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Title: Zoological Preservation and Conservation Techniques III. Injection, Corrosion and Miscellaneous techniques

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Source: Harris, R. H. (1990). Zoological Preservation and Conservation Techniques III. Injection, Corrosion and Miscellaneous techniques. *Journal of Biological Curation*, *Volume 1 Number 2*, 35 - 52.

URL: http://www.natsca.org/article/1041

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Zoological Preservation and Conservation Techniques III. Injection, Corrosion and Miscellaneous techniques

R. H. Harris

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Injection technique.

History

Before 1650 there existed only a few references to injection experiments in the bibliography of biology. Galen mentions the inflation of cerebral vessels by passing air through a tube. It has been stated that Giliani of Persceto, who died in 1326, filled blood vessels with liquids of different colours, which thickened and hardened after injection, but did not decompose. Unfortunately, it has not been possible to confirm this statement, made by Michele Medici.

The first reference to the use of injection, after the invention of printing, occurs in a commentary by Jacobus Berengarius published in 1521. He used a syringe and injected the renal veins with warm water. Massa in 1536 inflated the kidneys by forcing air into the renal vein, and Stephanus in 1545 devised a pump to inflate the vessels with air. Sylvius (Jaques Dubois), who arranged for the publication of his methods after his death, introduced "coloured fluids such as saffron and various wines", but he rejected the injection method because the liquids escaped whenever a vessel was cut through. In 1556, the Portuguese physician Amatus Lusitanius, filled vessels with a liquid through a siphon and also forced it through a tube filled by air pressure from the mouth.

Eustachius is no doubt one of the pioneers of the modern injection technique. Unfortunately, he made the error of thinking that by his technique there was a direct connection between the arteries and the uriniferous tubules and this mistake was taught in anatomical schools throughout the 18th century. It was not until Bowman published his work on the kidney in 1842, that the correct relationship between the renal arteries, veins and the uriniferous tubules was first demonstrated. Crook in 1615, suggested the use of quills, glass trunks and various reeds to blow up vessels.

At about this time, the possibility of diverting blood from the vessels of one animal to another, a transfusion, was first conceived. The earliest writers to mention transfusion seem to be Magnus Pegel in 1604, Andreas Libavius in 1615 and Johannes Cole in 1628. It is interesting to note that all this took place before Harvey's discovery of the circulation of the blood. It would have been most appropriate if Harvey had discussed injection technique but his treatise on circulation does not mention any methods (Singer,1957).

By 1666 Malpighi was investigating the structure of the kidney incorporating injection methods. He used ink and a black liquid mixed with spirits of wine and also urine coloured with ink. The first use of gelatine in injection media was made by Robert Boyle in 1663. Grew in 1681, asserted that Boyle had also used wax as an injection medium. Boyle most probably used lead acetate which has a waxy constituency. In 1672, Willis was likely to have been the first to inject an invertebrate when he treated the heart and gills of a lobster.

Swammerdam is generally regarded as the inventor of the solidifying injection media. Priority of publication must be given to Boyle and possibly Pequet but Swammerdam, probably one of the greatest anatomists of all time, really deserves the credit. The first wax injection of Swammerdam was carried out in a house in Leyden on the 22nd of January 1667. This work and other relevant information was communicated to a colleague, Frederick Ruysch and it was this worker, who as Professor of Anatomy at Amsterdam University developed what is known as the "Ruyschian Art".

He vastly improved the technique of injection and in 1666 by order of the Dutch Government was instructed to prepare the British Admiral Sir William Berkeley who had been killed in action on the 1st of June and return the body to England. The cadaver was stated at the time to be "as fresh as an infant". There is still little known about the formulae used by Ruysch. His great friend Boerhavve, who was present on many occasions when Ruysch was working, says nothing about the methods used.

When Peter the Great acquired Ruysch's collection in 1717 he stipulated that the preparations were to be accompanied by a full description of the methods used. In 1742 an account of this work based on a copy of Ruysch's handwriting was published by J. C. Rieger. Rieger had been in the employ of Peter the Great, after whose death in 1725 he retired to Holland, where he compiled a work which includes a description of Ruysch's methods. From this work we learn that a body was first placed in cold water for a day or two before injection. The aorta and the vena cava were slit and the blood "pressed out". The body was then immersed in hot water for several hours. The injection was carried out as follows:- in winter, suet or tallow was used and during summer a little white wax was added. The actual injecting mass seems to have been made with suet or wax added to turpentine and resin. The colouring agent was vermilion and wax was used to seal the injection sites to prevent escape of the injection medium. The injected specimen was preserved in diluted alcohol which Ruysch made from barley. The strength was stated to be around 60% which was far too weak for any permanent preservation. No obvious deterioration was noted in Ruysch's lifetime however. It is now known that dilute alcohol acts as a dissociating agent and would, in time, macerate the entire specimen. It was therefore obvious that Ruysch concealed much more than he disclosed.

