A RAPID METHOD FOR CLEARING AND STAINING SPECIMENS FOR THE DEMONSTRATION OF BONE

MARGARET C. GREEN

Department of Zoology and Entomology, The Ohio State University, Columbus 10

The potassium hydroxide clearing and alizarin staining method for the preparation of whole skeletons has proved very useful for the study of bones of embryos and small animals. It has certain advantages over methods in which the carcass is macerated and the bones are separated and dried. Among these advantages are: (1) there is no chance of losing the small bones, (2) all bones are retained in their original position, (3) there is no chance of wrongly identifying similar bones, (4) in the finished preparations the bones, after identification, may be disarticulated and examined from all angles, equally as well as in dried preparations, and (5) many animals may be processed together without danger of mixing their bones, a great saving in time and effort.

The method was originally developed by Schultze (1897) and has subsequently been modified by a number of investigators including Mall (1906), Dawson (1926), Lipman (1935), Cumley, Crow, and Griffen (1939), Gamble (1945), and True (1947). The steps usually employed include: (1) fixation in formalin or alcohol, (2) bleaching with hydrogen peroxide, (3) incomplete maceration in potassium hydroxide, (4) staining with alizarin red S, and (5) clearing in graded concentrations of glycerin, or dehydration and clearing in oil of wintergreen. Various authors have suggested the omission of one or more of these steps. In our genetic experiments it has been necessary to examine the skeletons of many thousands of mice, making the maximum possible simplification desirable. It has been found possible to omit fixation and bleaching and to transfer the specimens directly from the staining solution to undiluted glycerin without the intermediate steps.

Although the method is particularly useful for mass production of stained skeletons, the excellent quality of the preparations (fig. 1) makes it useful also for the preparation of exhibition specimens. It may be used successfully on small fish, amphibia, reptiles, birds, and mammals. Specimens thus prepared may subsequently be mounted in transparent plastic according to directions supplied with the plastic by biological supply companies.

The solutions needed are 1 percent potassium hydroxide, 0.5 percent alizarin red S in 1 percent potassium hydroxide, and glycerin. A schedule suitable for 30 day old mice is as follows.

1. Skin and eviscerate.
2. Place in 1 percent potassium hydroxide for 5 days.
3. Pour off solution and replace with fresh 1 percent potassium hydroxide. Add to this a few drops of 0.5 percent alizarin red S in 1 percent potassium hydroxide to make a medium pink solution. Leave in this for 5 days.
4. Pour off solution and replace with glycerin.

With a little practice the first step can be accomplished very rapidly. The following procedure for skinning has been found satisfactory for mice of all ages. With scissors a crosswise cut is made in the skin of the mid-dorsal region. This cut is then extended around to the ventral side by tearing. If the posterior part of the skin is now peeled back over the hind legs and tail, it will tear at the ankles and usually at the base of the tail and come off without further cutting. Sometimes the skin of the tail pulls off completely. A cut is now made in the anterior part of the skin running longitudinally from the mid-dorsal region to the tip of the nose. The skin can then be peeled off the head and front legs in one piece.

THE OHIO JOURNAL OF SCIENCE 52(1): 31, January, 1952.

Fixation of the specimens in 10 percent formalin or 95 percent alcohol has been recommended by many authors. In our experience this is not necessary for mice nor for the other animals listed below. Fixation prolongs the time of treatment with potassium hydroxide and does not improve the quality of the preparation. However, some animals, such as small salamanders, may disintegrate if placed directly in potassium hydroxide without prior fixation.

The potassium hydroxide solutions used in the second and third steps may be either 1 percent or 2 percent. The processing goes faster in the stronger solution but must be watched more closely, especially for young or small animals, for if the animals remain in the solution too long the bones will fall apart. The 1 percent solution is therefore more desirable unless extra speed is desired. It should also be remembered that higher temperatures speed up the reaction, so that a schedule that is satisfactory in winter may be too slow during hot weather in summer.

