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TECHNIQUES FOR FIXATION, PRESERVATION, AND CURATION OF CEPHALOPODS

CONTENTS

TECHNIQUES FOR FIXATION, PRESERVATION, AND CURATION OF CEPHALOPODS

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I. Introduction

The need for guidelines to techniques of fixation and preservation of cephalopods was recognized at the International Workshop on the Biology and Resource Potential of Cephalopods.[1] It was pointed out that, with a few exceptions, cephalopods traditionally have not been kept in large quantity from biological, fisheries, or environmental surveys. A few representative specimens might have been retained for qualitative (taxonomic) purposes, but this is of little help in retrospective determination of relative abundance, size ranges, maturity levels, populations, etc. To the biologist who is not a specialist in cephalopods the proper fixation and preservation of these animals might seem a difficult task with generally unsatisfactory results. Certainly many poorly prepared specimens exist, but with the increasing interest in the biology and fisheries of cephalopods on a world-wide scale, the necessity and demand for well-preserved material requires that non-specialists be able to fix and preserve cephalopods. Proper fixation is especially important for the identification of species and for biological (e.g., fecundity), histological, and anatomical examination.

We know of no published work that deals specifically with fixation, preservation and curatorial techniques of cephalopods. Although much literature is available for other specific groups of organisms, these techniques do not necessarily apply to cephalopods because of their large size, heavy musculature, lack of a skeleton, etc. The *Unesco* publication edited by Steedman (1976) is an excellent compilation of information for fixation and preservation of zooplankton, some of which is applicable to larger marine organisms including cephalopods. This work is highly recommended for anyone interested in experimenting with techniques of fixation and preservation in cephalopods.

The aim of this paper is to provide guidelines to the techniques of fixation, preservation, and curation of cephalopods for general systematic, morphological and biological purposes. Special techniques, e.g., those for preparation for electron microscopy, are not included. We recognize that we probably have omitted some techniques or chemicals that other biologists have found effective; we therefore solicit suggestions from these workers so that additional material can be incorporated into a future revision of this paper.

II. Materials

II.A. *Fixatives.* Fixation technically is defined as the process of coagulating the contents of cells into insoluable substances (usually by cross-linking proteins) to prevent autolysis and breakdown of tissue (Fink *et al.*, unpublished report, 1979). To be most effective fixation must be accomplished as soon as possible after specimens are captured, preferably on live (sometimes narcotized) material, because cephalopod tissues begin to break down very rapidly upon death. No amount of post-fixation manipulation and treatment will substitute or compensate for correct procedures during the initial phase of fixation. Generally, the process of fixation is accomplished in a relatively short time, usually a matter of hours or days, depending on the size and consistency of specimens and the volume of the fixative. Once proper fixation is achieved a second procedure is performed to insure long-term (permanent) storage of the specimen (i.e., preservation).

[1] The Workshop was conducted at Queenscliff and Melbourne, Australia, 8-14 March 1981.

II.A.1. FORMALIN. Formalin is the fixative of choice for most cephalopod applications. While it is the standard fixative, dilutions vary with different applications. Formalin is prepared by a dilution with water of formaldehyde, an organic compound with the following characteristics:

Formula—HCHO
Molecular weight—30.03
Boiling point—19.5°C
Flash point—30.0°C
Solubility—water, alcohol, ether
Highly reactive; polymerizes readily with various organic materials
Synonyms—Oxomethane, Oxymethylene, Methylene oxide, embalming fluid.

The function of formalin as a fixative appears to derive from the formation of cross-links between adjacent protein chains, resulting in their denaturization and deactivation. Autolysis (self-digestion) is inhibited, and proteins are coagulated; consequently the breakdown of tissues is prevented.

As a fixative for cephalopods, formalin is a dilution of stock formaldehyde (or "full strength" formalin), a 39% by volume (rounded to 40%) saturated water solution of formaldehyde gas. Dilutions vary from 4-10% of the full strength formaldehyde and are prepared in the following proportions:

% formalin	parts formaldehyde	parts distilled water
10	1	9.0
9	1	10.8
8	1	12.6
7	1	14.4
6	1	16.2
5	1	18.0
4	1	19.8

The decision of which dilution to use depends on the size of the specimen(s) and the consistency of the tissues. As a general rule, larger and more heavily muscled specimens require stronger dilutions, up to the 10% maximum. Thus the large, heavy-bodied squids, and octopuses, e.g., loliginids, ommastrephids, onychoteuthids, gonatids, octopodids, should be fixed in 8-10% formalin; medium-sized and muscled forms, e.g., enoploteuthids, histioteuthids, most cranchiids, sepiolids, in 6-8% formalin, and small, thinly muscled or gelatinous forms, e.g., some cranchiids, cirrate and bolitaenid octopods, larvae and many juveniles, in 4-6% formalin.

