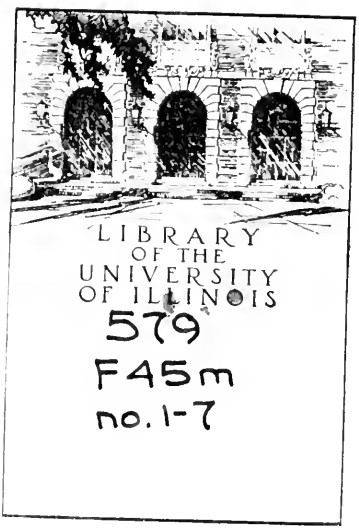

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NUMBER 4

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The preparation of osteological material has always been a source of annoyance to anatomists. With any of the smaller forms, from fishes to mammals, satisfactory results demand an expenditure of time out of proportion to the results obtained. A delicate skeleton, no matter how carefully handled, inevitably suffers more or less damage from continued use. Furthermore, with most small fishes and many amphibians and reptiles, even an expert finds it next to impossible to produce really satisfactory results. The abundant cartilage found in many forms, which dries into an extremely friable and almost shapeless mass, further complicates matters. In addition, since warping and distortion of slender or thin bones and calcified cartilage may make the exact form and relation of parts almost as much a matter of conjecture as of observation, work on the smaller vertebrates has been so impeded that the extremely interesting comparative osteology of these animals is still almost unknown.

The exclusive use of external characters in defining the major groups of the lower vertebrates is no longer practical. The extraordinary success which has been achieved in recent years by comparing the internal anatomy of these forms is perhaps an indication of what may be anticipated along these lines. Unfortunately, accurate results in this type of work demand an examination of extremely large series of specimens. In addition, the rapid rise of paleontology has given comparative osteology a new lease on life, and has at the same time made the imperfect state of existing knowledge all too apparent.

Cleared specimens cannot, of course, entirely supplant dry skeletons even of the smaller forms, except in certain types of work. Rather the cleared specimens should be viewed as supplementary ones. Some studies demand more or less completely disarticulated skeletons. Certain features of the skeleton can be observed with difficulty or not at all when the bones are surrounded by a layer of flesh, however transparent the latter may be. This is particularly true of the skull, where the general complexity of structure, the thickness of bony

masses, and the presence of such dense masses of flesh as the eyes[1] and the tongue make accurate observation of details quite impossible. For the post-cranial skeleton, however, cleared material may be employed to great advantage.

A list of the uses of the modified Schultze method could hardly be complete, but some of the more important applications may be pointed out. Unquestionably its greatest advantage lies in extraordinary saving of time. In the hands of a single careful technician, unlimited series of uniformly good specimens may be prepared in a surprisingly short time. A further general advantage is the fact that a skeleton is always *complete*. No small bones or delicate processes are lost during preparation, and none become detached and lost from careless handling later on. Glycerine-preserved specimens may also be manipulated to a certain extent, and actual dissection of parts may easily be carried out when necessary. More specifically, a host of special uses comes to mind. The preservation of cartilaginous parts in their normal condition has already been alluded to. The same applies to calcified cartilage, which is, if anything, even more brittle when dry. The entire pectoral girdles of amphibians and reptiles, the sternal and abdominal ribs of the latter, small isolated nodules of calcified cartilage (e.g. the hypoischium of lizards), may be examined in a complete and undisturbed condition. The small but highly important bones of the carpus and tarsus are not obscured by masses of dried ligaments, which unfortunately must be retained as a binder in dried preparations. The method has advantages when applied to the study of the vestiges of limbs and limb girdles in degenerate types, as anyone who has tried to dissect out these parts can testify. Equally obvious are its uses in the study of larval skeletons, and of the time and places of ossification in the ontogeny of individual bones. The intricate structure of the axial skeleton in the higher teleost fishes, with their complexities of extremely delicate rib and vertebral structure, is rendered beautifully apparent.

The application of this method to museum exhibition is almost a virgin field. Good preparations are by no means unattractive, and could be profitably introduced as a supplement to dried mounts. The curious question, so often encountered, "What do you make the bones of?" probably would not be asked on viewing an exhibit of this type. While the uses of the technique in this field are limited, they are none the less interesting, and could be explored to advantage.

[1] Of course the eyes may be removed. In reptiles, however, this destroys the sclerotic rings, which are an integral part of the skeleton. It is impossible to remove the tongue without damaging the hyoid apparatus.

FIG. 1. Horned lizard, *Phrynosoma cornutum*, photographed from the ventral side. Prepared from a freshly-killed specimen.

HISTORICAL SUMMARY

The use of various agents for clearing animal tissues is by no means new. Until recently this technique, usually in combination with bone stains or injections of blood vessels, has been almost exclusively employed in studies on human embryos and a few standard laboratory types. The possibility of utilizing it in studying the skeletons of various small vertebrates, where the preparation of dry skeletons is tedious and unsatisfactory, has only recently been exploited.

