefully removed together. This is facilitated by the hardening process and permits easy penetration of the stains.

Cartilage staining: The stock solution of toluidine blue was mixed with an equal part of 70% alcohol for staining purposes. Alcohol-hardened specimens were covered with toluidine blue and stained one week for small specimens (2.5-6.0 cm), and approximately two weeks for those above 10 cm. Effects of stain concentrations of 50, 75, and 100% stock solution were observed for periods of four to fourteen days. In general, the staining period varied directly as the size of the specimen.

After staining, the specimens were washed for 10-20 minutes in 70% alcohol. Care must be taken since rapid destaining may occur in the alcohol. Bensley and Bensley (1) describe a technique for destaining with 1% acid alcohol; however, this was found much too rapid in the present work. It should be noted that the toluidine stain has a strong affinity for flesh as well as cartilage; however, subsequent clearing procedures removes the stain from the flesh.

Initial clearing: Fresh material requires fixing and hardening for at least four days. In this technique 95% alcohol proved most effective. results in small-to-medium-sized specimens with a 1% solution for initial clearing. Consequently, after washing in alcohol, the specimens were placed in the lower concentration. It was observed that clearing very large specimens (above 18-20 cm.) was appreciably hastened by use of 3% KOH. It was observed also that a 0.5% solution was safer during clearing of very small specimens (8-10 mm) in which the process is very rapid. Toluidine blue becomes dark red in color when introduced into the KOH but after several hours assumes a characteristic deep blue color.

After 5-7 days in KOH, alizarin staining was begun. Four to six drops of stock alizarin stain were added to the 1% KOH bath until a clear ruby red color was attained. Solutions were changed daily and clearing and staining proceeded until the bones were stained the desired intensity. Because of the presence of toluidine stain in the tissues, clearing in 1% KOH was continued until the flesh became transparent.

Treatment of specimens with peroxide solutions during the clearing phase of the procedure was not necessary since the pigmented skin had been removed.

Though several authors recommend use of white trays, ultra-violet light, and sunlight to aid in clearing, Stokely did not find such necessary.

Final clearing: When specimens were sufficiently cleared they were passed in order through a series of aqueous glycerine solutions (20%, 50%, 75%) to pure glycerine. Very small specimens were passed through a more gradual glycerine series (5, 10, 20, 50, 75, 100%) to prevent bloating and distortion.

Clearing is often not complete until the specimen has been in pure glycerine for several days. In case it does not clear sufficiently it may be downgraded through the glycerine series and returned to the KOH solutions.

Though Davis and Gore recommend use of a small amount of KOH in the initial glycerine solution it was omitted in the present work since some KOH is carried over by the specimen. Furthermore it was observed to have no apparent effect on the final clearing.

All specimens were store din pure clear glycerine. A small amount of thymol should be added to prevent mold growth in the stored specimens. If the glycerine becomes colored on standing it should be replaced. Cork stoppers never should be used because they discolor glycerine and also the specimen.

Literature Cited

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