Because formaldehyde oxidizes rapidly in dilute solutions into formic acid, formalin should be mixed only as it is required, and it should be buffered to maintain near-neutral pH. Formalin undergoing oxidation turns yellow, then red-brown as decomposition progresses. Formalin also interacts with the proteins in animal tissue to form acidic solutions (Taylor, 1977). In cephalopods this acidity will result in dissolution of calcified structures and frequently these very characters are among the most important taxonomic features. Structures most adversely effected by acidic conditions in the fixative (and preservative) include the chitinous sucker rings and hooks, the cuttlebones of sepiids (cuttlefishes), the shells of *Spirula* and *Argonauta*, statoliths, and, to a lesser degree, the chitinous beaks, radulae, and gladii. Acidity also tends to clear tissues and turn them semi-gelatinous. Frequently used buffers include sodium borate (borax), calcium carbonate, and hexamine. A more detailed discussion of buffers appears in a following section.

The amount of fixative used also has bearing on the results. The fluid volume should exceed the tissue volume; a 2:1 to 4:1 ratio generally is sufficient, and never should be less than 1:1.

Duration of fixation in formalin depends on the size and musculature consistency of the specimen and the temperature of the solution. While penetration of formalin is enhanced at warmer temperatures, it is *neither advisable nor recommended* to heat formalin to achieve more rapid fixation. Heating the formalin also will accelerate autolysis and decomposition of the specimen. Large, heavily muscled specimens require longer times for fixation, perhaps several days to two weeks, while small, light-bodied forms are well fixed in one to two days. Specimens should remain in the formalin until the tissues have been completely penetrated and are "hardened". Degree of "hardness" is quite subjective, but experience will indicate the correct stiffness and rigidity. If one is in doubt, it is

better to retain the material in the buffered fixative for a longer rather than a shorter time.

Complete fixation requires complete, preferably rapid, penetration of the tissues. The penetration of formalin is blocked or retarded by presence of lipids in the tissue (Steedman, 1976); therefore, the incorporation of a lipid solvent greatly enhances penetration of the fixative. A suitable solution (Steedman, 1976) consists of:

propylene phenoxetol—1.5 parts
propylene glycol—5.0 parts
formaldehyde (full strength; 40%)—10.0 parts
distilled water—83.5 parts

While this solution is used in preservation of plankton, we are unaware of its application to cephalopods. However, because of the high lipid content in some families, e.g., Ommastrephidae, Gonatidae, we believe this procedure would be worth trying. Empirical observations, in fact, indicate that these are the very families that frequently are the poorest-preserved in collections.

Warning: Formalin in any dilution used for fixation of specimens is noxious at the least and can be dangerous. Extreme care should always be exercised whenever handling or working with this fixative, and vigorous ventilation should be used. Among symptoms due to exposure to formalin are skin, eye, and respiratory irritation, including dermatitis, hives, conjunctivitis, rhinitis, bronchitis, pulmonary adema, headache. Ingestion can cause burning of mouth and oesophagus, nausea and vomiting, abdominal pain, vertigo, unconsciousness. Formalin or formaldehyde is suspected to be carcinogenic in human lungs after long and excessive exposure in industrial settings, but this has not been confirmed (Sax, 1981).

Formalin should be stored in unbreakable containers protected from damage in temperatures not exceeding 16-35°C. It should not be stored in confined spaces or near open flames.

First aid procedures include copious irrigation of eyes with water, washing exposed skin with large amounts of water and soap, and gastric lavage if swallowed, using 1% ammonium carbonate followed by saline catharsis (ITII, 1975).

Spills and leakage should be absorbed with rags and absorbent materials and the area washed down several times with water until the odor disappears. For massive spills, gloves and a gas mask must be used for protection.

