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Zoological Preservation and Conservation Techniques IV. Skeletal Preparation

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Contents

Skeleton preparation technique.

Skeletal material found in different animal groups

Protozoa - Many protozoa have skeletons of some kind. The Radiolarians have delicate siliceous skeletons. The Foraminifera are enclosed in shells of calcium carbonate (tests), usually composed of calcite, or they may have skeletons formed of cemented grains of sand. Some make use of other materials such as barium sulphate and strontium sulphate.

Porifera - There is a diversity of materials to be found in the sponge skeleton. Some have a horny collagen-like protein material called spongin, others calcium carbonate spicules, others silica spicules and still others, a mixture of spongin and silica.

Coelenterata - The corals have a skeleton of calcium carbonate while other members of this group have a main skeleton of aragonite. There are chitinous threads running through many of these matrices. Some gorgonian corals have a skeletal component of a collagen-like protein called gorgonin. Many hydroids have a chitinous perisarc and some have a stiffening of calcium deposits.

Worm-like lower invertebrates - Some secrete hard calcite tubes, while others have bristles of chitin or tanned protein.

Bryozoa - These animals secrete variable amounts of calcite in their tubes and the cases that surround them.

Mollusca - The external shell shows a great variety of forms in this group. There is sometimes an outer proteinaceous layer called the periostracum, inside which is a layer of calcite or aragonite. Cephalopods mostly have internal shells of horn or calcite etc.

Arthropoda - The exoskeleton found in this group is formed from the protein chitin. This material is very often heavily impregnated with calcium salts in the Crustacea and Myriopoda.

Brachiopoda - These animals look very similar to the Molluscs but they are totally unrelated to this group. They have two main types of external shells, one chitino-phosphatic in the smaller species, the other larger groups, having a protein-calcium carbonate basis.

Echinodermata - These animals have external tests made up of calcite-like material with magnesium taking the place of some of the calcium. There is virtually no organic matrix in the mineral skeleton.

Vertebrata - The bone forming the skeletal material of many of the vertebrates is essentially phosphates of calcium laid down in a matrix of collagen fibres. Other substances present are water, proteins and certain polysaccharides, with the remaining bone component being calcium phosphate.

The other important skeletal material found in vertebrates is cartilage. This is a complex and variable substance, consisting of cells lying in a matrix of collagen fibres, mucopolysaccarides and water. Cartilage also often occurs in invertebrates. It is the support of the tentacles of many sedentary polychaetes, and in some molluscs it supports the radula. In these examples the cartilage is formed of collagen and polysaccarides and never become calcified

The various techniques of invertebrate skeletal preparation:

Protozoa - The skeletons of radiolarians are prepared according to whether recent or fossil material is being prepared. For recent material, place in clean water and decant several times to remove debris. Finally add to the fresh water sample a few drops of nitric acid. When the skeletons, or tests,

are clean and free from any tissue, transfer to fresh water and either store in distilled water with a few crystals of thymol (to prevent mould formation) until they can be examined, or place into 80% alcohol. These samples can be mounted for microscopic examination in the dry state, but it is now more usual to dehydrate through the alcohols clear in xylene and to mount under a coverslip in Canada balsam or some other resinous mountant.

When dealing with fossil samples of Radiolaria, the earth containing them is broken into small fragments and boiled in water to which a little sodium carbonate has been added. Boil for 30 minutes and then decant several times, before transferring to fresh water. Add a few drops of nitric acid and when the tests are clean, transfer to distilled water or alcohol, as for the recent samples.

For foraminiferans, separation and cleaning of fossil samples is carried out by boiling in a dilute sodium carbonate solution. The organic material from recent samples is best removed by treating in hot 10% sodium hydroxide. Free either recent or fossil material from sand by washing through a series of sieves gauged at so many meshes to the inch. The series 40, 80, 100, 150, 200, 300 and 400 are the most suitable mesh sizes to use. Sometimes the bolting silk used for plankton trawls is used to sieve, and this can go down to a few microns in mesh size. When sorting, it is often found that sand grains will still pass through a sieve mesh and a spinning method may be adopted for the final separation of the tests. Place the sample in a little water and gently swirl the water round and round the container. The lighter tests will collect at the sides of the vessel (watch glass or petri dish), leaving the heavier sand grains in the centre at the bottom of the container.