There have been many conjectures by anatomists as to the exact composition of Ruysch's media but none carry the authority of the definite but meagre statements of Rieger. Jesse Foot published a statement in 1794 in which he says "I saw the preparations belonging to Ruysch, which are deposited in the museum in St. Petersburg, going apace in decay". Before this in 1748, Lieberkuhn had examined examples of Ruysch's injection masses. He considered them too fluid to last, also that the preparations would not stand microscopic examination. Besides injection technique, Ruysch was also a pioneer in the inflation of lymphatic vessels with subsequent drying.

From Ruysch to Lieberkuhn, who first established the methods for micro-injection, there were few further advances made at the time. Lieberkuhn shares with Ruysch some of the most important advances in the practice of technical anatomical methods. He carried Ruysch's work still further and was one of the first to inject microscopic vessels. Most of his material was human in origin and sixty of his microscopical preparations are still in the Museum of Human Anatomy in Vienna University. A few preparations have been made from fish, frog, tortoise, as well as some from ox and horse material. Other preparations are in the Berlin University Museum and there are said to be a few of Lieberkuhn's specimens in the Museum of the University of St. Petersburg, being described by Burdach in 1817. Lieberkuhn's work was so good that a hundred years later Henle was using them for the purposes of original research. The period of the two Monro articles and the Hunter brothers is now reached.

Alexander Monro wrote two articles on preparation, neither of which contained much original material but they were very influential. The first was an account of the injection methods of the time and the second a comprehensive synopsis of current basic methods. Nevertheless he produced an incredible amount of work. He carried out probably the first, or one of the first, triple injections. He was certainly the first to inject an echinoderm. Some of these preparations may be seen in the Museum of Anatomy in Edinburgh, although there is now some doubt as to the preparator authenticity of some of some of the specimens. With very few exceptions, up to the time of Monro all material was mammal in origin.

In 1752 William and John Hunter injected the epididymis with mercury. William Hunter taught anatomy from 1746 to 1783 and four of his lectures in eighty two were devoted to the techniques of injection, a proportion large enough to emphasise the importance of such work in those days. William Hunter's work appears to be too similar to that of Lieberkuhn to have evolved independently.

John Hunter in some notes, written about 1770 but not published until 1861, seemed to prefer stale or unpreserved material for his injection methods. Occasionally he would use alcohol preserved specimens. His injection media consisted of resin, tallow, turpentine, hog's lard, butter, glue and isinglass. Colours were provided by hydrated copper (blue), vermilion and flake white. The first general treatise on anatomical injection was published by Pole in 1790. No original research was embodied in the work but four types of injection media were described: coarse injection, seven formulae; fine injection, six formulae; minute injection, six formulae; and mercurial injection. A cold injection which sets after some hours was added on the authority of William Hunter. The coarse injections were wax based, the fine injections varnish based and the minute injections were based on gelatin. According to Pole, mercury injections were going out of fashion and seldom used but in 1819 Rudolphi injected a liver fluke with this reagent. In 1843 the distinguished anatomist Straus-Durchheim published a careful review of

work based on first-hand investigations. He also classified methods into three main groups: coarse injection media, fifteen formulae; fine injection media, nine formulae and in addition corrosion methods. Substances tested were many and various, yellow and white wax, tallow, lard, spermaceti, fatty oils, essential oils especially turpentine and lavender, resin, plaster, gelatin, egg white, water, alcohol, fusible metal and mercury. He regarded mercury as one of the worst methods and soon after this publication it ceased to be used seriously. His fusible metal consisted of bismuth 3 parts, lead 5 parts, tin 3 parts and a small amount of mercury to lower the melting point. He considered the gelatin method probably the best and liked the isinglass method, coagulating the watery medium with ferric sulphate.

The work of Strauss-Durchheim is said to mark the termination of the historical period in which injection methods were developed and tested. From 1843 onwards the modern techniques developed rapidly (Cole,1921).

Sites for injection.

Invertebrates - In all cases the vascular system, if present, is the usual site for injection, good examples being the vascular systems of crayfish and freshwater mussel. Sometimes in very small invertebrates, such as liver flukes, chance injection is tried with considerable success. A small incision is made in the median line near the hind end of the animal. The alimentary tract is injected by using a site about 1mm from the median line. With a little practice very successful preparations can be made. Earthworms and related species may be injected through the main blood vessels remembering to inject from the hind end forward. Echinoderms and related animals may be successfully injected in a similar way.

Fish - The arteries and veins in the animal, the afferent and efferent branchial systems are used, mainly through the region presented when the tail is cut off to reveal the caudal vessels.

Amphibians and Reptiles: In these animals the arterial arches, heart and abdominal vein are the normal sites for injection.

Birds - These are very vascular animals. Sites in the legs are the best for injection but with experience, both the neck vessels and the heart may also be used.

Mammals - Depending on the size of the specimen it is usual to attempt injection through the femoral arteries and veins. Arteries are usually empty after death and are therefore quite easy to inject. Veins, on the other hand, are usually overfull with blood. Here, drainage, followed by perfusion is often necessary. It may be possible to force injection media through the vein contents, but the results are not usually very successful.

