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CLEARING AND DYEING FISH FOR BONE STUDY*

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OUTLINE

Intrdouction

(Figs. 18-21 incl.)

During my work in the Department of Tropical Research of the New York Zoological Society in connection with the shallow-water and deep-sea fishes of Bermuda I have prepared, with clearing fluids and bone stain, approximately three thousand transparent specimens in order to facilitate the study of the skeleton. A necessary requisite was a rapid and, at the same time, adequate technique which would render the material ready for immediate study. I have had to adapt old techniques not only to fish in general but to individual species. An entirely new improvement is the use of ultra-violet light. (Hollister, 1932).

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This work, in the Department of Tropical Research, began at Kartabo, British Guiana in 1922 when Dr. Beebe made many transparencies of embryo birds, bats, frogs, and lizards.

While at Columbia University in 1924 and 1925 I studied and experimented with various methods and prepared a series of human embryos, small mammals, chicks, and snake embryos. Since 1927 I have continued to develop a technique especially for fish, both in our field laboratory at Nonsuch Island, Bermuda, and at the Zoological Park in New York City.

This paper is in answer to many requests from interested workers for details and new adaptations.

I take this opportunity to thank for their cooperation Dr. William Beebe, Dr. H. J. Conn, Dr. W. K. Gregory, Dr. A. Pollister, and Dr. R. M. Strong.

Technique

Material and fixing: The use of distilled water in the preparation of all solutions and dyes is advisable. It is absolutely necessary with solutions of alizarin.

The best cleared preparations of small and medium sized fish result from fresh specimens that have had no preliminary fixing. If the tissues should become too soft in the beginning of the clearing process, add increased amounts of 70 per cent alcohol to the potassium hydroxide solution to control this condition. Fix large, fleshy specimens in 70 per cent alcohol for several days.

When it is necessary to keep specimens for any prolonged length of time before running them through the clearing process, the least injurious preservative to the bony tissues is alcohol, which reacts equally well with delicate deep-sea fish and tough shallow water forms. Fixing in 70 per cent alcohol is, in one way, an advantageous preliminary step because it helps to remove green and blue colors from the skin. But, better than any fluid, is ultra-violet light which extracts all colors, even black pigment. Specimens should be irradiated after the staining, along with the clearing.

The use of formalin is dangerous because of the decalcifying action of formic acid on the bony tissues. If for any reason formalin must be

used, it seems less harmful in its acid form than after it has been neutralized.

It is advisable to puncture the sides of the fish in several places with a fine needle before attempting to dye or clear specimens having tough, fibrous flesh, such, for example, as found in the Gobiidae and Blennidae. The removal of scales from large specimens will hasten the penetration of the dye and the clearing of the tissues.