Porifera - The skeletal structure of the sponges is usually left untouched as the samples are generally kept in the dry state. It is, however, also necessary to prepare the skeletal supporting material for identification and general taxonomic research. The spicules of silica and calcium carbonate are separated and mounted as microscopical preparations for this purpose. Sponge samples are boiled in 10% sodium hydroxide and after careful washing, are treated according to the spicule content.

For siliceous spicules the samples are treated in 10% hydrochloric acid to clean away debris, before final mounting. The calcareous spicules are not treated with acid, as they would dissolve and disappear in this reagent. At the outset, small samples are tested to ascertain the spicule content of the sponge. Great care must be taken to keep all the spicules of a sample, as many are extremely minute and might be missed in the rather crude technique

Coelenterata - The corals are usually kept in the dry state with a few small samples, fluid preserved, to show the expanded polyps and other features of the entire animals. Hydroids are usually fluid preserved and are often stained on microscope slides for identification and taxonomy.

Worm-like lower invertebrates - The calcareous tubes of specimens are usually kept in the dried state, with no special treatment. If containing entire

specimens, especially if they are expanded, the tubes may be fluid preserved. Bristles and other supporting features are often demonstrated by preparing microscopical preparations (wholemounts) of either the entire animal removed from the tube, or various parts of the animal. Transparencies may also be prepared of this type of material by dehydrating through the alcohols into benzyl alcohol in which the bristles show up clearly against the cleared tissues of the body.

Bryozoa - Large colonies are usually kept in the dry state, with small samples fluid preserved for fine detail and identification purposes.

Mollusca - Shell preparations are made by removing the animal from the surrounding shell, with minimal treatment. The radula, if present, should be kept for preparation, also the operculum, when present. Dried shells of freshwater and marine animals are usually lightly smeared with a little vaseline for better preservation and prevention of cracking, but this should not be done to on the shells of terrestrial forms.

Arthropoda - Insect cuticle, forming the exoskeleton, is usually preserved in the dry state, although some samples are fluid preserved for research. Some of the larger Crustacea, also examples of the Arachnida, Myriopoda, Chilopoda and Onychophora may also preserved dry, though the bulk of the collections of these groups will be fluid preserved.

Brachiopoda - The very delicate shells of these creatures require great care in preparation. Specimens are placed in dilute enzyme solution. A few grains of papain are added to the water, in a small watch glass containing the specimen. When digestion of the tissues has taken place, leaving the branchial skeleton intact for removal, very careful drying is essential.

Echinodermata - These animals are all marine and any preparation of the skeletal structure is best carried out by immersion in fresh water after collection. This is a most effective narcotising and finally killing agent. All examples can be then injected with formol-acetic acid and allowed to dry in the dark for a few weeks. In almost all cases, good colour preservation of the external skeleton is maintained.

Various techniques of vertebrate skeletal preparation

For the preparation of bony skeletons, proceed as follows:-

1. dissection
2. maceration; natural, chemical, enzyme, antiformin, animal
3. bleaching
4. degreasing

Dissection

Removal of the skin followed by very careful evisceration. It is advantageous to remove as much muscular tissue as possible without damaging ligamentary attachments. The best preparations are generally the result of very careful dissection and tissue removal.