General considerations - Obtain the experimental animal as soon as possible after death. Incisions are then made in the selected spots. Cannulae or needles are fixed in position in the femoral arteries, veins, or sometimes

the carotid artery and jugular vein. Warm saline is then placed in an aspirator which is suspended above the animal and the vascular system is flushed through until there is no more blood present. Five to ten litres are required for an animal of rabbit to dog size.

Triple injections of small vertebrates.

Usually a rabbit, pigeon, small cat or dog is selected for this purpose. The thorax is opened and the heart exposed. After removing the pericardium, cannulae are inserted in the right and left ventricles. The system is then flushed out with warm saline. If using an injection medium containing gelatin, the animal must be kept warm, usually in a tank or similar container. Inject red media into the left ventricle, noticing the reddening of the feet area (pads), claw bases, and lips and ear regions. Blue media will be injected into the right ventricle, noting the blue colour appearing in the liver and adjacent vessels. The two colours will not mix for the fine capillaries form a barrier between the two injected systems. The third injection is made into the hepatic portal vein in a direction away from the liver. The whole injection is then coagulated by cooling or other chemical means and left for some days. Final dissection, either to display the fine detail, or for general demonstration, may then be carried out.

Points to note - Injection technique is not difficult but requires a good basic knowledge of anatomy and a great deal of patience. Do not, on any account, attempt to use rubber latex as an injection medium until thoroughly competent with the gelatin based media. Constant practice will provide valuable teaching material.

Recent developments in injection technique.

Some interesting work has been carried out in Kenya whereby animals in the entire state have been injected with fixatives in the normal standing anatomical position. By using suitable frames and selecting sites for injection with care, it has been possible to treat full grown ostriches, antelopes, zebras etc. As the organs have been injected in normal anatomical positions, all the organs are in their correct situations and may be considered to represent the lifelike appearance in all respects. Many injections and dissections are spoiled by the pressures exerted on the organs at post mortem when the animal is worked on in the prone or other supine position.

Knower, in 1908, recommended the use of glass bulbs with the finest capillary points. He comments, "If a gentle warmth is applied to a capillary tube, while a fine point of the tube is held below the surface of some fluid, such as Indian ink, air will be driven out of the bulb and the ink will run up to replace the water as the bulb cools. If the point of the tube is inserted into the desired blood vessel the ink may then be injected by warming the bulb."

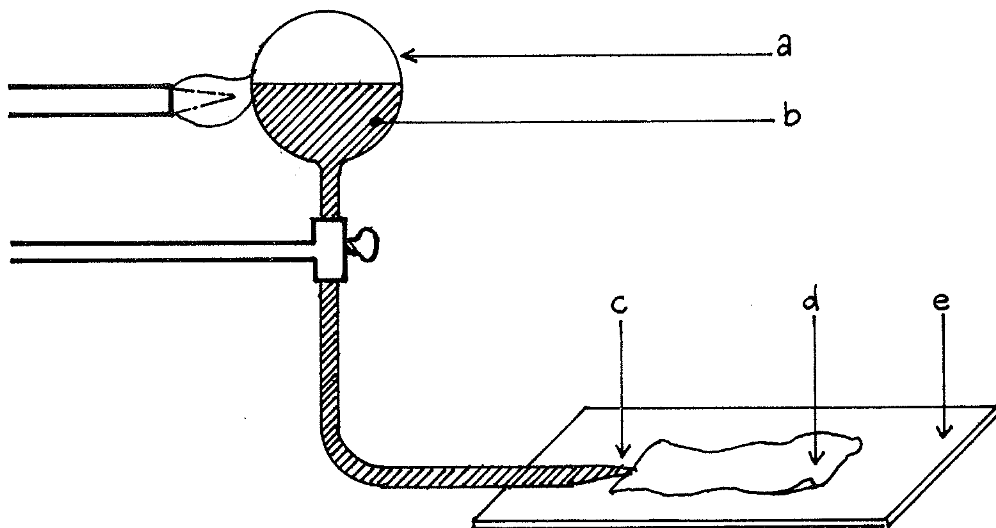

Fig 1. A simple technique for small specimens.
a. glass bulb; b. injection fluid; c. fine-drawn capillary glass tube;
d. specimen; e. glass slide.

He used the method very successfully on small fish, amphibia, reptiles, birds and mammals. Its best application is for small embryonic specimens.

Injection masses used at the present time.

1 Starch
2 Milk
3 Plaster of Paris
4 Gelatin
5 Celloidin
6 Wood's metal
7 Latex
8 Phenol-glycerin-alcohol

Celloidin and Wood's metal are used for corrosion following injection, phenol-glycerin-alcohol for embalming following injection.

1. **Starch** - An excellent method which is still used, as follows:-.