II.A.2. BOUIN'S FIXATIVE. This solution is the most extensively used picric acid fixative for general histological preparations. The tendency of picric acid to cause shrinking in tissues is counterbalanced by the swelling affect imparted by glacial acetic acid. Bouin's fixative consists of:

picric acid (saturated aqueous)—15 parts (150 ml)
formalin (full strength =40% aqueous)—5 parts (50 ml)
glacial acetic acid—1 part (10 ml)

Bouin's solution is recognized as one of the best fixatives for general purposes and for histological preparations, because it penetrates tissues rapidly, preserves soft and delicate structures well, and acts as a mordant for certain histological stains. Bouin's solution should be used with soft tissues; due to its high acidity it is not suitable for calcium structures. Also, it destroys red blood cells (not a problem in cephalopods) and cytoplasmic structures, so that cytoplasmic staining is less well defined. Bouin's solution does not interfere with the staining qualities of tissues when the picric acid is removed through successive changes of 70% ETOH. Ideally, Bouin's-fixed specimens (tissue) should be removed and stored in 70% ETOH after fixation is complete.

Since relatively small specimens or individual organs or pieces of tissue normally are fixed, the amount of Bouin's solution used should be at least 10 times the volume of tissue being fixed. Duration of fixation varies between 4 and 24 hours, depending on the size and density of the specimen (tissue). Overfixation or extended exposure in Bouin's solution may cause undesirable effects, so, once fixed, material should be transferred to 70% ETOH. For example, long periods of storage of tissue in Bouin's fixative results in poor staining characteristics of nuclei.

II.A.3. ETHYL ALCOHOL. Alcohols generally do not make good fixatives for cephalopod tissues, but they can be used in an "emergency" in the absence of formalin. Alcohol-fixed specimens tend to become dehydrated and brittle, and they may decompose gradually. If alcohol must be used as a fixative in the field, specimens subsequently can be refixed in the laboratory using the standard buffered formalin technique. Preservation then can be continued in ethyl or isopropyl alcohol.

Ethyl alcohol has the following characteristics:

Formula—C_2H_5OH
Molecular weight—46.07
Flash point—15.8°C (open cup)
Boiling point—78.5°C
Solubility—water, methyl alcohol, ether, acetone, etc.
Highly hygroscopic and volatile liquid; flammable
Synonym—ethanol, ETOH

The hygroscopic property of ethanol allows it to function as a fixative (and preservative) by reducing the concentration of water in cells and tissues. Since enzymatic activity in tissues requires water, the loss of water reduces or stops this activity. Fixation through dehydration of tissues, however, is not as effective or complete as it is through the denaturizing of the adjacent protein chains caused by formalin.

If ethyl alcohol is used as a fixative, it should be in concentrations of 70-75%. Ethanol available for general fixation and preservation is not absolute, that is it consists of 95% alcohol and 5% water. Therefore, to dilute ETOH to 75% requires 3.7 parts of 95% ETOH to 1 part water and a 70% dilution requires 2.4 parts of 95% ETOH to 1 part water. Because ordinary tap water contains impurities that frequently create precipitates that may damage specimens, distilled water should be used for diluting alcohols (Hochberg *et al.*, in press).

A major problem with alcohol as a fixative appears to be its slow and/or incomplete properties of penetration. Specimens, especially those larger than a few centimetres, may appear fixed externally, but their internal structures generally are so poorly fixed as to be nearly indistinguishable because of tissue degradation. The larger the specimen, the poorer the fixation.

The ratio of fluid volume to tissue volume should be as high as possible, at least 4:1 or 5:1, when using alcohol as a fixative, because the water liberated from the specimen(s) significantly dilutes the alcohol. Specimens should remain in alcohol only until they can be refixed in formalin. Acidity may be a problem with long-term storage of specimens, especially with oily forms (e.g., gonatids, ommastrephids). It is recommended that acidity be reduced by several changes of alcohol until the pH approaches neutral (Dingerkus, 1982), but it must be remembered that several changes of alcohol will contribute to excessive dessication of the specimen until it becomes brittle and unmanageable (see Preservation section).

Ethanol is flammable, so it should not be used in enclosed places devoid of adequate ventilation where fumes can build up to the extent that open flame or sparks could ignite them. Irritation of the eyes and respiratory tract can result from exposure to ethyl alcohol fumes; more extreme exposure may produce headache, dizziness, drowsiness, mental confusion, or nausea. Proper ventilation should prevent most fume-induced symptoms. First aid for contamination of the eyes includes liberal irrigation with water and for ingestion, gastric lavage followed by saline catharsis is required.