Maceration, natural

Cold water maceration is the oldest and remains one of the most efficient means of tissue removal for skeletal preparation. Unfortunately, the conditions under which the technique is carried out are very disagreeable. The nauseating smell and the possible hazard from bacterial infection are the two major drawbacks. Hot water maceration has many advantages. The method is equally applicable to freshly killed animals, deep frozen and also dried specimens. As is the case in cold water maceration, the animal is carefully skinned and fleshed at the outset. It is essential to make sure that rudimentary clavicles, hyoid apparatus, etc. are not lost and that where they occur, penis bones (bacula), and the epipubic bones which are found in marsupials and some reptiles, are not discarded unnoticed with the waste tissues. It is a good plan to become very familiar with the skeleton of the subject animal before proceeding. If possible consult drawings, mounted skeletons and alizarin transparencies, at the outset, especially where rare or valuable material is concerned.

Maceration, chemical

The smaller vertebrates may be processed in the articulated state; rat, mouse, vole, shrew, mole, lizard, amphibia and fish (Harris,1959). Larger forms such as guinea pig, hedgehog, rabbit, cat, dog, pigeon, chicken etc., should be divided thus:- head and neck region, ribcage (entire abdominal skeleton in birds), lumbar-sacral-caudal region with pelvic girdle, fore-limbs with scapulae and lastly the hind-limbs. The animal or portion of animal is simmered in a 5% solution of sodium carbonate until the flesh is tender and can be stripped from the bones easily. There will be great variation in the times of simmering according to the size and age of the specimen. The removal of the adherent tissue can be facilitated by gently brushing with a paste of calcium hypochlorite (bleaching powder) in water, using an old toothbrush. If it seems that the bones are separating too readily the sample may be placed in a bath of 80% alcohol to reharden the ligamentary attachments after which it may be further simmered till all the flesh has been removed.

Maceration, enzymatic

Size and fragility are sources of much difficulty in the successful preparation of osteological material. These difficulties can be largely overcome by the use of enzymes, pancreatin and papain and the macerator antiformin. Most small vertebrates with the exception of fish and some amphibians, may be prepared by the following methods (Luther,1949).

Pancreatin - The specimen to be treated with pancreatin is carefully skinned, eviscerated and defleshed, leaving the skeleton attached by cartilaginous and ligamentary means. Limbs and skull are not usually separated unless this happens accidentally.

Transfer the specimen to ***Rowley's fluid:***

Sodium sulphide	1gm
Pancreatin	2gm
Normal saline	1L

The normal saline is made up according to the animal to be treated. Mammal saline is 0.9% NaCl in distilled water, bird and reptile saline 0.75% and amphibian saline 0.65%. The specimen is gently simmered in this solution. The times taken will be varied for the size of the animal and also the varying strengths of the enzyme batches. usually after 30 minutes it is advisable to remove the specimen and examine for signs of maceration. As soon as the flesh adherent to the bones is seen to come away easily the process is halted and the sample transferred to a bowl of warm water, any remaining flesh being removed either manually with a blunt seeker or an old tooth brush, according to the size of the specimen. Note: this method, although making use of an enzyme, does not follow the usual practice with these reagents of incubation in the appropriate saline. With papain, however, the normal procedure is adopted. Procedures for defatting and bleaching are as described below.

Papain - The specimen is skinned, eviscerated, etc. as before and is then placed in the appropriate saline and papain added, 0.5gm per 100ml of saline. The specimen is then incubated in the resultant fluid for 24 hours. The specimen will, after a few hours, disintegrate into its separate bones. The method is used for complete disarticulation and is so complete that it is not recommended for juvenile material, as the epiphyses of long bones and other attachments will separate. The bones are cleaned without handling and for very small vertebrates this can be a distinct advantage. The vertebrae of shrews, for example, may be prepared to show a clean flesh-free technique. The incubating papain has a foul smell so that the container should be placed in a fume chamber or be well stoppered. An important point to remember is that papain solutions may be used repeatedly at slightly higher temperatures. A mouse can be macerated completely in 8 hours at 40°C. The sludge forming at the bottom of the container is simply filtered off. Defatting and bleaching are seldom required after papain treatment. The bones are air dried and show no signs of cracking or fragility.

