

# PREPARATION OF VOUCHER SPECIMENS

## Amphibians & Reptiles

### VOUCHERS

#### *Why Vouchers?*

Voucher specimens are animals or plants that biologists gather to preserve for future documentary and scientific uses. These vouchers serve multiple uses within these two categories. First and foremost, a specimen documents the occurrence of a particular species at a particular location at a particular time. A sight or photographic record is useful for recording the occurrence of a species, but it is inadequate for confirmation. The major scientific importance of an actual voucher specimen is that it offers other people an opportunity to examine the entire plant and animal, and thereby, allows them to confirm or correct the identification of the specimen. The specimens are also a source of biological data. Through their anatomy, biologists are able to study the genetic relationships of population by the variation in different traits. Even as preserved specimens, they yield information on behavior, ecology, and reproduction, also through an examination of their anatomy.

Voucher specimens serve the conservation and management of wildlife and floras. They do this by allowing us to identify accurately what occurs in wildlife sanctuaries and elsewhere, and if our sampling protocols are properly designed, we obtain population and other ecological data when we collect specimens. Without sampling and the retention of vouchers, you cannot protect or manage a plant or animal species or their biological community if you are unaware of what species occur in a sanctuary and the nature of their interactions. Vouchers are critical, because many plants and animals cannot be identified accurately from photographs, and many are simply very difficult to identify even by a trained biologist; these require an “expert” to identify them. Thus, it is necessary to retain sufficient vouchers in order to retain some as part of a synoptic collection at the sanctuary and to have other specimens to send to experts for identification. Further, only with voucher specimens can we accurately map the distribution of each species within a region or country, and this task requires a systematic regional sampling of plants and animals. The mapping of a species’ occurrence identifies the commonness or rarity of a species, identifies the preferred and avoided habitats, and provides actual data to estimate a species management and protection requirements. This wider sampling requires the retention of voucher as well. These specimens can become part of a sanctuary’s or a national park’s synoptic collection or are placed in a central depository, such as a national collection. Each sanctuary benefits by maintenance of a synoptic collection, that is a collection containing representatives of each species. The major benefit of a synoptic collection is that it improves the ability of the staff to recognize [identify] their flora and fauna. The presence of a well-maintained collection is essential, because new staff arrive without knowledge of the fauna and flora, and knowledgeable staff transfer to other sanctuaries. The synoptic collection remains to teach the new staff.

### *How Many?*

A fundamental question is how many animals should be captured and preserved. I recommend a minimum of four specimens. For scientific study, a sample of 15 specimens is barely adequate for statistical reliability and 20-30 specimens are ideal. However, scientific analysis is not always the goal for a voucher collection. Rather for the development of on-site synoptic collections, four specimens is adequate. That number allows you to retain two specimens for the synoptic collection and send two specimens to an expert for identifications.

For some species, obtaining even a single specimen will be difficult because of the elusiveness, rarity, or dangerousness of the species. In those cases, a single specimen when captured should be maintained in the synoptic collection, until a second voucher is obtained in order to forward a specimen for identification. I note here that all specimens which you send me become part of the Smithsonian Institution's permanent collection. Our retention of specimens provides a second permanent depository for voucher specimens and their records. It also makes the specimens readily available for study by research scholars throughout the world and will permit the recognition of new species that may be endemic to Myanmar.

Salvage of specimens is a good method to obtain voucher specimens. Turtle shells from kitchen dumps or elsewhere should be retrieved. Similarly, snakes killed by villagers or any other amphibian or reptile discovered dead should be salvaged. Specimens discovered dead, even if dried [mummified], are collected, data recorded, and tagged by the standard voucher technique. To avoid insect pests in your collection area, each dried specimens should be individually stored in a plastic bag with a fumigant. Animals found freshly killed [typically 24 hr or less from death] can also be prepared in the standard voucher technique. You will, however, need to examine salvaged specimens closely during preservation to ensure that the formalin has stopped the rotting. Even an animal that is very rotten might prove to be an important voucher and should be considered for salvage. In such situations, you may wish to remove the animal's head and preserve it or perhaps move the carcass to a location where it is somewhat protected from marauding animals and later retrieve the skeleton.