Water	100ml
Glycerin	20ml
Formaldehyde	20ml
Powdered starch	75gm

Add the water and the glycerine to the starch rubbing out all the lumps. For colouring use :- for yellow - 10 gms chrome yellow; green - add 10 gms of chrome green; and red - 10gms of vermilion. Strain through cheese cloth and add the formaldehyde. There are many aqueous solvent dyes available from I.C.I. which may be used as alternatives to the dyes mentioned above.

2. **Milk** - Fresh milk may be injected and the following formula used as a coagulating agent.

Formaldehyde	75 parts
Acetic acid	15 parts
Water	1000 parts

I.C.I. dyes may be used for colouring purposes

3. **Plaster of Paris** - The finest dental plaster is used. A very dilute solution is made up and mixed with dilute Seccotine. This lengthens the time of coagulation and makes the resulting injection mass harder and less brittle.

4a. **Cold gelatine injection masses.**

Metagelatin - If a small amount of ammonia is added to gelatin and the solution heated for several hours, the mixture passes into the state of metagelatin. In this state it no longer coagulates on cooling and thus may be injected without warming either the injection mass or the specimen being injected. Colouring agents may be added to this medium and it may be thinned with weak (30%) alcohol. After injection specimens are placed into strong alcohol which sets the mass.

Tantler's gelatine injection mass - 5gms of the finest gelatin (it is usual to use Coignet's Gold Label sheet gelatin for this purpose) soaked in 100ml of distilled water, melted, and any dye required added. 5 to 6gms of potassium iodide is added slowly to the melted medium. After injection coagulation is carried out by immersing in 5% formaldehyde. Specimens injected in this fashion withstand decalcification.

Mejeko's injection mass - 10% sodium salicylate will retard setting of gelatin for hours. Coagulation is effected in 2 to 4% formaldehyde.

4b. **Hot gelatin injection methods**

It is usual make up a 15% solution of gelatin in water to which chromopaque or I.C.I. dyes may be added as required.

5. **Celloidin** - Dilutions may be formulated from the histological reagent 'Necoloidine', a 4% solution in a mixture of ether and absolute alcohol. Inject this reagent to prepare a corrosion specimen. The resultant injection mass is resistant to decalcification and to most strong acids and alkalies.

6. **Wood's metal** - This is a low melting point alloy, of many different formulae, containing bismuth, tin, lead and (usually) cadmium, in variable proportion. It melts at 66 to 72°F.and is used in lung preparations and for other vascular tissues.

7. **Latex** - Usually obtained as a rubber solution in strong ammonia. It is coagulated in solutions of acetic acid. Is capable of great dilution but technique must be good for the reagent will permit no mistakes. Gelatin, however, can in most cases, be remelted for a fresh attempt at injection, provided of course, this is done before coagulation reagents have been added to solidify the mass. Latex once set, forms an irreversible gel. It is resistant to dilute acids and alkalis and can be used for transparency preparations.

8. **Phenol-glycerin-alcohol** - This is an embalming agent with the formula:-

Liquor phenol	4 parts
Formaldehyde	1 part
Glycerin	4 parts
96% alcohol	4 parts
Water	12 parts

Specimens may be injected and then left to dry in the atmosphere of the injection medium or in a tank of the solution as required.

Volumes needed for various animals:-

Rabbit	1000mls
Dog	2 - 5000mls
Cat	1000mls
Gazelles	5 - 10,000mls
Cattle	20 - 30,000mls
Horse	40 - 50,000mls
Human	5000mls
Dogfish	20 - 50mls
Frog	5 - 10mls
Crayfish	2 - 5mls
Earthworm	0.5ml
Liver fluke	very small drop

Corrosion Techniques

History

In 1882 Paul Schieffendecker described a technique using celloidin (cellulose nitrate) dissolved in ether. After injection the evaporation of the ether left a cast of celloidin. The original tissues could then be dissolved away in strong hydrochloric acid which did not dissolve the cast. As ether does not mix with water the evaporation was slow. In addition, the dry celloidin cast tended to crumble so it was found necessary to keep the cast in a fluid preservative.

In 1899 Carl Storch introduced the use of celluloid (cellulose nitrate) which was highly inflammable and dangerous to use. Most authors refer to the use of X-ray film as part of the technique and this means that they probably meant celluloid. The X-ray film were taken from used stock and dissolved in acetone. The modern X-ray film is non-flammable and not suitable for corrosion formulae.

In 1936 Narat introduced the use of a vinyl resin called Vinylite dissolved in acetone as a substitute for cellulose acetate, and in 1948 the cold setting polyester resins Marco resin and Castolite were introduced successfully. The

histological reagent celloidin, used in differing dilutions to suit the size of the corrosion specimen, is also a very successful corrosion reagent.

Technique - Celloidin is usually diluted in acetone, and as this reagent is miscible with water the celloidin will precipitate out of solution to form a cast on the site of the injection.

For fine capillaries or vessels the following solution is suggested:-

Acetone	100ml	
Celloidin	3gm	
Camphor	3gm	(This reagent is added to make the cast more pliable and less liable to cracking etc.)