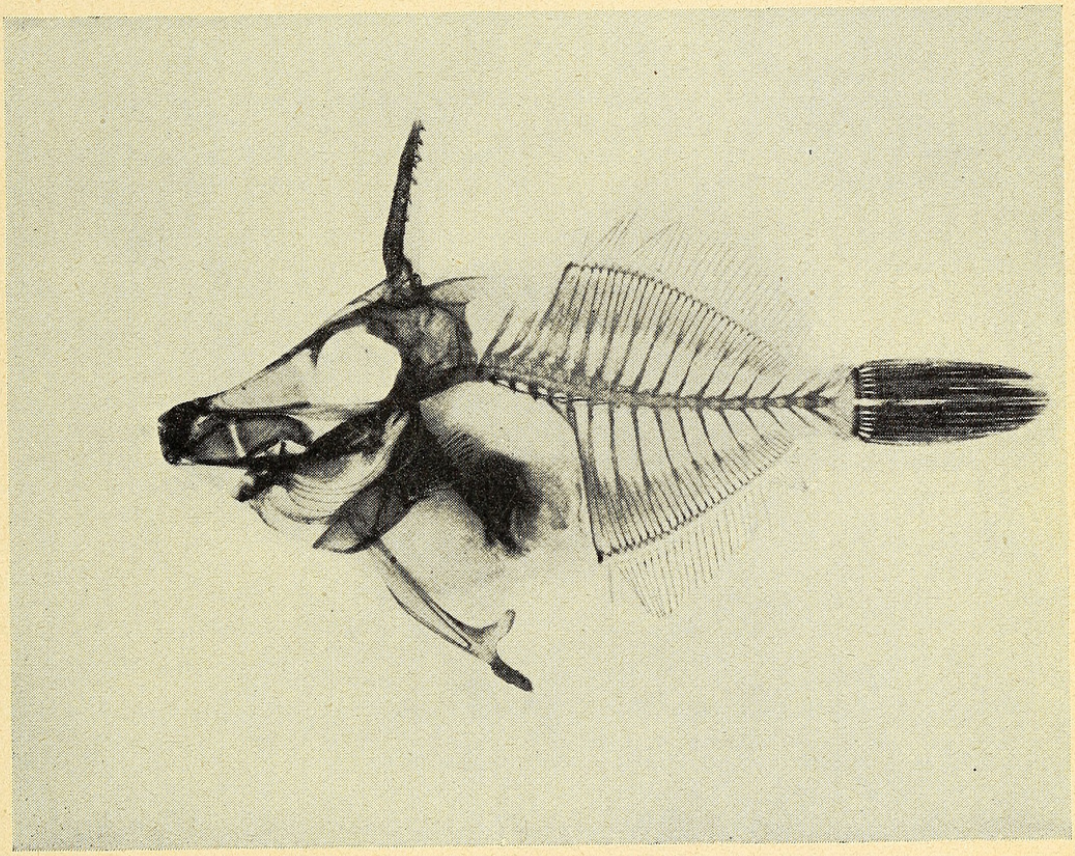

Fig. 18. *Monacanthus ciliatus* (Mitchill), showing every detail of bone clearly etched in red in a transparent body. The mechanism for depressing and raising the dorsal trigger spine can be demonstrated.

Alizarin

Brief history of alizarin: The earliest record was published in 1581 by Laevinius Lemnius, who mentioned in De Miracules Occultis Naturae that madder root had the property of being a vital dye for bone. Over a century and a half later, in 1736, John Belchier (Phil. Trans. Royal Society of London. 1735-36, Vol. 39 Page. 287) re-established this fact and experimented with the properties of madder

root by feeding it to animals. In 1868 Grabe and Liebeman first produced alizarin synthetically. It is now widely used in the form of its soluble salt, Alizarin Red S, Alizarin S, Alizarin Sodium Monosulfonate, and Alizarin Monosulphonate.

There has been a great deal of confusion caused by manufacturers giving their particular alizarin product a slightly different title with no formula to guide the biologist and experimenting zoologist. All these alizarins react alike with bone and are apparently alike in chemical formula.

Dyes used: There are several alizarin stains on the market which include Alizarin Red S, Alizarin S, Alizarin Sodium Monosulphonate, and Alizarin Monosulphonate. In addition to these are the following which have been used also by the author with repeated excellent results. —Alizarinsulfosaur Natron Grubler, Alizarin Sodium Sulphonate Eimer and Amend. According to Schultz and Julius all these dyes have the formula $C_{14}H_7O_7SNa$.

Alizarin is soluble in water and alcohol and it is an acid dye in an alkaline solution and a vital stain for bone. It belongs to the oxyquinone group of biological dyes.

Preparation of alizarin dye:

Alizarin, saturated solution in:—	
Glacial Acetic Acid c.p., 50 per cent	5 cc
Glycerine c.p., white	10 cc
Chloral Hydrate crystals, c.p., 1 per cent sol.	60 cc

The staining qualities of alizarin, made in the usual saturated solution in alcohol, is less permanent and the results more variable than when this formula, without alcohol, is used.

The addition of acetic acid in the dye solution counteracts partially the discoloration of the soft tissues by alizarin and facilitates clearing.

Keep materials for making alizarin dye solution in ordinary room temperature. Colder temperatures are detrimental.

Preparation of alizarin dye in staining solution:

Alizarin dye	1 cc
Potassium hydroxide, 1 or 2 per cent	1,000 cc

Alizarin dye is misible in dilute and concentrated solutions of potassium hydroxide prepared from white sticks, U.S.P., with distilled water. When tap water is used a flocculent suspension of the dye often results.