Fires can be extinguished with dry chemical, alcohol foam, and carbon dioxide retardants (class B and C fire extinguishers). Spills should be absorbed with paper or other absorbant which can be burned.

Stored ethanol must be held in nonbreakable containers in well-ventilated, designated storage rooms, away from all sources of ignition, including static electricity.

A detailed discussion of alcohol(s) appears in the section on Preservation.

II.A.4. FREEZING. Freezing can be a very convenient and effective method of fixing cephalopods, especially large specimens for which no containers are available and in situa-

tions where it is undesirable to have the standard fixatives and preservatives. Best results are achieved with flash-freezing of fresh specimens at −24°C (−10°F) so that the entire specimen is completely frozen before autolysis begins. If possible, specimens should be sealed in air-tight polyethylene bags with the air squeezed out to prevent freezer-burn during storage.

Freezing cephalopods for most biological/systematic uses should be considered only a temporary technique that will fix and preserve the material until it can be fixed permanently in the laboratory.

Techniques for freezing and for thawing frozen cephalopods are given in the Procedures section.

Other Techniques. Several other methods of fixation are used in other groups of animals and are listed here to provide workers with alternative techniques in the event the more preferred techniques cannot be used.

II.A.5. PARAFORMALDEHYDE. Paraformaldehyde has been used in the field for fixing fishes (Fink *et al.*, 1979). It is inexpensive and convenient to handle, as it comes in powder form. A 10% solution is prepared by dissolving 35 g of paraformaldehyde powder in 1.0 l of water (Taub, 1962). To prevent polymerization and precipitation a base, preferably sodium carbonate ($NaCO_3$), should be added to the water which is then heated or boiled prior to the addition of the paraformaldehyde powder.

A 10% solution of buffered formalin can be made from paraformaldehyde by mixing 4 parts paraformaldehyde powder with 1 part anhydrous sodium carbonate, a small amount of powdered wetting agent (e.g., alconox), and 100 parts of water (heating is unnecessary). The powder mixture should be sealed in an air-tight container for storage and transport.

Paraformaldehyde seems to have several advantages over formaldehyde as a fixative: (1) convenience of transport in the field and reduced storage space, (2) no problems with air shipments (1 and 2 refer to the powdered form), (3) purity (stock formaldehyde frequently contains impurities, e.g., methanol, which can adversely effect the specimens). Disadvantages also are noted: (1) a rapid clearing of specimens occurs after preservation (Saul, 1981) when sodium carbonate is used as a catalyst to drive paraformaldehyde into aqueous solution. Paraformaldehyde is difficult to dissolve in water because the powder is a long chain polymer not a crystal. Saul suggests that sodium hydroxide (NaOH) pellets be substituted for the sodium carbonate and that the water be heated or boiled (143 g paraformaldehyde, 7-8 NaOH pellets, 1 gallon H_2O (heated or boiled) = 1 gal. 10% formalin). (2) the pure formalin derived from dissolution of paraformaldehyde is unstable and polymerization occurs with temperature fluctuations, thus inhibiting the fixative properties. There, another chemical, such as a methanol (as in stock solutions of formalin) or ethanol, must be added for stabilization.

II.A.6. GLUTERALDEHYDE. Gluteraldehyde is used as a fixative for preparing specimens (or small pieces) for electron microscopy. This fixative needs to be treated with the utmost care because of its toxic properties. It is a cross-linking agent much superior to formaldehyde. Gluteraldehyde and formalin together appear to create a fixative superior to formalin (Taylor, 1977), but experimentation needs to be carried out on its effectiveness for cephalopods. Apparently gluteraldehyde penetrates well in cool temperatures but not in warm temperatures. Bouin's and Gilson's solutions are used for standard histological preparations.

Certainly other fixatives are available for specialized applications and workers are encouraged to consult modern histological texts and specialists concerning these solutions.

II.B. *Buffers.* A buffer is a chemical system that prevents changes in hydrogen ion concentration. For example, proton donor and acceptor systems act as buffers to prevent marked changes in hydrogen ion concentration. In the context of fixation and preservation buffers are employed to stabilize solutions at an acceptable pH. Both formalin and alcohols develop low pHs, either innately in formalin or in alcohol through exposure to breakdown products from specimens (e.g., fatty acids).