Maceration, Antiformin

For samples long preserved in formaldehyde or alcohol, there is a useful technique, in which small skulls and skeletons may be satisfactorily prepared. This is by using antiformin, a microbiological reagent used for the removal of fats and mucins in tissue culture work. It has been found that small lizard, fish, snake and mammal skulls are particularly well prepared by this method. A 10% solution of antiformin in distilled water is made up and the specimen immersed in this, after a few hours in running water to remove the preservative. After some hours the specimen is examined for signs of

maceration. Ear ossicles, teeth and other fragile elements are usually found attached to their points of contact. The bones may need bleaching and defatting, although in a majority of cases bleaching only is necessary. The antiformin stock solution is prepared as follows:-

Sodium carbonate	150gm
Bleaching powder	100gm
Distilled water	1L

Place the sodium carbonate in 250ml of the water and the bleaching powder in the remainder. Mix the two solutions and shake for 2 to 4 hours at intervals. Filter, and add to the filtrate an equal quantity of 15% sodium hydroxide in distilled water.

Maceration, animal

The animals used for maceration purposes are mainly the following:- arthropods; dermestid beetle larvae, cockroaches and crayfish; Vertebrates; fish and amphibian larvae

Dermestid beetles - Various species of *Dermestes* are used. One of the most common is *Dermestes maculatus* (Hall and Russell,1933). These are kept in an escape proof box at a temperature of 75 to 80°F. The bottom of the box is covered with meatmeal to which a pinch or two of yeast is added. This helps egg laying and ensures the continuation of the colony. Pieces of cotton wool, to provide cover should also be included. The colonies should be transferred to fresh boxes every few months and it is advisable to give the beetles small pieces of raw meat, from time to time. Skulls and skeletons are usually coated with a meat extract solution to interest the larvae and so start the maceration. Preserved material may also be treated but all preserving agents must be thoroughly removed and the bones dried and then coated with bacon grease or other similar substance, to start the colony feeding. In general remove as much skin and meat as possible, giving the colony the opportunity of getting to the bone surface fairly easily. The usual techniques for bleaching and defatting will be necessary.

Cockroaches - These have been used extensively in the U.S.A. but not in the U.K. Bones coated with bacon grease give good results although cockroach colonies are not to easy to maintain in an eating situation as they have long periods when they do not eat in any case.

Crayfish - Maceration by these animals is usually carried out in an aquarium, which must be well aerated because a good deal of pollution is likely to occur in the water during maceration (Sealander and Leonard,1954). The bones, skulls etc. are placed in open ended tubes, so that the crayfish can crawl in to feed. Small fish skeletons are prepared quite well by this technique, as are frogs and toads.

Fish - The guppy, a common tropical fresh water fish kept in aquaria, is a very good macerator when used for preparing skeletons of frog and toad

tadpoles, etc. The specimen will need to be removed when seen to be nearly clean. Excellent tadpole skeletons can be produced by this means. The fish are not fed during the maceration period but are well fed between macerations. Amphibian larvae will macerate in a similar way to guppies but are only available for a short time each year.

Bleaching

This is carried out by immersing the specimen in 10 volume strength hydrogen peroxide solution with the addition of a drop of .0880 ammonia to aid and catalyse the reaction. When the evolution of bubbles ceases and the skeleton is white, carefully wash in tap water. At this point it may be necessary to remove the fat from the bones as many vertebrates tend to leak oil globules from the bone marrow after the simmering treatment.

Degreasing

Specimens are placed in a bath of acetone overnight; larger bones may need drilling to remove excess grease. They may then be dried out in an incubator at 35°C, no hotter, for at higher temperatures the bones have a tendency to twist and split.