Alizarin dye should not be mixed in volumes less than 500 cc of potassium hydroxide. A violet-purple color as shown in Ridgway's Color Standards gives excellent staining reesults. If the bones of delicate specimens are to be stained, such as species which will not withstand much time immersed in potassium hydroxide, good staining results from using alizarin in a solution of potassium hydroxide with a high percentage of pure glycerine.

Make up staining solution with materials at ordinary room temperature. Colder temperatures are detrimental.

USE OF STAINING SOLUTION: Place specimen in the staining solution immediately after a bath in weak potassium hydroxide, 1 to 4 per cent, which is the first step in the clearing process. The length of time to keep specimens in this preliminary bath of KOH and in the staining solution varies according to the size of the specimens,—whether the tissues are delicate or tough, and the amount of ossification in the skeleton. Careful observation must be made of the progress of each individual. For example, a delicate deep-sea specimen, *Sternoptyx diaphana,* 8 mm in length and previously fixed in 3 per cent formalin, was stained in six hours, and completely cleared and ready to be photographed and studied in less than twenty-four hours. But a tough shallow-water eel, *Gymnothorax moringa,* 285 mm in length and previously fixed in 3 per cent formalin, took six days to stain and over six months to clear.

It is important to immerse specimens in volumes of 200 cc or more of the alizarin staining solution.

Use staining solution at ordinary room temperature. Colder temperatures are detrimental.

ALIZARIN AND BONE: Calcium salts absorb alizarin according to the amount of accumulation in the developing bone of young and maturing fish. In large, shallow-water fish, where bone is dense and penetration of the dye is slow, it is often necessary to keep adding alizarin to the potassium hydroxide until no more dye is taken up and the solution remains its original color. In some deep-sea fish, especially the Isospondylids, certain parts of the skeleton, such as the fins, take

up only a trace of alizarin, and often none. These little known fish are always prepared with a control specimen.

Otoliths do not react to alizarin. Stained otoliths have never been observed in hundreds of specimens prepared, including shallow water and deep-sea forms from Bermuda, Hudson Gorge, and the Pacific. According to Signor Bercighi, of the Florence Museum of Palaeontology, otoliths are 94.87 per cent calcium carbonate, with only .35 per cent calcium phosphate which latter is the chief constituent of bone. The analysis