Formalin oxidizes into formic acid and, in the dilute solutions used as a fixative the oxidation takes place rapidly. Also, formalin reacts with proteins leached from specimens to form acidic compounds. Acidic conditions contribute to dissolution of calcified tissues (see section on formalin, above) such as sucker rings and cuttlebones that are important taxonomic characters. Therefore, buffering agents must be added to the fixatives upon preparation to maintain neutral pH. Caution must be exercised, however, because some commonly used buffers such as borax cause adverse effects in specimens, e.g., excessive clearing of tissues (Taylor, 1977). Oxidation can be deterred by filling the specimen container completely to the top, thereby excluding any air/formaldehyde contact.

Alcohols appear to acidify over time for two reasons: (1) oils dissolve out of the tissues and break down into fatty acids, and (2) residual formalin remaining in the specimen from the time of fixation breaks down into formic acid. Initial acidification of alcohol can be prevented by using either distilled or deionized water for dilution or at least tap water with minimal minerals and contaminants. Formalin-fixed specimens should be drained of formalin and rinsed with water to remove all "free" formalin. Specimens should not be soaked to completely remove all formalin from the tissues, as this will lead to deterioration of the specimens. Several changes of alcohol may be necessary in order to achieve neutrality.

II.B.1. CALCIUM CARBONATE. Calcium carbonate ($CaCO_3$) is the buffer of choice for formalin; it occurs naturally as aragonite or calcite. It has several advantages: (1) inexpensive, (2) readily available, (3) does not induce clearing of tissues. Obtainable either as marble chips or marble dust or powder, $CaCO_3$ is added to formalin to excess, beyond a saturated solution, which should maintain a pH of 6.0 (Taylor, 1977). As a buffering agent marble powder is much preferred over marble chips because of its greater surface area and more rapid dissolution that prevents layering of the solutions (Steedman, 1976). It is important to note that Taylor (1977) observed a layering effect of pH in formalin solutions when specimens were fixed (preserved), so that formalin in the bottom of the container had a pH of 6.4, while at the surface the pH was 8.4. Therefore, it is necessary occasionally to invert bottles several times to homogenize the solution or to stir or agitate the solution if fixation is done in trays or basins.

II.B.2. SODIUM BORATE. This base, often called borax, is a commonly (and traditionally) used buffer of formalin used for fixation. The formula for sodium (tetra) borate is $Na_2B_4O_7 \cdot 10\ H_2O$. It is more soluble in water than $CaCO_3$.

A "rule of thumb" indicates that sodium borate powder should be added to the undiluted formaldehyde until saturation is achieved, a 1 to 2% solution, giving a pH of 8.0-8.3. But this may be excessive for other reasons, since even when diluted it causes lysis of tissues and clearing of pigments within 1-2 years (when formalin is used as the preservative) and this effect may be more undesirable than slightly acidic conditions. So borax should be used only for short periods of time. A less concentrated solution can be made by dissolving 5 g of sodium borate in 1.0 l of full-strength formalin. This should suffice for short-term neutralization (a maximum of 1 year). Borax that has been added to saturation eventually will precipitate out as white, sticky globules that may adhere to specimens and obscure the finer morphological details.

II.B.3. HEXAMINE. Hexamine, $C_6H_{12}N_4$, is an organic compound also known as hexamethylenetetramine, urotropine, and methenamine (molecular weight 140.19). As a formalin buffer, hexamine maintains a stable, non-acidic pH of near 7 (depending on solutions used), a level not achievable with chalk or borax. A solution of 200 g of hexamine in 1 litre of full strength formalin should provide a constant neutrality (pH 6.2 to 6.9) of the 10% formalin fixative diluted with distilled or sea water. That is, the final dilution in 10% formalin would be 2% hexamine. Hexamine works in 3 ways in formaldehyde solutions: (1) as a mild base, (2)

as an anti-oxidant, (3) to remove acid in solution (Smith, in Steedman, 1976).

Concentrations above 2% are not advisable as they may result in damage to the specimens by softening and swelling of proteinaceous tissues. Warm storage conditions also will cause degradation of calcareous tissue, therefore cool to cold conditions are recommended.

While a number of compounds will buffer fixatives and preservatives to pHs well above 7, the concentrations required to maintain neutral or above pHs in themselves are deleterious to animal tissues, as is the case with borax and hexamine.