### *Record Keeping*

There is a minimum of two items that must be recorded and maintained in association with each voucher specimen: 1) when was the specimen captured [day/month/year] and 2) where was it found [name of sanctuary and precisely where in the sanctuary]. You should also attempt a tentative identification. Even listing a specimen as a ranid frog or an agamid lizard in your record books helps if an accident occurs and specimens or labels are temporarily lost; the identity helps reassociate records and specimens. Other data, such as who collected, time of day, in what specific microhabitat, what it was doing and others can be recorded, because these data help you understand the biology of that species and in association with the specimen help you remember its scientific name.

For best record keeping, each specimen should have a uniquely numbered tag. For amphibians and reptiles attach a tag to the specimen as it is preserved [tied either around the waist or right hindlimb above the knee]. This number **must** be recorded in your field book in direct association with the capture date and locality data. Typically each species is stored in a separate jar; each jar may contain several specimens, perhaps collected at different times and/or locations, but they are all the same species. It is also useful to place a label with the tag number, species identification, and general locality data in the jar with the specimens, because this label helps you locate specimens without taking them out of the jars.

## SOLUTIONS

### *Formalin*

Use.-- Formalin is the best preservative known for amphibians and reptiles. It is used at a dilution of approximately 4%. Only short-term storage in formalin is recommended.

Dilution & management.-- Please handle formalin with care. Paraformaldehyde crystals provide a relatively safe form for transport, but paraformaldehyde is still an irritant if it gets on your clothes or skin. It is also a very light powder and easily blown about; it will cause considerable damage if inhaled or blown into your eyes.

Paraformaldehyde dissolves slowly in "room-temperature" water so it should be mixed with water heated to 50-60°C -- no hotter. If the water contains any sediment, the water must be filter. The sediment coats the paraformaldehyde crystals and prevents them from fully dissolving; instead a semicolloidal solution results that is too dilute for proper preservation. If you are on the coast and have access to seawater, mix the paraformaldehyde with seawater. The seawater salts naturally buffer the formalin and produce an excellent preservative.

If you obtain commercial grade Formaldehyde, it will be stock solution at a concentration of 37%. This solution is diluted 1 part formaldehyde to 9 parts water. Then buffered with magnesium carbonate, about a third teaspoon/liter of formalin.

Voucher specimens can be stored in 4% formalin, but when possible, they should be transferred to a 70% ethyl alcohol solution. If such transfer is not possible, the formalin solution must be buffered otherwise its naturally high acidity will decalcify the bones of the voucher specimens.

### *Alcohol*

Use.-- Ethyl alcohol is the best known solution for the long-term storage of amphibian and reptile specimens. Methyl and isopropyl alcohol are also acceptable for storage although not as effective as ethanol. Any of the alcohols at a dilution of about 25 - 30% can be used as a killing agent for reptiles [injection of small amount into the heart or heart area].

Dilution & management.-- The ideal concentration for long term storage is 65-70%. Lower than 65%, the concentration is inadequate to retard bacterial and fungal growth. Above 80%, the alcohol becomes a dehydrating agent and removes too much water from the specimen and makes it brittle and distorts the shape.

### *“Chlorotone”*

Use.-- An aqueous solution of chlorobutanol makes an excellent anesthetizing agent for amphibians. An overdose/overexposure in solution kills amphibians gently and humanely.

Dilution & management.-- Making a chlorotone solution is somewhat like making a good soup, you have to test [No, do not taste!] the effectiveness of the solution. Your goal is to obtain a solution that totally anesthetizes your frogs in 3 to 5 minutes. You will need a container of about 2 liter capacity with a lid. Fill half full with water [filtered] and add a large pinch of the chlorobutanol crystals. Then add a frog and watch its behavior. If it is still very active after three minutes, add another pinch of crystals, and so on until the frog begins to relax and stop its swimming.

Once made, the solution can be kept for months -- even more than a year -- and remain an effective anesthetizing agent if the container is kept closed. For reference, a liter of water will require less than 1/8 teaspoon of chlorobutanol crystals to be effective.

### *Sodium pentobarbital*

Use.-- An anesthetizing agent for killing reptiles.

Dilution & management.—This chemical is classed as narcotic in the United States. I have a special license to purchase and use it, and I cannot dispense it to others. I mention it, because it is regularly used by veterinarians here and may be available to you. My sodium pentobarbital [nembutal] is an aqueous solution (65mg/ml). I dilute it 50% with distilled or filtered water for injection into reptiles.