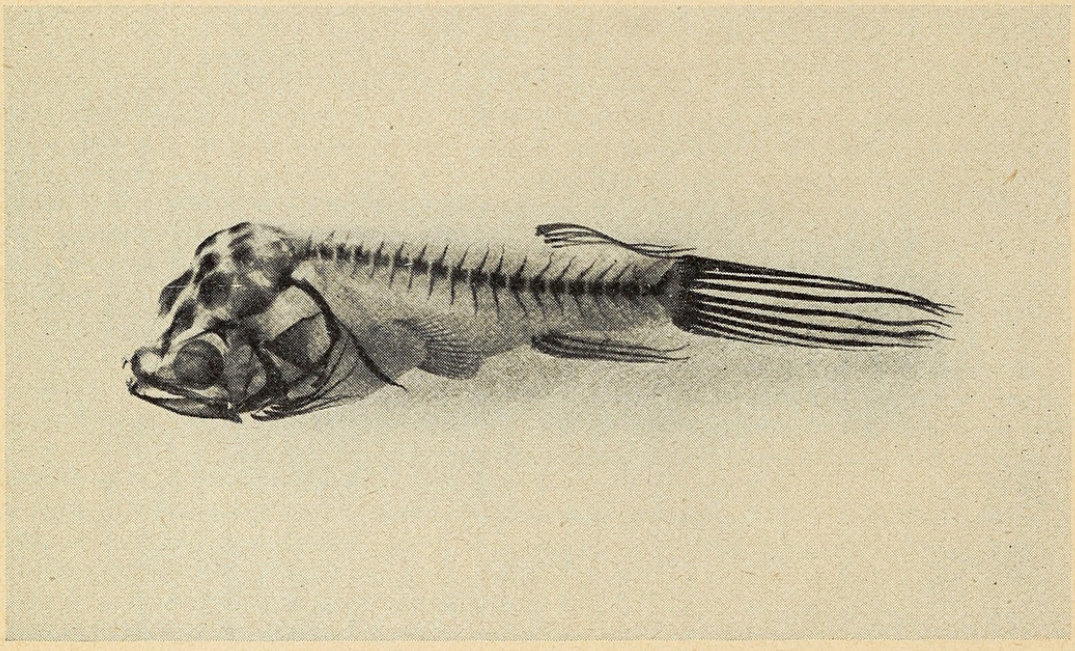

Fig. 19. *Haplophryne hudsonius* Beebe. Through the dermal balloon-like covering of the body (15 mm standard length) the exact number of hitherto obscure fin rays, teeth and delicate illicium are distinct.

of bone shows its major constituent to be calcium phosphate with a small amount of calcium carbonate. Human bone is 85 per cent calcium phosphate and only 10 per cent calcium carbonate.

The vertebrae of eels, pikes, salmon, and cod have 32.46, 38.70, 36.64, and 57.65 per cent respectively of calcium phosphate, and 3.64, 14.30, 1.01, and 4.81 per cent of calcium carbonate. (Animal Chemistry with Reference to the Physiology and Pathology of Man. By J. Franz Simon 1846.)

These facts show a certain correlation concerning the chemical affinity of alizarin and calcium phosphate of bone and also the apparent in-

sufficiency of calcium phosphate to absorb alizarin in certain parts of deep-sea fish skeletons.

In our collection there are specimens that were stained with alizarin ten years ago. Some of these preparations have been kept in the dark and others in the light and there is no evidence that the skeletons have lost any of their original color tone.

Clearing process

Clearing in conjunction with alizarin stain, has been tried with several oils, glycerine, sodium hydroxide, and potassium hydroxide. The most effective and harmless solutions are glycerine, and potassium hydroxide used separately and in per cents varying in strength with the kind of tissues to be cleared.

The most transparent and the most perfect preparations have resulted from immersing specimens in a pure dilute solution of KOH for a short time only. Prepare KOH from white sticks U.S.P. I have found it important, especially when the tissues are delicate, to immerse the specimens in a weak solution of KOH, 1 to 4 per cent, with a large percentage of pure glycerine early in the clearing process. In general, the addition of at least 40 parts of glycerine to the KOH, immediately after the removal of the specimen from the alizarin solution, gives excellent results. The most effective time to use the KOH without glycerine is immediately before and after the specimens are immersed in alizarin solution. The length of time in the KOH must be determined entirely by the size and the character of the specimen to be cleared, and its reaction and constant change. In the first step of using KOH before the alizarin solution, the specimens should be transferred to the alizarin as soon as the tissues show signs of clearing. After staining is complete, transfer specimens to a bath of weak potassium hydroxide just long enough to remove most of the alizarin from the tissues, then change to a solution of part KOH and part glycerine, as mentioned before. Use from 1 to 4 per cent, ordinarily, and up to 20 per cent for a short time only with large, tough, stubborn individuals.

Individual variation in clearing is very noticeable even in fish of the same size, and each specimen must be watched and the solution varied according to individual changes and progress.

After specimens are removed from the alizarin solution to the potas-

sium hydroxide clearing solution, ultra-violet light from an alpine sun lamp hastens the clearing perceptibly. Irradiation with ultra-violet can continue, if necessary, while the specimens are run up gradually into pure glycerine which is the final step. (Mall's solution is not satisfactory with fish. F. P. Mall, 1906.)

Ultra-violet light

Uses for decoloration and depigmentation: Instead of acids and bleaching fluids to remove pigment and excess alizarin from the