For coarse vessels use the following:-

Acetone	100ml
Celloidin	10gm
Camphor	8gm

After injection the specimen is placed in cold water in a refrigerator at 4°C for at least 24 hours and then placed in a bath of concentrated hydrochloric acid. This will macerate all the tissue away leaving the injection cast revealed. The method can be used for lung, liver, blood vessels in fact all vascular systems. In recent years great strides have been made by Tompsett working at the Royal College of Surgeons who has successfully prepared entire juvenile human material. He worked with plastic injection media using mainly the polyester resins. It is not advisable to try these reagents until fully proficient with celloidin corrosion. The advantage of the plastic media is that it can be kept in the dry state. Celloidin corrosion preparations are usually kept in a jar of fluid (alcohol or formaldehyde based formulae).

Specific examples of the technique

Corrosion preparation of the Lungs - The organs are obtained fresh and are washed out in warm saline and if no leaks show after inflation, the specimen is considered suitable for treatment. The thin solution is run in first, the time and amount depending on the size of the specimen. Only the alveoli area and base of the lungs are filled with the fine solution and then the thicker solution is run in on top. This may take several hours and the lung must be palpated to remove air bubbles. The injected lung is placed in cold water in a refrigerator, as previously mentioned, and left for 24 hours or so. The specimen is then placed in concentrated hydrochloric acid and the tissues allowed to digest away. After 24 hours direct a stream of water over the half digested tissue to wash it gently away. Although the casts are usually kept in a fluid condition it is sometimes convenient to have a dry preparation to hand, even if eventual breakdown is a risk. In such a case allow the specimen to dry thoroughly and then spray it with a fine transparent lacquer or shellac.

Pettigrew's method for a corrosion technique applied to the kidneys.

Procedure - The injection mass consists of a solution of celloidin in acetone. As acetone solutions are miscible with water the celloidin is rapidly precipitated out of solution, whenever water is encountered. Thus, by injecting the celloidin solution into the lumen of blood vessels or any other vascular system, when moisture is present, one soon obtains a deposition of celloidin forming a cast. When the parenchyma of an organ with the vessels so injected is macerated, the celloidin cast is revealed.

Materials for the injection mass - Acetone, Celloidin (Scherings) and camphor. For fine injections it is essential to use celloidin but for more coarse work old used X-ray film was used with success. This is no longer available as the film is now plastic based. The reason for this preference was simply one of expense, as the celloidin, a form of gun cotton dissolved in an ether alcohol mixture, is very expensive. The masses used are very similar to the method described earlier but a source of pressure is used with Pettigrew's technique.

Fine injection mass A.

Acetone 100ml
Celloidin 3gm
Camphor 2gm

Coarse injection mass B.

Acetone 100ml
Celloidin 4gm
Camphor 3gm

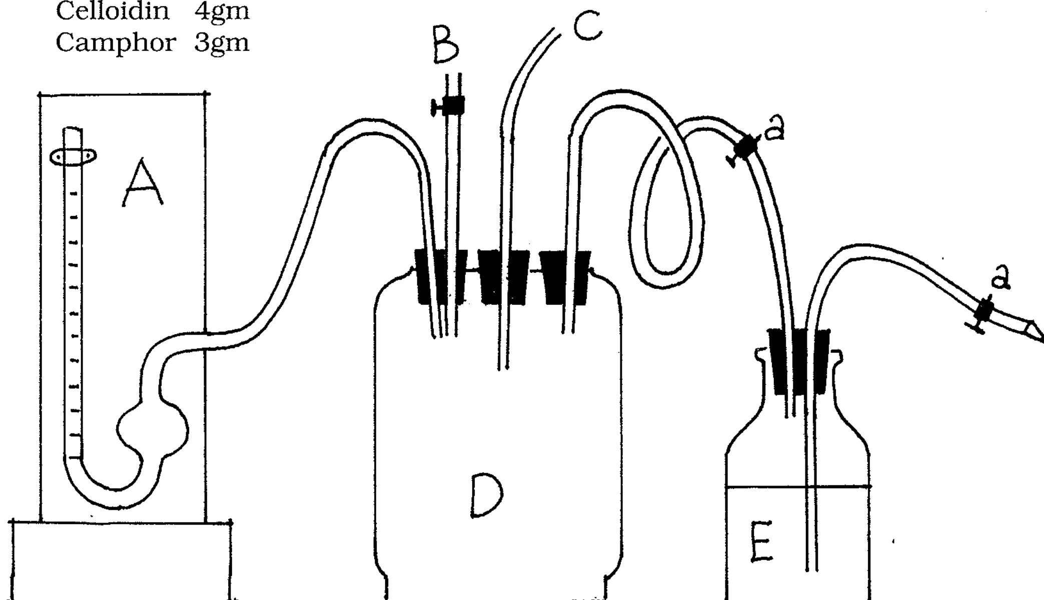

Fig 2 Apparatus for injecting masses under pressure using Pettigrew's method. A. manometer; b. safety valve; C.source of pressure; D. Wolff bottle; E. pressure bottle; F. canula; a. screw clamps.