## **KILLING SPECIMENS**

The technical literature abounds with supposedly nicer terms [euthanizing , sacrificing] for killing the captured animals. But if we are to obtain voucher specimens, we take the lives of the animals. We must, however, kill them as painlessly and nontraumatically as possible, and as few as possible to obtain our goals for inventory and biological study. The following agents and techniques accomplish that.

Agents.-- Chlorotone for amphibians; sodium pentobarbital for reptiles and if not available a 30-40% solution of alcohol. All killing-agents are administered as drug-overdoes to relax and then stop the heart beat and brain function of an animal prior to its preservation.

Protocols.-- As noted above, amphibians are placed in a container of chlorotone and are fully anesthetized and dead in 5-6 minutes. Reptiles are injected in the heart with alcohol and typically die within less than 30 seconds. Dosage is variable for the different reptiles, but generally 0.1 ml for every centimeter of snout-vent length [SVL] in lizards and snakes, and 1 cc for every cm of plastron length in turtles.

You recognize death in your voucher specimens by a total relaxed and limp state. No muscular reaction if you pinch or prick a limb. Tails still twitch in totally dead snakes so tail movements are not good indicators of death. If you see a muscular reaction, return the frog to the chlorotone solution and repeat the injection for the reptile. Preservation should not begin until you are certain that the animal is dead. Please note that with venomous snakes, you must be especially alert at this stage and during initial preservation, because the reflex-action of a nearly dead snake can produce a fatal bite. Always immobilize the snake's head when testing its state of relaxation and during the initial preservation stages.

## **PRESERVATION OF SPECIMENS**

Preservation requires the use of formalin. Formalin is a toxic chemical that must be used carefully. Use it only in a well ventilated area and avoid breathing the fumes as much as possible when working with it.

Please use rubber gloves when preserving the specimens. Formalin can cause skin damage and some people are more sensitive than others. Use baby-powder when putting on the gloves. Rub some of it onto your hands before inserting them into the gloves; turn the gloves inside out when you are finished so that the insides may dry and then lightly powder. The powder greatly prolongs the life of the gloves and enables you to put them on and take them off easily.

### *Preservation*

Preservation or fixation is the critical step in producing voucher specimens that are useful for identification and for long-term use and retention in your synoptic collection. Preservation should begin as soon as the animal has died. First affix a tag to the specimen; the position of the tag is shown in the following diagrams. All specimens [except tadpoles and no matter how small] are injected with a 4% formalin solution. Frogs and lizards less than 150 mm SVL are injected by inserting the syringe-needle into and through the cloacal wall into the body cavity and sufficient formalin is injected to return the animal to the volume of a well-fed specimens, not until it looks like an over-inflated ballon. For lizards, you should also inject some formalin into the base of the tail and where the tail is too narrow for injection, use a needle to perforate the bottom surface to allow the absorption of formalin. Small snakes are prepared the same way as lizards, but as the length of the snake increases, you will need to move the needle and injections sequentially along the body to ensure that all parts of the body cavity receive an adequate volume of formalin. As you inject snakes, you will see the body swell as the fluid enters and generally you will need to move the