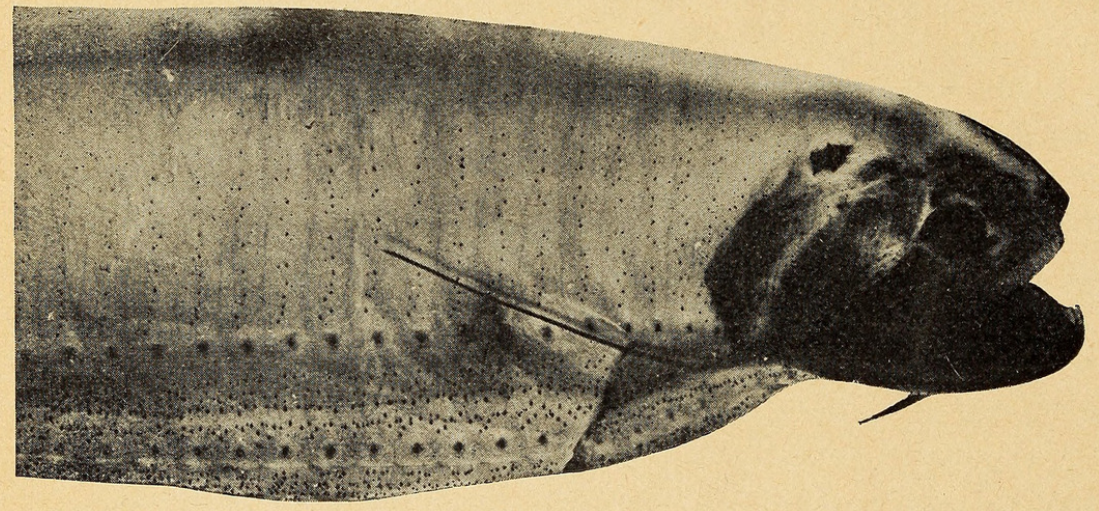

Fig. 20. A jet black deep sea fish, *Echiostoma ctenobarba* Parr, after irradiation, showing the number and arrangement of minute skin photophores which are almost indistinguishable until the black pigment is bleached before the final clearing.

flesh, ultra-violet light from an alpine sun lamp has been used with success for several years. It is doubly valuable because, along with its power to bleach, it speeds up the clearing of the tissues and often makes possible immediate study of prepared material.

During a certain phase of the clearing process, sunlight is a necessary agent to extract or bleach pigment from the tissues. Many times just when the specimens had reached this critical stage, a prolonged spell of sunless days would make progress in depigmentation impossible. In the absence of the sun's rays I experimented with incubator heat and bleaching agents, such as hydrogen peroxide, ammonium hydroxide, and various acids. For some time a controlled substitute for the rays of the sun was sought. Finally, exposing the specimens to ultra-violet rays

was tried. This is the only treatment which, without damaging the specimens, will extract the black pigment from deep-sea fish and render the tissues crystal clear with every detail of bone visible. Good results were had in combination with several solutions but by far the best was weak sodium or potassium hydroxide.

The great convenience of having the essence of sunlight under control in the laboratory ready for use when the specimens need bleaching cannot be overestimated.

Use ultra-violet immediately after removing specimens from alizarin staining solution and for as long as necessary to thoroughly bleach and clear.

Sulfuric and hydrochloric acids are dangerous because they attack not only the dye in the bones but the bones themselves. Also, ammonium hydroxide is much too potent for bleaching deep-sea fish and most shallow water fish. Many specimens became opaque and the tissues swollen and flaky when immersed in this fluid. Acetone, to remove fat, is injurious and not necessary when ultra-violet is used.

Permanent preservation

The preservation of cleared and alizarin stained specimens is permanent when they are kept in chemically pure white glycerine with a small thymol crystal. Thymol is vital to prevent mould if specimens are to be kept in a warm temperature. Glycerine should be renewed with the slightest indication of discoloration.

Another very important factor in the preservation of cleared specimens is the use of glass or rubber stoppers instead of cork. After a short time cork discolors the glycerine which in turn affects the tissues of the specimens, turning them dark brown.

Specimens with controls have been kept in the light and in the dark for experimental purposes with no apparent difference. Indirect sunlight does not seem to have any affect, but in order to be certain of no further change in completed preparations our collection is kept in semi-darkness.

Anatomical study

Value of cleared and stained specimens: The most important value of cleared specimens is the elimination of dissection and with the

possibility of injury, derangement, and loss of bones and organs. Cleared specimens that have been stained for bone with alizarin are not only valuable for a detailed study of the skeleton, but for a study of the various organs with their structural relationships. The following characters are some of those of which the study is greatly simplified in cleared specimens.

EXTERNAL: The delicate devolepment of barbels, or filaments on the head, and especially the tentacles in certain deep-sea fishes.