Disarticulation technique

This method is applicable to skull preparation, especially medium sized vertebrate skulls. Prepare the skull by any of the usual methods and then thoroughly dry. Fill the skull with dried peas, through the foramen magnum, and cork the foramen securely with a rubber bung. Cover the skull loosely, with a wide (3") bandage wrapped round, to keep the separating bones together. Immerse the skull in tepid water and keep the temperature as steady as possible. The peas swell, causing the bones to separate at their suture lines. After a few specimens have been prepared, it will be possible to work to a definite time schedule. The separated bones are then carefully dried and usually wired together, leaving a gap of ⅛ to ¼ of an inch between the bones. This is a long and painstaking task but it is very well worth attempting. Care must be taken to see that very delicate bones are not split and it might be necessary to help the process along manually, by using a mounted needle or forceps to loosen attachments.

Decalcification technique

Bones may be decalcified to show how soft and pliable they may become when the mineral component is removed, for comparison with the normal specimens. The method is especially useful for brain preparation. Decalcification fluids such as Gooding and Stuarts solution and 10% hydrochloric acid are the most suitable. Take for example the preparation of a sheep brain. Remove the skin from the head and deflesh as much as possible leaving the eyes intact. Remove the lower jaw etc. Place in a solution of 10 %

formaldehyde and leave for at least two weeks. Wash out the preservative thoroughly, in running tap water and place in a solution of 10% hydrochloric acid. After two days, the skull bone will be of the consistency of thin cardboard and may be easily cut away with scissors. Careful removal of the skull bones will leave the brain intact. Place the brain into a 5% formaldehyde solution for 12 hours to ensure that preservation is complete.

Cartilaginous Skeletons

These may be prepared in the fluid preserved or dry state.

Fluid preserved skeletons

The dogfish is taken here as an example. Dissect out the pectoral and pelvic fins and girdles, and remove the head with the gills attached from the cartilaginous backbone and caudal fin. Place the various parts in a solution of 3% hydrochloric acid. Leave for 12 to 24 hours. Test with a finger to see if softening and maceration have commenced. If not, leave for a few more hours. When ready, gently remove the major portions of flesh and finally neutralise in a bath of 2% ammonia. Leave for an hour. Do not clean any further at this stage. Prepare alcohol baths of 25, 50, 75 and 90%. Place for 5 hours in 25%, 24 hours in each of 50 and 75% and a week in 90% alcohol. Then commence the final cleaning. Use a tooth brush (bristle not nylon) and gently brush on a cream made of bleaching powder and water to remove the final flesh remnants. Bleach after this stage in 3% hydrogen peroxide. Store or mount in 70% alcohol. A great deal of experience and skill is required to achieve a reasonable result.

Rapid technique useful for teaching - The specimen is roughly fleshed by placing under running hotwater tap, transferring to 70% alcohol from time to time to prevent separation. Brush away flesh with a paste of bleaching powder in water and when completed store in 70% alcohol

Dry preserved skeletons

Fluid preserved cartilage skeletons may be carefully dehydrated into paraffin wax and polyethylene glycols, to store in the dry state. The method is useful for the demonstration of the chondrocranium of elasmobranchs, axial skeletons of elasmobranchs and holosteans, persistent notochords of cyclostomes, chimaeras and the dipnoans. Embryological material of all the vertebrates is well demonstrated by this method.

Technique - After dehydration, place in equal parts of absolute alcohol and toluene for a day and then into pure toluene for 2 days. Transfer to toluene and paraffin wax equal parts at 45°C for a day and from there into several changes of pure wax for 2 hours each change at 60°C and then remove the specimen and allow to cool and set.

Skeletal Transparencies

Alizarin transparencies for bone

This stain is calcium specific and is used to stain bone and other calcium deposits in vertebrates and some invertebrate animals.

History - It has been known since the eighteenth century that the bones of animals fed on madder would become stained red. Madder is obtained from the roots of *Rubia tinctorum L.* and *R. perigrina*, which are found growing in Western Europe. An Indian variety *R. cordifolia*, is used for making the dye Turkey red. This was one of the dyes used by the ancient Egyptians and in the 15th century, dyers used it with Brazil red, to make a richer shade of red. Pliny describes it as a very common dye used for wool and leather and as a paint made from the leaves and fibres of the plant. It was one of the best dyes for wool and cotton but it has now been superseded by the synthetic product alizarin. Madder is, however, still valuable to physiologists, to enable them to make an accurate study of bone development. Animals fed on it, as stated above, show a red colouration of bones, and in addition, the claws and beaks of birds become coloured. Two related compounds occur in madder. They are alizarin and purpurin. The synthesis of alizarin, in 1869, gradually led to the end of the cultivation of the plant and to the synthesis of related compounds, some of which are very important in the dyeing industry.