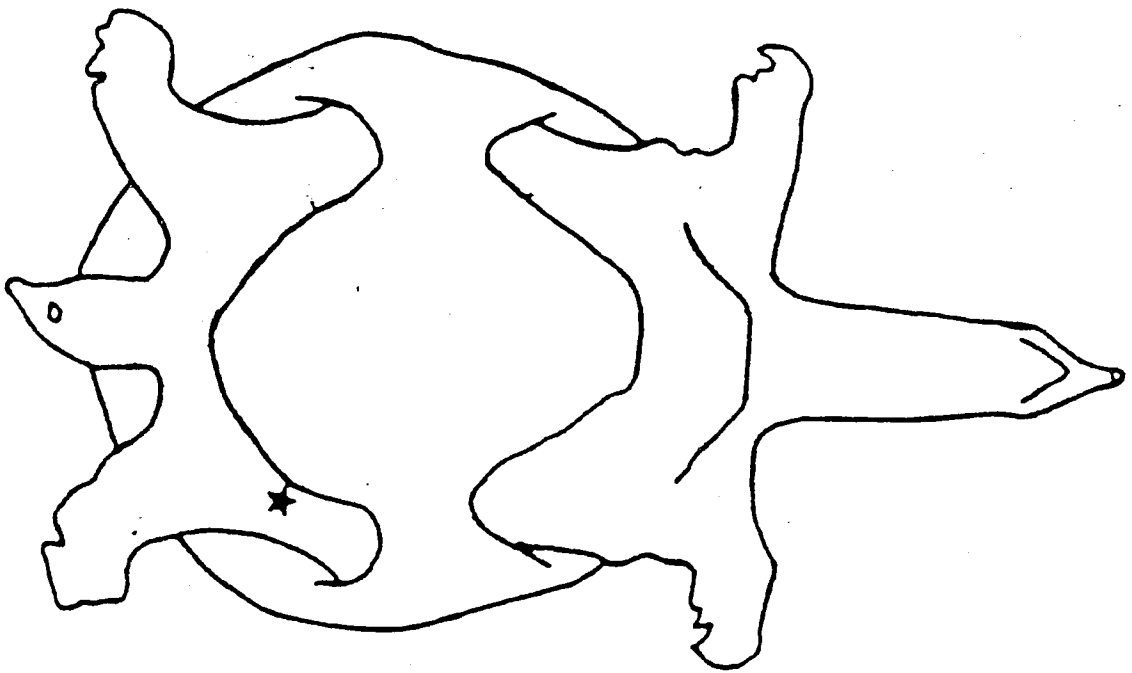
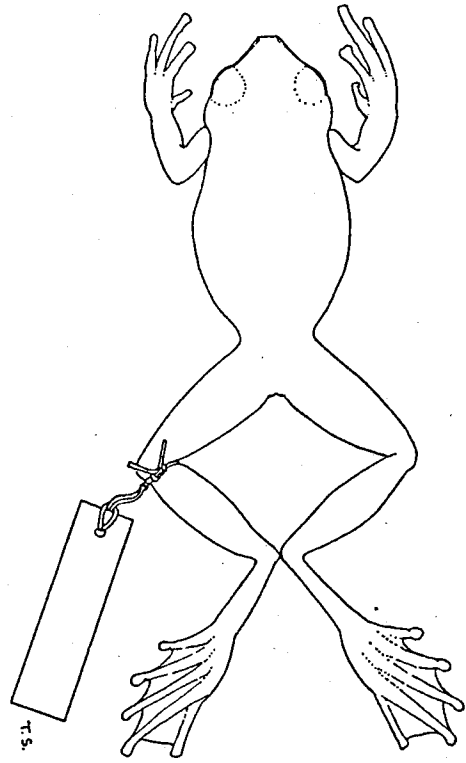
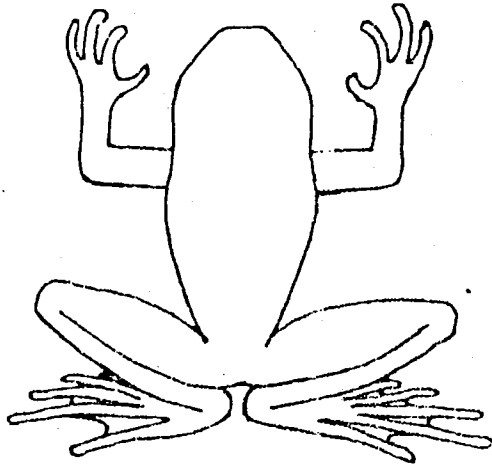
needle about every 20 cm; also inject from the cloaca toward the head, and inject and perforate the tail as in lizards.