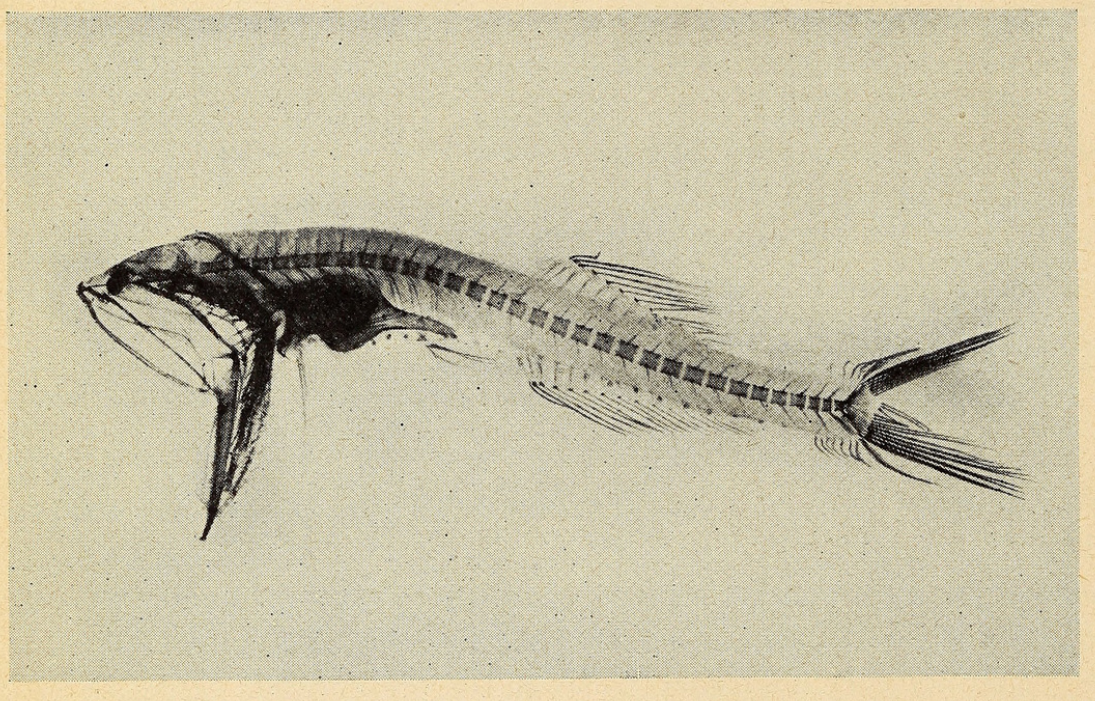

Fig. 21. *Cyclothone microdon* Günther, showing besides the skeleton, the shape of the alimentary canal system and food contents thus eliminating the need for dissection.

The accurate count of spines and soft rays in the paired and median fins even when not composed of bone. It has been found in many cases that the shorter spines and rays are hidden completely, and visible only when the flesh is transparent. In certain deep-sea fishes the fins are submerged in membrane which makes recognition of the rays impossible.

The course and intricate structure of the lateral line is traced more easily.

The photophores, their structure, number, arrangement and distribution in the skin and fins, some being visible in no other way.

Identification of the type of scales; whether placoid, ganoid, cycloid, ctenoid and the specialization of the lateral line scales. Growth striae are easily traced when stained with alizarin. *Dolichopteryx* was considered scaleless until stained and cleared.

INTERNAL: The number and development of teeth, and their attachment, whether implanted in sockets of the jaw bone, or ankylosed with it, or imbedded in the mucous membrane. The presence of enamel covering the dentine is indicated by remaining uncolored by alizarin.

The shape of the stomach, whether prolonged into a blind pouch, or bulbous, or leading directly into the intestine.

The food content of the stomach can be identified often without dissection.

The presence of eggs can often be determined.

Otoliths, which have never been observed to stain with alizarin, are easily located, standing out in contrast to the red skull bones. The sagitta is always evident and often the capillus and asteriscus.

Development of cartilage and bone and areas of deposition.

Delicate structures such as the details of the anterior dorsal spines of the trigger fish, and the migration of the eye of the flounder with the change in the skull bones.