Apparatus - From the stock bottle the solutions are transferred to pressure bottles of 250ml capacity.

Pressures required:

A. fine structures:	1. Arterial	350	to	600mm Hg.
	2. Venous	100	to	200mm Hg.
B. coarse structures:	1. Arterial	200	to	300mm Hg.
	2. Venous	80	to	100mm Hg.
	3. Renal pelvis	50	to	80mm Hg.

Hochstetter's wax impregnation technique.

History

Wolhard (1914-1915) wrote a paper on the impregnation of heart tissue for anatomical purposes, followed by Voss (1926) who described methods for wax infiltration of gross anatomical specimens of various kinds.

In 1927, Hochstetter, who may be regarded as the originator of the technique concerning impregnation of plants and animals, wrote a paper in which he used ether as an intermediate reagent between the alcohol and wax. Briefly, the technique is similar to block preparation for paraffin wax histology except that in the final phase all excess wax is removed from the external surface of the tissue. The use of ether with molten wax is not recommended because of fire risks and general safety hazard considerations.

In 1951 Rack wrote a paper in which he suggested the use of stearine with paraffin wax to keep the final impregnation softer and to prevent cracking. Although this sometimes occurs, most impregnations are successfully carried out, without recourse to this refinement.

Method

Taking a small grass snake as an example, the reptile is narcotised and killed, arranged in the required position and then injected with a suitable fixative such as 70% alcohol. Inject in several places along the body to be certain of complete penetration and preservation. After 24 hours fixation the specimen is transferred to ascending grades of alcohol, 2 days or so in each. Either industrial methylated spirit or isopropyl alcohols may be used. The grades are made up in 10% dilutions starting at 70% and thence by degrees to 90%. From 90% the specimen is placed in absolute alcohol and after 24 hours into a mixture of equal parts of chloroform and absolute alcohol. After 12 hours transfer to pure chloroform to complete the clearing. The tissues of the specimen are now completely impregnated with chloroform which is miscible with molten paraffin wax. From pure chloroform the specimen is placed in a bath of equal parts of chloroform and molten paraffin wax at 56°C melting point and after 8 hours to a bath of the pure wax. After 12 hours

immersion in this the specimen may be injected with molten wax if necessary. The needles used for the injection of fixative may be left in place for this purpose.

After thorough impregnation remove the specimen from the wax bath, place it on a filter paper in a dish in the oven and allow the excess wax to drip off. The specimen should be removed from the oven and cooled before any of the wax in the tissues can escape. Before the specimen has cooled completely it should be wiped with a cloth moistened with Inhibisol. It may be necessary to brush specimens with hair or feathers with an Inhibisol moistened brush.

Points arising from the technique. A well impregnated specimen will probably not need any injection as no shrinkage will be seen but in some cases it might be necessary to inflate the specimen slightly to give it a natural, lifelike appearance. The method is suitable for all small zoological specimens and many plants, In addition it has many applications to archaeology.

Brain and nervous system techniques

Preparation of whole brains.

The head is removed from the animal as soon as possible after death. The flesh and fur is then dissected away from the skull leaving it as clean as possible. A small portion of skull bone is removed to allow access of the fixative to the general brain area. Place for at least a week in 10% formaldehyde.

Wash in running tapwater for 12 hours and then transfer to a solution of 10% hydrochloric acid and leave for 4 days to one week. Note: because of the carcinogenic nature of the combination of hydrochloric acid and formaldehyde it is suggested that Gooding and Stewart's solution is used as an alternative to hydrochloric acid. The formula for this decalcifying agent is:-

Formic acid	5 - 15ml
Formaldehyde	5ml
Distilled water to	100ml

Test the consistency of the skull bone by probing with the point of a scalpel. The bone removal can begin when the skull is as soft as a thin card. A fine pair of scissors may be used for this purpose. Brains when prepared in this way are often quite soft and will need further hardening. The specimen is placed in the following solution:-

Chrome alum	2.5gm
Copper acetate	5.0gm
Glacial acetic acid	5.0ml
4% formaldehyde	10.0ml
Distilled water	77.5ml

Boil the alum in the distilled water and when dissolved add the copper acetate and then the acetic acid. Allow to cool and then add the formaldehyde. This is a useful alternative to the technique previously mentioned where 5% formaldehyde is used as the final storage solution. Brains may be successfully freeze dried or wax impregnated if required.

Mounting of the entire brain.

The hardened brain is now ready for mounting as a museum or study specimen. To mount it in a firm and stable position attach the organ to a glass or plastic slip that can be placed inside the museum jar. A glass rod of suitable size is pushed into the brain far enough to disappear from view. It will then possible to sew through the brain to attach it through small holes previously bored in the glass or plastic slip.