A soluble sulphonate, alizarin red S, can be made from alizarin and is the reagent used in the preparation of transparencies. There are also various colour shades now available. Alizarin is too insoluble for microtechnique but purpurin, although only sparingly soluble in alcohol or water, is used for histo-chemical tests for calcium. The alizarin red S is prepared as a saturated alcoholic solution. The addition of an alkali causes the formation of an insoluble dye lake. This principle is involved when preparing whole transparencies of vertebrates and for certain calcium specific structures in some invertebrates. There are numerous ways in which to carry out this technique, but one of the most successful methods is as follows (Cumley, *et al*, Gray,1939; Harris,1960).

Technique - The specimen is first prepared for treatment, then fixed and rendered partially transparent in a solution of either sodium or potassium hydroxide. Staining with alizarin then takes place, followed by differentiation and clearing, with final storage in a selected preservative.

Before fixation it is necessary in the case of vertebrate specimens to skin and usually eviscerate the animal concerned; it may also be prudent to remove excess muscular tissue in some cases. Nevertheless, it is often quite possible to obtain satisfactory results from small specimens in the entire condition. Initial fixation is in 70% alcohol and takes several days. The specimen is then transferred to a solution of 2% sodium or potassium hydroxide, until the extremities of the limbs appear through the tissue. This is a matter of practice and experience.

The staining in a saturated solution of alizarin red S in absolute alcohol can be either progressive or regressive. In the former method, the stain is added drop by drop until the maximum staining effect is obtained. It should be borne in mind that clearing is continuing all the time in the hydroxide solution. In the latter method, the specimen is placed in a deeply stained hydroxide solution and after a period of time removed. Excess stain is then removed from the tissues by immersion in fresh hydroxide solution. Of the two methods the first, or progressive technique is probably the best for a beginner to commence with. After some experience has been obtained, several specimens can be stained by the regressive method, at the same time. The colour of the stain, rich port wine, only occurs when the saturated alcoholic dye is added to the alkaline hydroxide solution; the alizarin stock solution being a light brown colour.

The staining of the bones and the clearing of the specimen takes place at the same time, and after several changes of fresh hydroxide solution the differentiation of the clearing specimen becomes much more obvious. The actual time of staining is related to the size of the specimen and the amount of calcium in the skeleton; usually, staining has taken place in a few hours. The hydroxide solution is mixed with glycerin, so that after a few changes the specimen is fully cleared and transparent in pure glycerine. The procedure can be carried out as follows:-

Staining and differentiation are followed by removal of excess stain, using a freshly prepared hydroxide solution. When no more stain comes out of the specimen, transfer to equal parts of 2% aqueous hydroxide and glycerine and leave for several days. Then, transfer to a 50% glycerine solution in distilled water, for 2 to 3 days. By now the specimen should be almost clear. Finally, store in pure glycerine in a container with a well fitting stopper or lid. The most commonly used alternative method of clearing is to dehydrate through the alcohols to methyl benzoate, storing in a second change of this reagent. The glycerine method is, however, generally considered to be superior.

The alizarin transparency technique is a most useful method in genetics, embryology and taxonomic research. Small specimens which would be extremely difficult to prepare as dry osteological preparations such as small amphibians, reptiles, and juvenile specimens of mammals and birds are beautifully prepared by this method.

Examples of invertebrate animals containing calcareous structures, which make valuable alizarin transparencies are molluscs with internal shells, decapod crustaceans with a gastric mill, and sea urchins with the Aristotle's lantern.