The several processes now in use are all adaptations of one of two original techniques. The *Spalteholz method*, which is essentially a modification of standard histological procedure, is open to several objections when applied to skeletons, and is no longer in general use. The most serious disadvantage, in addition to the time involved and the expense of some of the materials used, is the impermanence of the specimens, which discolor rapidly unless they are mounted in balsam. Schultze (1897) first formulated the technique of using potash and glycerine in clearing human embryos, and any technique involving clearing with potassium hydroxide is generally referred to as the *Schultze method*. He used KOH solutions as strong as 20 per cent, but subsequent investigation has reduced this concentration considerably. Later, Mall (1906) introduced the technique into this country, where it has been widely used, in more or less modified forms, in studies on ossification. An important addition to the original Schultze technique was made by Lundvall (1905), who introduced the use of alizarin for staining the bones. Although various stains have been used, alizarin has been almost universally adopted because of its selective properties.

The Schultze process is essentially one of rendering all the fleshy parts of an animal more or less transparent by means of a strong alkaline solution and glycerine and by removing pigment with hydrogen peroxide, ultra-violet light, or other bleaching agents. At the same time the calcified structures are brought into sharp relief by staining them with a selective dye.

Thomas Barbour has published evidence which indicates that he was the first to employ this process on small vertebrates. The figure of *Sminthillus limbatus* that appears on Plate I of a paper by him and Noble (1920) bears the caption "Specimen cleared after Schultze's method by Thomas Barbour in 1913." Noble (1917) used a modification of the technique on the toes of frogs and in his "Herpetology of the Belgian Congo" (1924) figures a series of pectoral

girdles prepared by this method. More recently Beebe (1929), and particularly his assistant, Hollister (1932, 1934), have used it on a mass production basis on fishes. Although Hollister (1934) has given an outline of the process she employs on fresh fishes, an adequate and detailed discussion of the method with reference to the various vertebrate groups has long been needed, especially with reference to preserved material, and its lack has doubtless impeded the wide application that the process merits.

The present study was undertaken in an effort to fill the need for a simple, reasonably rapid method of making the large series of good osteological preparations so necessary in various types of taxonomic and anatomical studies. An important consideration in this connection is the fact that studies of this nature can rarely, if ever, be made on fresh material. Museum specimens, which are used in most investigations of this type, are universally preserved in 60–65 per cent alcohol. This type of material has been used in this study, together with enough fresh material to serve as a basis for comparison.

While preserved material rarely yields the crystal-clear preparations that may be made with fresh specimens, this objection is by no means a serious one, since in most instances the results are sufficiently transparent to photograph well.

MATERIALS AND METHODS

In view of Hollister's account of the treatment of fishes, our work centered largely on amphibians and reptiles, the only other vertebrate groups that are habitually preserved *in toto*. It included, however, a number of fishes and several nestling birds and mammalian embryos. An interesting possibility was opened up by the unusual success achieved with a small bat, a form in which the delicate skeleton is exceedingly difficult to prepare in the orthodox way. A wide selection of sizes and degrees of ossification was made among the salamanders, frogs, and lizards.

On general principles, a good grade of chemicals was used in making up the solutions. C.P. potassium hydroxide sticks are preferable for making the KOH solutions. Distilled water was used throughout as a precautionary measure. According to Hollister its use is especially desirable in making up the stain, since flocculent suspensions may result if tap water is used. A stock solution of 10 per cent KOH was prepared, and the lower percentages were made up from it as necessary. U.S.P. white glycerine was used, with excel-

lent results. The stock dye was made up according to the formula recommended by Hollister:

Alizarin, sat. sol., in:	cc.
Glacial acetic acid	5
Glycerine, white	10
Chloral hydrate, c.p., 1 per cent sol.	60

Hollister claims that the staining qualities of alizarin made up according to this formula are superior to the alcoholic alizarin stain recommended by others. She also advises the addition of the acetic acid on the grounds that it has a tendency to counteract the absorption of the stain by the softer tissues and facilitates final clearing. These points were not checked by us. Her formula gave uniformly satisfactory results, and was used throughout this work. As needed, this stock dye solution was added to 4 per cent KOH solution in the proportions of 1 part of the dye to 1,000 of the KOH.

PROCEDURE

The process falls naturally into four parts, and in order to simplify the discussion as much as possible each part is taken up step by step, with a discussion of points of special interest. A brief summary of the entire procedure is then inserted for easy reference. It is believed that this method of presentation, if followed carefully, will enable anyone to produce satisfactory results with a minimum of failures. Unless otherwise stated, statements in the following pages refer to preserved material.