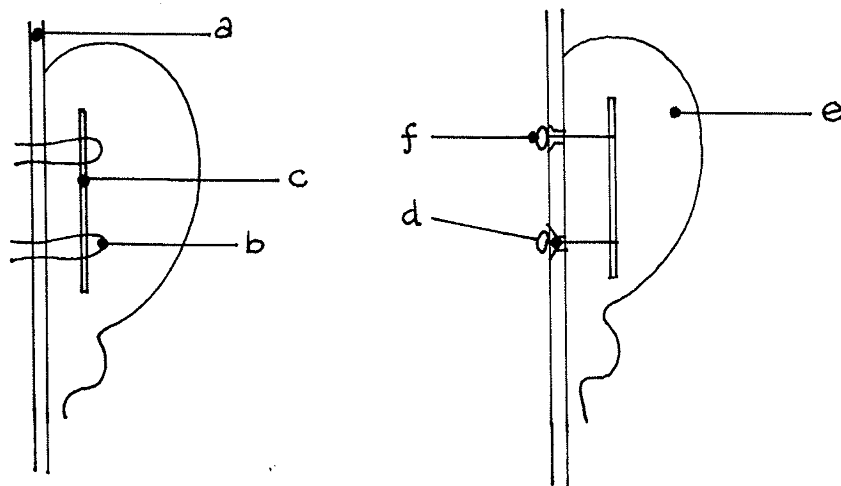

Fig 3 Attachment of preserved brain to glass slip in museum jar.
a. glass slip; b. nylon monofilament; c glass rod; d. countersunk hole;
e. brain; f. small glass bead

Brain decortication technique.

This interesting method of dissecting the grey matter from the white matter of the brain was first described by the Swiss anatomist J. Klinger in 1935 (Meyer,1954). The larger brains of dog, sheep cow or horse are most suitable. Human brains are not so readily available but usually give excellent results. The brain is removed from the skull by the techniques of fixation and decalcification as described above and then placed in 10% formaldehyde for at least 3 months. Klinger stated that the best contrast was achieved if some blood remained in the external vessels. Some form of perfusion of the vessels is necessary before initial fixation can be tried.

The actual method is quite simple. The specimen is washed in running water for a few days and then frozen for several days at 10°C. Freezing

appears to separate and loosen tissue interfaces. After thawing the grey matter is peeled from the white matter with forceps or small rounded cocktail sticks. This work should be carried out in a bowl of water in order to wash away fragments as they break free from the brain surface. The cerebral cortex can be removed entirely to reveal the white cores of the gyri. Most of the major tracts can also be exposed. It takes considerable time to clear all the debris away but the results are very well worth while. The finished dissection is fragile and can be freeze dried with success or wax impregnated.

Brain slice staining technique.

This is a useful method for the demonstration of the grey and white matter of brain tissue (Mulligan,1931).

Grey matter is defined as the neurons or nerve cells and their fibres and dendrites.

White matter is defined as the myelinated nerve fibres and practically no neurons.

Technique - Cut sections of the brain as required. Place in Mulligan's mordant for 2 minutes.

Mulligan's mordant:

Phenol	40gm
Copper sulphate	5gm
Distilled water to	1000ml

Wash in running tap water for 1 minute. Place in 2% iron alum and watch the development of the black colour in the grey matter, which takes about 1 minute for a rat brain section. Wash in running tap water and store in 5% formaldehyde. If left too long everything will be stained. As this is a surface stain no harm will have been done. Simply remove another thin slice and try again.

There is a dry method for this technique whereby the stained slices are taken up through the alcohols and then into absolute alcohol, paraffin wax and naphthalene, equal parts. This mixture is liquid at oven temperatures of between 50 to 60°C. Allow the impregnated slice to solidify, and when dry it should be varnished to prevent loss of the naphthalene. This impregnated tissue is quite satisfactory for general histological work.

A variation of this technique to give a blue and white comparison instead of black and white is as follows. Brain slices are immersed in the mordant as described above for 5 minutes at 60°C. The slice is then placed in iced water for 10 seconds and immersed in freshly made up 2% ferric chloride in distilled water for 45 seconds to 1 minute according to the intensity of the staining required. The slice is then immersed in 1% potassium ferrocyanide for 4 minutes during which time it will turn blue in the grey matter areas.

The specimen should be stored in the following solution:—

Distilled water	75ml
Glycerine	35ml
Formaldehyde	10ml
Citric acid	0.2gm

Other methods of staining the grey matter areas of the brain are as follows.

Orange - Tartar emetic, 5%. Immerse for 6 minutes, wash in running tap water for 1 minute. Place in a solution of hydrogen sulphide (saturated solution of hydrogen sulphide gas dissolved in water plus an equal volume of water) until there is a good strong stain. Wash and preserve in 5% formaldehyde or dry as required, either by wax impregnation or freeze drying.

Yellow - Lead nitrate, 1%. Stain section for 6 minutes, wash in running water for 1 minute. Place in 5% Potassium iodide solution for sufficient time to give the required intensity of staining. Wash and preserve or dry.