The alizarin transparency may be described as an X-ray in three dimensions, with the added advantage that it may be handled and freely manipulated during examination.

Cartilage transparencies

The whole technique depends on the use of an acid stain and is, in every way the exact opposite of the alizarin transparency method. It is for this reason that it is extremely difficult to combine a calcium and cartilage specific staining technique in one specimen although it is occasionally successful.

Van Wijhe (1902) technique for cartilage transparencies

Embryos, small cartilaginous fish, amphibian larvae and similar specimens intended for this technique should be formalin fixed. This seems to give a sharper staining reaction. If, however, the specimen is already in alcohol, the technique can still be attempted and quite good results will be achieved. Following fixation, the specimen is placed in acid alcohol (1% hydrochloric acid in 70% alcohol), for one week. It is then transferred to a solution of 0.25% methylene blue in acid alcohol, for 2 weeks. The excess stain is washed out in 1% aqueous hydrochloric acid, and the time to stop destaining is immediately the entire specimen seems to be white in colour. Any staining of the cartilage will not be seen at this stage. Dehydration now commences, up through the alcohols, into toluene as the final storage solution. If the specimen is small enough for microscopic examination on a glass slide it can be mounted in Canada balsam. Alternatively, the larger specimens may be cleared through aqueous dilute acid and glycerine, and finally stored in glycerine.

Modifications of this technique, using victoria blue and toluidine blue instead of methylene blue, are available. Victoria blue is generally considered to give the best results of any of the techniques for demonstrating cartilage. The same schedule is followed as for the classical method of Van Wijhe except that 1% Victoria blue is the staining solution, and also the clearing solution is different. When differentiation is complete dehydrate in 90% alcohol for 12 hours, then into several changes of absolute alcohol for the same times, followed by clearing in methyl benzoate. Store in fresh methyl benzoate. Alternatively, oil of wintergreen (methyl salicylate) may be used as the clearing agent.

Specimens fixed in formaldehyde, to which acetic acid has been added, give brilliant results with toluidine blue but only fair results with methylene blue. In this case, specimens are cleared into glycerine, which is the final storage solution. If, however, a specimen is small enough for microscopy, it may be dehydrated via the alcohols, cleared in xylene and mounted in Canada balsam.

Technique combining bone and cartilage staining in one specimen

Fix the fresh specimen in 10% formaldehyde for 2 to 3 days or longer. The technique will also work for material which is already formalin or alcohol fixed (Dingerkus and Uhler,1977). Wash in several changes of distilled water

for 2 to 3 days. Skin and eviscerate the specimen as far as possible. Place in the following solution for 24 to 48 hours.:-

Alcian blue 8 GN	10gm
95% Alcohol	80mls
Glacial acetic acid	20mls

Then transfer to 2 changes of 95% alcohol, 2 to 3 hours in each. Hydrate through 70%, 50%, 30% and 15% alcohol, 2 to 3 hours in each until the specimen sinks. Then transfer to the enzyme solution:-

Trypsin	1gm
Sodium borate	30mls saturated aqueous
Distilled water	70mls

Change this solution every 2 to 3 days, or sooner if the solution takes on a bluish colour. Continue until the bones and cartilage are clearly visible and the flesh retains no blue colour; this may take 2 to 3 weeks. Transfer to 0.5 aqueous potassium hydroxide solution, to which enough alizarin red S has been added to make the solution deep purple. Leave for 24 hours, or until the bones are distinctly red. Transfer for a few days in each of the following solutions: a mixture of 3 parts of 0.5% potassium hydroxide to 1 part of glycerine, then equal parts of hydroxide and glycerine, followed by 1 part hydroxide solution to 3 parts of glycerine, and finally into pure glycerine. Note: 3 or 4 drops of 3% hydrogen peroxide per 100ml of solution may be added to the final two hydroxide/glycerine solutions to bleach away the pigments of dark specimens. Store the specimens in pure glycerine to which a few crystals of thymol have been added.

Results - Cartilage, blue; bone, red.