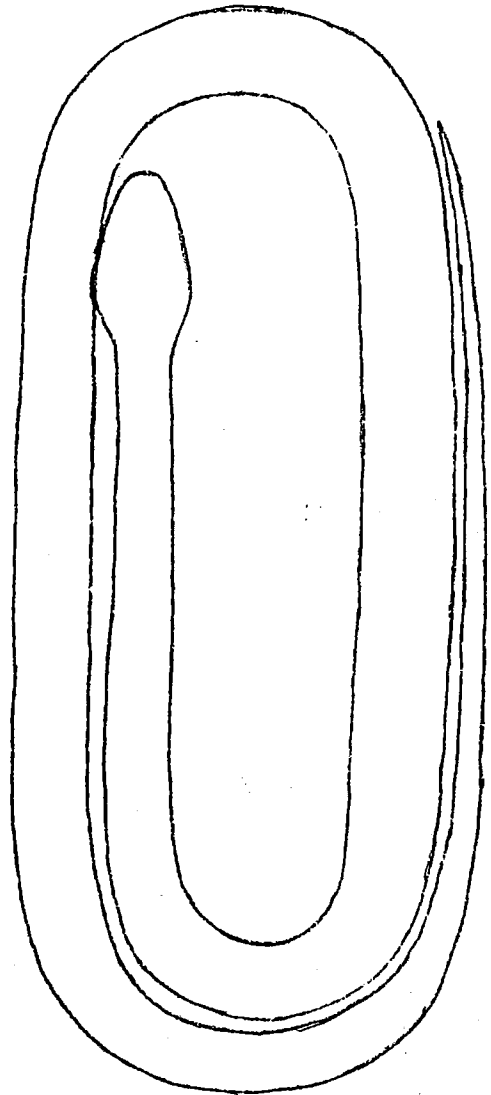
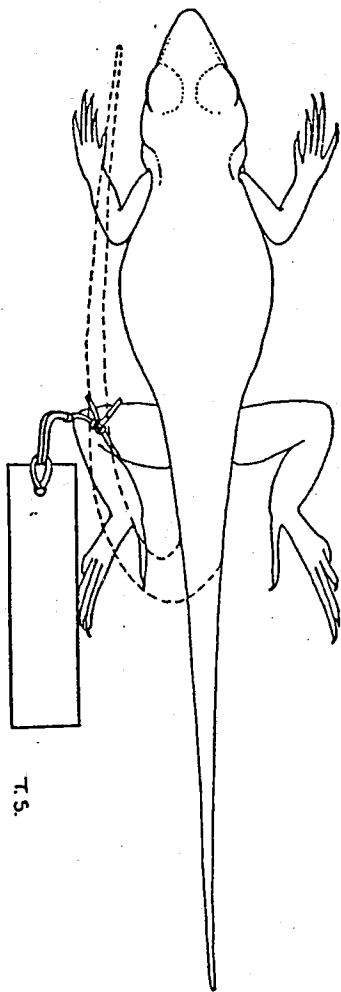
Turtles, because of their shell, offer a special challenge in preservation. First, turtles are difficult to kill and even when they appear dead, they may only be deeply anesthetized. A dead turtle will be totally relaxed! Pick the turtle by the bottom shell [plastron], and holding horizontal, gently shake to and fro. If dead, the head & neck and legs will all fall outward and swing limply. Then inject formalin into the body cavity by inserting the needle into the body cavity immediately in front of the hindlimb. Estimate the volume of the shell and insert about 1/3 to 1/2 that amount of formalin into the body cavity. Each limb, tail, and head-neck also require separate injections. For the limbs insert the needle in the palm/sole of the foot [carefully because skin is thick and you may bend the needle] and inject; then moving needle to the lower leg area to inject the remainder of the limb. The head-neck requires small multiple injections [somewhat like injecting a snake], first at base of neck at the juncture of the carapace and then moving forward with a final injection at the base of the skull. For the tail, typically only an injection at its base is required for females; in males, an additional injection in the middle of the tail may be required to ensure formalin penetration for satisfactory preservation.

Injection is not the final step in preservation. Layout is also required. The following illustrations show the various layout postures for amphibians and reptiles. Once the animal is fixed, the posture during fixation becomes permanent. By carefully adjusting the body and limbs immediately after injection of formalin, the voucher specimen will have a posture that permits easy use for identification and study. I urge careful layout in trays; the additional moments spent at this time, greatly enhances the usefulness of the specimens; thus proper layout must not be neglected. The following illustrations show better than I can describe the preservation postures. Note that for snakes, the specimen should be turned on its back to ensure the penetration of the formalin from the body cavity into the thick muscle mass along the vertebral column; after 24 hours of fixation turn the snake on to its stomach to move the formalin to the ventral surface of the viscera. For turtles, the reverse rotation is recommended, like frogs and lizards, turtles are initially fixed/hardened on their plastron/ventral surface; then after 24 hrs, turtles are laid on their backs to ensure formalin penetration into the dorsal viscera mass.