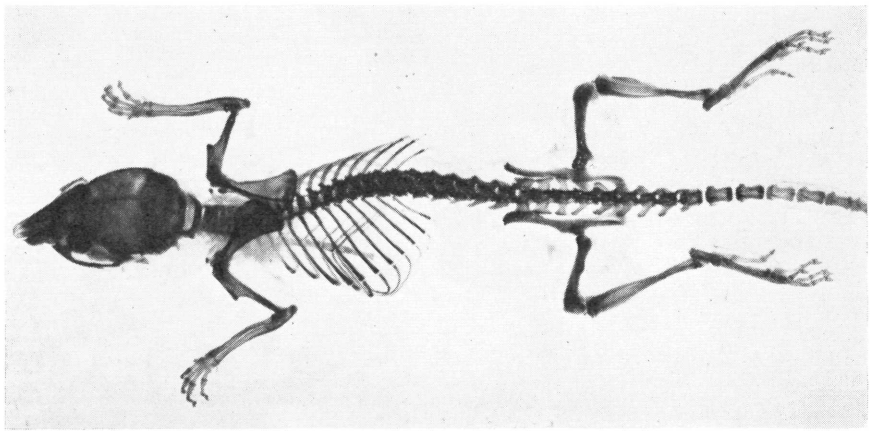

FIGURE 1. Skeleton of a 30 day old mouse. (By Department of Photography, The Ohio State University.)

Too many animals should not be crowded together in a single container. Crowding slows the reaction and if severe may result in some spoilage. Experience has shown that about eight 30-day old mice can be processed in a pint jar. When several animals are processed together, it is well to stir them once or twice during the staining process.

The concentrated solution of alizarin does not keep well for longer than one or two months and should therefore be made up in small quantities. It is conveniently stored in a dropping bottle. In practice the concentration need not be accurately controlled. After a little experience the appropriate amount of alizarin for a given amount of fluid can be judged without weighing. The concentration in actual contact with the mice can be regulated by adding enough of the concentrated solution to produce the correct medium pink color. Too much alizarin in the solution will stain the soft tissues as well as the bones. This does not render the preparations useless but detracts from their appearance. Excess stain can be removed by immersing the mice in plain 1 percent potassium hydroxide for a few days. If the bones have stained only faintly at the end of 5 days, more alizarin may be added and the mice left in the solution for another 24 hours. When the concentration of alizarin used has been just right, the skeleton at the end of the staining period will be strongly stained and the solution will be practically colorless.

The total length of time in potassium hydroxide varies with the size of the animal, the strength of the solution, and the temperature. For mice one can judge

whether the action of the alkali has proceded far enough by lifting a carcass out of the solution with a pair of forceps. If it trails a viscous slimy material, it is not done. It should be replaced in the potassium hydroxide and left until the solution runs off in drops when the carcass is lifted out. Other animals may require longer treatment than this. A more general criterion for the end point of this stage is the deeply stained appearance of the bone through the semitransparent muscle. When a 1 percent solution is used there is, for most animals, no danger of disintegration before this point is reached.

The approximate total time in 1 percent potassium hydroxide necessary for a few representative animals is given below. Fresh potassium hydroxide and alizarin are used for the last half of the period.

One day old mouse	4 days
Goldfish, 2 inches long	7 days
Adult leopard frog	15 days
Adult fence swift (lizard)	7 days
Adult junco	15 days
Adult rat	30 days

If skeletons are to be examined in some routine fashion and discarded one soaking in glycerin will suffice. For exhibition specimens one or two changes to fresh glycerin should be made. Specimens may be stored in glycerin indefinitely.

For routine examination of cleared and stained specimens a viewing light is helpful. One can be easily and cheaply constructed by mounting a number 2½ can, with the ends cut out, on a board over a light socket. A piece of white paper laid across the top of the can serves to diffuse the light and a piece of window glass on top of the paper supports the specimen being examined. A switch to control the light may be mounted on the board for added convenience. A 25 watt bulb is about the right size to fit the lamp and produces enough light without too much heat.

LITERATURE CITED

Cumley, R. W., J. F. Crow, and **A. B. Griffen.** 1939. Clearing specimens for the demonstration of bone. Stain Tech., 14: 7–11.

Dawson, A. B. 1926. A note on the staining of the skeleton of cleared specimens with alizarin red S. Stain Tech., 1: 123–124.

Gamble, J. T. 1945. A combination bleaching-clearing agent and its use in the processing of "Spalteholtz" preparations. Stain Tech., 20: 127–128.

Lipman, H. J. 1935. Staining the skeleton of cleared embryos with alizarin red S. Stain Tech., 10: 61–63.

Mall, F. P. 1906. On ossification centers in human embryos less than 100 days old. Amer. Jour. Anat., 5: 433–458.

Schultze, O. 1897. Uber Herstellung and Conservirung durchsichtigen Embryonen zum Stadium der Skeletbildung. In Verhandlungen der Anatomischen Gesellschaft; Anat. Anz., 13: 3–5.

True, R. M. 1947. Staining of embryonic and small mammalian skeletal systems. Stain Tech., 22: 107–108.