INITIAL TREATMENT

Fresh material.—Fresh fish require fixing and hardening in alcohol for several days before they are started through the clearing process. For this purpose, 95 per cent alcohol hardens quickly and is generally more satisfactory than lower percentages. Any shrinkage or distortion resulting from this concentration seems to be temporary, since specimens assume their normal proportions during the clearing process. In spite of Hollister's excellent results with fresh fish without any preliminary fixing, all our specimens so treated macerated within a short time in 1 per cent KOH, and had to be discarded. Addition of alcohol to the KOH which she recommends failed to stop maceration. Hardening of fresh frogs, lizards, and snakes was also found to be desirable. All material should be carefully and completely eviscerated.

Preserved material.—Evisceration of all material is highly desirable, since otherwise details of the girdles and axial skeleton are always more or less obscured. Where the digestive tract contains

FIG. 2. Above: Small African ranid frog, *Phrynobatrachus francisci*, photographed from the dorsal side (from preserved specimen). Below: Cricket frog, *Acris gryllus*, photographed from the ventral side (from fresh specimen).

much undigested matter it is imperative, and with material that is to be photographed it is always necessary. Abdominal ribs are present in many lizards, and the incision and subsequent removal of the viscera must be done with great care to avoid damaging them. Where these structures are well developed, it is almost impossible to avoid injuring them. Our procedure in these cases was to make a clean cut with a pair of scissors to one side of the ventral midline. While this severs all the abdominal ribs on one side, the cut is clean and uniform, and the region of the midline as well as the ribs on the opposite side are left intact.

The stamped tags of treated paper in current use at Field Museum (Schmidt, 1932) were found to be ideally suited to marking specimens, both the tags and the linen thread used to tie them going through the entire process without serious damage. Stamped metal tags may also be used, since none of the reagents contain corrosive agents, but are less desirable because of their weight and sharp edges.

After evisceration, specimens are soaked in water over night or longer, to remove the alcohol. Skinning, although it speeds up the process considerably, is not desirable, since it is apt to leave the specimens in a flabby and extremely fragile condition. In such lizards as skinks, where the skin contains a heavy armor of osteoderms, it is necessary, however, since the osteoderms stain heavily and obscure all structures lying beneath them. The scales of many fish also stain to a greater or less degree, but in these cases removal of the scales rather than skinning is recommended. This is more easily done after treatment with KOH.

It must be remembered that formaldehyde is a strong decalcifier of bone. Material that has been preserved in it for any length of time is not likely to yield good preparations.

Dried specimens.—Air-dried specimens were put directly into 1 per cent KOH without any preliminary treatment, and have yielded some surprisingly excellent preparations. A desiccated moonfish produced one of the most transparent specimens in the entire series. The general use of dried material can not, of course, be recommended. There is nothing to indicate that the specimens so treated are in any way superior to those made from preserved material. In addition there is always danger of serious maceration.

CLEARING

Specimens previously preserved in alcohol are put into a bath of 3–4 per cent KOH. Of all the strengths of potash tested, this

FIG. 3. Buffalo fish, *Carpiodes tumidus* (preserved specimen).

resulted in the best and quickest clearing with the least maceration of tissues in alcoholic material. On the other hand, a 1 per cent solution is sufficiently strong for fresh material, and higher concentrations are likely to result in maceration. After 24 to 48 hours in the clearing agent, heavily pigmented specimens are transferred to full strength hydrogen peroxide until thoroughly bleached. Bleaching usually takes several hours. The use of peroxide after KOH treatment rather than before is advantageous in that good penetration of the peroxide is insured and more thorough bleaching obtained. The principal objection to the use of peroxide is its tendency to produce air bubbles in the flesh of the specimen. To remove these one by one with a needle requires time and patience. Actually, unless they are extremely large and numerous, they do not interfere to any great degree with the utility of the specimens, except where material is to be photographed. Treatment with KOH before bleaching seems to counteract the formation of bubbles to a considerable extent.

With the exception of the time in peroxide, the specimens are exposed to full sunlight in shallow, white, porcelain-lined photographic trays. Average sunlight is sufficiently strong to make good transparent preparations with three or four weeks' exposure, and in even shorter times for some material.

After bleaching, the material is returned to 4 per cent KOH, where it remains until it is quite transparent. The bones of the toes, the pectoral girdle, and other parts directly under the surface should be readily visible, although deeper parts may still be obscure. The time required for this clearing varies from a day for small frogs to a week or more for fair-sized lizards.

Greasy bones will not take up the dye. Dehydration and treatment with acetone has been recommended, but in our experience this was not necessary. Alcohol is a fair degreasing agent, and specimens that have been preserved in it for some time are usually fairly free from grease. What little may remain seems to be removed by the KOH.