For all animals, use a piece of plastic or wood to hold the mouth open during fixation. Some characters used for species-identification are in the mouth. If the animal's mouth is shut when preserved, the jaw usually must be broken to open it, thus preserve your voucher specimens with open mouths.

*"Fixation Postures"*





*Initial maintenance.*

The specimens should be allowed to harden/fix in and on the preservation trays for a minimum of 48 hours, but if you have adequate tray-space, three or four days are recommended. During this time the specimens are covered with formalin-moistened paper or cloth toweling to ensure the specimen remains moist. The specimens are maintained in a lidded tray, or if large turtle or snake, on a larger tray within a large, closed plastic bag.

After 48-96 hours, the specimen should be transferred to a container and fully immersed in formalin for another week. After that time, the specimen can be transferred to its permanent storage container and solution. Note the container and formalin for this final fixation-immersion can be



used repeatedly [recycled] for subsequent specimens. Only when you observe that it has become diluted or polluted should you discard it. Because formalin, even when diluted, is toxic, please discard only in the area where you burn or bury your trash. Keep away from your drinking water, foods, and access by domesticated animals and wildlife.

## STORAGE & MAINTENANCE OF SPECIMENS

Whether maintaining amphibian and reptile specimens for a brief or a long time, the specimens must always be kept moist and ideally fully immersed in either formalin or alcohol. Specimens should also be kept in the dark, because light, even indirect light, bleaches specimens. Specimens should never be transferred or held even briefly in water; water dilutes the fixative in the tissue and permits bacterial and fungal growth. And specimens always should be maintained with a label, ideally with the label/tag tied to the specimen, and the collection-number and voucher data recorded in a ledger or field-book.

### *Short-term.*

Short-term storage is for less than one or two months. Formalin is a satisfactory fluid for such storage. The specimens can be held fully immersed or wrapped in formalin-moistened cloth. In all cases, the specimens must be maintained in evaporation-proof containers.

### *Long-term.*

Alcohol is the preferred fluid for long-term storage, and of the alcohols, ethanol is the best. No matter which alcohol is used, a 70% solution is recommended. Formalin [4%] is adequate, but it must be buffered to prevent decalcification of the skeleton and to retard its darkening effect on the skin of the specimens. As noted above, storage containers must be evaporation-proof and maintained in dark conditions. Aside from the specimens bearing data tags, a bottle label with the collecting data is useful to have inside the jar. Also I recommend keeping only the same species in each jar. If that is not possible, attempt to segregate amphibians and reptiles in different jars and also attempt to keep species of equivalent sizes together. Mixing sizes and amphibians & reptiles will damage the smaller and softer-skinned species.

Whether using alcohol or formalin as a storage solution, do not use metal containers or glass/plastic containers with metal lids. Both liquids rapidly rust the lids, and commonly within a year the container loses fluid via evaporation. A regular [at least every six months] examination of your voucher or synoptic collection is recommended to ensure that all containers are preventing evaporation. If a container is less than 3/4 full of liquid replace totally with new storage fluid, because the remaining fluid is much diluted by evaporation and simply adding new fluid will not return the container's fluid to full and adequate concentration. Where evaporation is excessive, there is a problem with the container and it should not be used for long-term storage.

*Transport and shipping.*

Whether moving/shipping specimens within country or sending them outside of your nation, the specimens must be removed from their storage containers and wrapped in alcohol moistened cloth. Wrapping the specimens so that they do not touch other specimens prevents their damage and the moisture prevents their drying out. Alcohol, even a solution as weak as 30%, is preferable to shipping specimens in formalin, because any leakage of the formalin creates noxious odors and transport officials -- especially postal authorities -- are likely to discard the package without considering its importance and value. The wrapped specimens are placed inside a plastic bag, and the bag is sealed to prevent leakage. The bagged specimens are then placed inside a second plastic bag to ensure no leakage. Note that only droplets of free liquid should be visible within the first bag. As the volume of liquid increases in a bag, the greater is the probability that it will leak during transport. The double plastic bag protocol is adequate to prevent the dehydration of a specimen wrapped in moist cloth for at least six months.

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Version: Jan 1999