To demonstrate staining in both grey and white areas.

Place section in soluble starch solution for 24 hours. Wash in running water for 3 minutes. Place in Gram's iodine until there is a good differentiation, and store in 70% alcohol with a little iodine. The grey matter will be dark purple, the white matter yellow. It is not usually possible to prepare suitable dry sections using this method.

Preparation of intact nervous systems.

This technique was described by Cornwell in 1934. The intact nervous system may be removed from small vertebrates by acid maceration. Non-myelinated autonomic fibres are generally lost together with a few abdominal spinal nerves which extend into the body wall and a few caudal and terminal fibres in the limbs. The technique is not a substitute for dissection but does reveal the surprising bulk of the nervous system.

Technique - Skin and eviscerate the animal. Place in a pan of 30% nitric acid. Agitate from time to time with a glass rod. Bone is quickly decalcified and connective tissue destroyed, muscles will fray and separate. Nerves are protected by their fatty myelin sheaths. After 12 hours immersion clean away with a gentle flow of running tap water. Return to acid if it is difficult to remove eroded material. Save any major nerve that separates and this may be joined to the main system when the preparation is mounted.

Mounting - This is done on a film of gelatine attached to a glass slip in a formaldehyde based preservative. Or alternatively the specimen may be freeze dried, or displayed as a Riker mount on dark coloured cotton wool or similar bedding media.

Staining nerve tissue in entire specimens.

This technique, described by Guyer in 1953, may be applied with considerable success to samples of fish and other small vertebrates.

Three solutions are required:-

1	Potassium hydroxide	1% aqueous solution
2	Glacial acetic acid	1 part
	Glycerin	1 part
	Chloral hydrate 1% aqueous	6 parts
3	Glycerin	1 part
	Ehrlich's haematoxylin	1 part
	Chloral hydrate 1% aqueous	6 parts

A small fish for example is killed in 95% alcohol and left for 72 hours. After evisceration, it is then transferred to the potash solution for 1 to 3 days. By this time the specimen should be more or less transparent. Place in solution 2 for 73 hours and then without washing into solution 3 for one week. Destain in solution 2 for 24 hours and then clear in glycerin. The nervous tissue shows up dark purple against the transparent muscular tissue. The technique can also be applied to a number of invertebrates especially those with a complex nervous system.

Agar support technique for small transparencies and other demonstration specimens.

Some of these items are really too large for conventional microscope slide preparation and at the same time too small for a museum jar or similar container.

The writer has developed a technique for supporting such small or delicate objects and at the same time affording them complete protection. (Harris 1965).

Technique - A 4% agar solution is prepared. Only the best powdered agar is used for this purpose. Using a steamer or perhaps a pressure cooker or small autoclave, remembering not to put the agar under steam pressure but use only free steam. Add a crystal or two of thymol to prevent mould growth. The agar solution hardens on cooling into a translucent mass. To prepare an agar block use a solid watch glass, pour in the melted agar and allow it to set.

Place the specimen, washed clean in several changes of distilled water and gently dried with a paper tissue, on the surface of the set agar. Pour melted agar over the specimen until completely covered and allow to set. The semi opaque block may be 'shelled' out of the watch glass and then trimmed into a small cube. This is then placed into 70% alcohol for a day or so and then slow dehydration is initiated. 80, 90 and absolute alcohols, a few days in each, and then into a mixture of equal parts of absolute alcohol and benzyl alcohols and finally into pure benzyl alcohol.

From the time the block is immersed in the 50:50 absolute and benzyl alcohols the block will have begun to show signs of becoming transparent and

by the time it is in neat benzyl alcohol it will be nearly completely transparent. This cube supports the alizarin or cartilage stained specimen as though it were embedded in plastic. The great advantage of this technique is that the specimen can be removed from the agar by careful dissection if required.

The cubes are kept in benzyl alcohol as a final storage mixture. They can be removed and studied under the microscope or lens for 1 or 3 hours before any drying becomes apparent. There is a tendency for the blocks to tinge a light brown colour over the years but this may be postponed indefinitely by keeping them in the dark between examinations.

Use of agar to demonstrate the internal organs of echinodermata.

All the globular echinoderms are well demonstrated by this technique. The fresh specimen is injected with melted 4% agar and the mixture allowed to set. The specimen is then placed in 70% alcohol for a few days. After washing well in running water, to get rid of the excess alcohol, the specimen is placed in a bath of 5% hydrochloric acid. The test will slowly erode away leaving the internal organs intact in their correct anatomical position. The opaque mass of agar is cleared in the same way as the small transparency blocks already mentioned. The final preserving fluid being benzyl alcohol.

Preserved material may be attempted using the technique but there may be difficulties in making a successful complete injection with the agar.

Holothurians may also be treated in this way. There is no need to use acid but it may be necessary to bleach the external wall of the animal using a 10 vol. strength hydrogen peroxide solution after the agar injection and before the dehydration in alcohol.