Beebe and Hollister used an alpine sun lamp in place of sunlight, with good results. Its advantages, particularly during cloudy weather, are obvious. Hollister claims that the use of ultra-violet light makes the use of bleaching agents for depigmentation unnecessary. We were unable to test its efficiency on preserved material.

STAINING

The most satisfactory dye solution for preserved specimens was made with 10 cc. of the stock dye solution (p. 8) per liter of 4 per cent KOH. Material is transferred directly to this solution from the KOH bath. The time required for staining varies somewhat, but with a solution of this strength twenty-four hours is sufficient for most specimens. The flesh takes up a good deal of stain if specimens are allowed to remain in the dye too long. Although most of this stain eventually comes away, it makes subsequent clearing needlessly long and complex, without adding anything to the usefulness of the preparations. In many instances the stained flesh is extraordinarily resistant to clearing. On the other hand, immersion in the dye for too short a time results in a light stain which does not differentiate satisfactorily. Hollister recommends a 1–1000 alizarin solution in 1 per cent KOH for staining fish, and Dawson used the same formula of alcoholic alizarin on legs of rats with good results. This concentration is not strong enough for preserved amphibians and reptiles since specimens immersed for several days in 1 per cent KOH, 1–1000, were stained an unsatisfactory light red.

FINAL CLEARING

Material is transferred from the stain to a 4 per cent KOH solution where it remains twenty-four hours. By this time the solution is usually deeply colored from the excess stain that has come away from the specimen, and transfer to a fresh solution is necessary. This process should be repeated as long as the KOH discolors. After the material is free from excess stain and fairly transparent, it may be transferred through the following solutions to pure glycerine:

Glycerine (Parts)	KOH 4 per cent (Parts)	HOH (Parts)
20	3	77
50	3	47
75	0	25
100	0	0

Transfer of material directly into pure glycerine causes great shrinkage and distortion.

Exposure to sunlight in white-lined trays should continue all along. Even in pure glycerine it is beneficial in final clearing. A specimen that is not wholly transparent frequently becomes remarkably clear in one or two days when finally placed in pure glycerine. If specimens are not sufficiently transparent at the end of the treatment, they may, of course, be returned to KOH for further treatment.

STORING

Specimens should be stored in chemically pure, white glycerine. Glass-stoppered bottles are advisable. If the glycerine shows signs of discoloration at any time, it should be replaced immediately, since the discoloration is rapidly taken up by the specimen. Under any circumstances this impairs their transparency, and in extreme instances may render them useless. Under no circumstances should cork be used, since it inevitably discolors the glycerine, and this discoloration is taken up by the specimen. A thymol crystal should be added to each jar to prevent the growth of molds, which is frequently very rapid and destructive. Material should be stored away from light, although Hollister claims she has left specimens in the light for ten years without any signs of deterioration.

SUMMARY OF TREATMENTS

(1) Fresh specimens are hardened for about a week in strong alcohol (95 per cent). Material preserved in alcohol is washed in water over night or longer. All material is completely eviscerated.

(2) Transfer fresh specimens to 1 per cent KOH in distilled water; use 4 per cent KOH for preserved material and expose to strong sunlight in shallow, white-lined trays for two days.

(3) Bleach thoroughly in full strength hydrogen peroxide.

(4) Return to 4 per cent KOH. Expose to sunlight until specimen is transparent and bones are easily visible through the skin.

(5) Stain twenty-four hours or longer in 10 cc. stock dye solution to 1,000 cc. 4 per cent KOH.

(6) Return to KOH (1 per cent or 4 per cent, as stated above), exposing to sunlight and changing solution until stain ceases to come away and specimen is transparent.

(7) Transfer gradually to pure glycerine.

(8) Store in glass-stoppered bottles, away from light. Add a crystal of thymol to the glycerine.

It is important to remember that each specimen presents its own individual problems. Variations in type of preservation, in the length of time the material has been stored in alcohol, and in the size and consistency of the specimen itself, are such that any but the most general rules would be valueless and misleading. Each specimen demands careful watching in all stages of the process, and unquestionably the factor of personal experience on the part of the technician is an important one in producing results that are uniform

in quality. Some idea of the variations encountered may be gained from the following notes:

An adult, freshly killed horned toad (*Phrynosoma*) was cleared in four days in 3 per cent KOH, was stained in twenty-four hours, and was quite transparent in two weeks' time (fig. 1).

A series of fresh cricket frogs (*Acris gryllus*) was cleared and stained in a week, resulting in almost crystal-clear preparations (fig. 2).

Preserved specimens of the larger ranids (*Rana pipiens, R. clamitans*) required about three weeks for the entire process.

Fresh lizards (*Eumeces, Crotaphytus*) were ready for study in three weeks. A series of twenty preserved iguanid lizards were cleared in from two to five days, stained in twenty-four hours, and cleared satisfactorily in from one to two months' time.

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