SUMMARY OF TECHNIQUE

1. Fix large specimens in 70 per cent alcohol.
2. Bath in distilled water, few minutes.
3. Bath in potassium hydroxide, KOH, 1 to 4 per cent according to size of specimen and quality of tissues. Length of time according to individual. Prepare KOH from white sticks U.S.P.
4. Alizarin dye solution in potassium hydroxide, KOH, of 1 to 4 per cent. KOH per cent and length of time according to individual.
5. Potassium hydroxide, KOH, 1 to 20 per cent with or without pure glycerine, according to individual. Irradiation with ultra-violet light.
6. Irradiation with ultra-violet light and specimen in potassium hydroxide, KOH, with 40 per cent glycerine. Length of time according to the individual.

7. Potassium hydroxide, KOH, decreasing amounts with increasing amounts of pure glycerine, and ultra-violet light if still necessary. Length of time for each change according to individual progress.

8. Chemically pure, white glycerine with thymol in rubber or glass topped container.

Bibliography

These few references are restricted to techniques of potassium hydroxide, and potassium hydroxide with alizarin, in an attempt to simplify the literature.

It is interesting to note that Schultze mentions Beale as the first to realize the value of potash and glycerine for clearing. Schultze, however, was the first to set forth a definite adequate technique in the use of these solutions.

Potassium hydroxide without dye

Beale, Lionel S.

1858 King's College, London.
Dr. Beale's Archives of Medicine, No. 11, 1858.
On Making Transparent Tissues more Opaque, and Opaque Tissues more Transparent.
Material; Human Embryos.
Mentions value of glycerine, also alcohol with caustic soda to make tissues transparent. Prepared first human embryo in 1853, clearing it in alcohol with caustic soda.

Schultze, Oscar

1897 Professor at Würzburg Universität.
Anat. Anzeiger, Vol. 13. April, 1897.
Verhandlungen der Anatomischen Gesellschaft. Pages 3-5.
Ueber Herstellung und Conservirung durchsichtiger Embryonen zum Studium der Skeletbildung.
Material; Human Embryos.
In the above reference the technique is stated in greater detail than in the following reference which is often given.
Gundriss der Entwicklungsgeschichte des Menchen und der Säugethiere. 1897. Page 459.
Material; Human Embryos.

MALL, FRANKLIN P.

1906 Johns Hopkins.
The American Journal of Anatomy.
Vol. 5 1906, No. 4; pages 433-458.
On Ossification Centers in Human Embryos Less Than One Hundred Days Old.
Material; Human Embryos.
Mall is said to be the first to introduce the Schultze method into America.
Mall's technique is slightly modified by using weaker solutions.

STRONG, REUBEN MYRON

1925 Loyola University School of Medicine, Chicago.
The American Journal of Anatomy.
Vol. 36 1925, pages 313-355.
The Order, Time and Rate of Ossification of the Albino Rat Skeleton.
Material; Rats.
Mall's modification of Schultze's technique.

POTASSIUM HYDROXIDE WITH ALIZARIN DYE

LUNDVALL, VON HALVAR

1905 Institute der Universität Lund.
Anat. Anzeiger, Vol. 27, 1905, pages 520-523.
Weiteres über Demonstration embryonaler Skelette.
Material; Human Embryos.
Schultze's method plus alizarin.

DAWSON, ALDEN B.

1926 New York University.
Stain Technology, Vol. 1, No. 4, October, 1926.
A Note on the Staining of the Skeleton of Cleared Specimens with Alizarin Red S.
Material; Rats.
Mall's modification of Schultze's method plus alizarin.

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1929 New York Zoological Society.
Bulletin New York Zoological Society.
March—April, 1929. Vol. XXXII, No. 2.
The Architectural Beauty of Fish.
New Adaptations of an Old Method.
Material; Fish.

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Bulletin New York Zoological Society.
March—April, 1930. Vol. XXXIII, No. 2.
Fish Magic.
Material; Fish.

1932 Bulletin New York Zoological Society.
March—April, 1932, Vol. XXXV, No. 2.
A New Use of Ultra-violet Radiation.
Material; Fish.