II.C. *Preservatives.* Preservation is the process of permanently maintaining the fixed state of specimens and tissues. Several chemicals are used as preservatives and in general they are less toxic (therefore less dangerous for people to work with) and they avoid the harsh side effects of initial fixatives (e.g., the decalcification of hard tissues caused by formalin). The most common preservatives for cephalopods are ethyl alcohol, isopropyl alcohol, and formalin. The decision about which chemical to use is typically based on a number of factors: (1) availability, (2) cost, (3) tradition, (4) regulations, (5) human considerations (e.g., allergies), (6) type of material (e.g., size and consistency of specimens), (7) anticipated use of material, etc.

Interestingly, no long term comparative experiments have been conducted to determine objectivity which preservative is the best for permanent preservation of cephalopods. While for most applications alcohol is accepted as superior to formalin for preservation, little experimental information exists to indicate which alcohol, ethyl or isopropyl, is superior. Whichever preservative is used, it should have the following characteristics:

(1) provide permanent preservation so that material is available in a state suitable for systematic and specimen-oriented examination by future generations of researchers.

(2) allow as many systematic (taxonomic) characters as possible (ideally all) to be preserved.

(3) allow as many biological and anatomical features as possible to be maintained, e.g., spermatophores, ovaries, photophores, internal organs and systems.

These characteristics imply that acceptable preservatives should not: dehydrate or distort soft tissues; decalcify, distort, clear, or dissolve hard tissues; allow biological or chemical activity to occur (e.g., bacterial growth, autolytic enzyme activity or acidification).

II.C.1. ETHYL ALCOHOL. Ethyl alcohol (or ethanol or ETOH), C_2H_5OH, molecular weight 46.07; boiling point 78.5°C traditionally has been the preservative of choice for cephalopods in concentrations of primarily 70 to 75%. It has several advantages over isopropyl alcohol, the other alcohol preservative used for cephalopods (see below): (1) it is relatively more pleasant to work with, both in odor and effect on skin, (2) specimens are firmer, (3) the higher concentrations used, 70-75%, reduce concern of dilution below levels safe for preservation, (4) specimens in ethanol are preferable for most histological techniques.

Several disadvantages also are noted for ethyl alcohol: (1) the higher concentrations of ethanol result in a greater rate of evaporation especially if air space occurs between the surface of the fluid and the closure of the bottle and if closures are inadequate, allowing leakage of evaporated alcohol, these effects result in the necessity for greater curatorial attention, (2) specimens are more dehydrated in 70-75% solutions, consequently are harder and more brittle, especially if alcohol must be added or changed, (3) when tap water or sea water is used for dilution an undesirable precipitate may be formed; so distilled or deionized water is necessary, (4) ethanol is very flammable (flash point 15.8°C, open cup) so great caution is required in the storage of undiluted alcohol, and even with diluted solutions in the collections area and laboratory, (5) it tends to be more expensive than isopropanol, (6) since ethanol is a stimulant and intoxicant its purchase, handling, and use are rather strictly controlled by Federal and State regulations which require a considerable amount of records-keeping and secure, fireproof storage facilities.

Permanent preservation of well-fixed

cephalopods in ethanol requires a volume of alcohol to volume of specimen(s) of at least 2 to 1 (33% specimens) and preferably greater, e.g., 3 to 1 (25% specimens). Practical considerations of availability of large containers or of storage space may force the use of containers too small for the size (volume) of the specimen(s). In such cases frequent checking of the material is mandatory to ensure that fluid concentrations do not drop. Alternatively and preferably, if more than one specimen is involved, the lot should be divided into several containers (appropriately labelled). Since the free water content of the specimens dilutes the alcohol significantly, one or more changes of alcohol may be necessary to bring the concentration up to the desired permanent strength. The final (shelf) concentrations of alcohol that result from initial preservation with subsequent changes of alcohol were calculated by Taylor (1981) for three different volumes occupied by specimens and two different water contents of the tissues. The results of which are presented below:

Volume of specimens in container	25%		50%		75%	
Water content of specimens	65%	90%	65%	90%	65%	90%
Alcohol percentages after the following changes:						
Add 40% alcohol	32.9	30.8	24.2	21.1	13.6	10.8
Change to second 40% alcohol	38.7	37.9	33.8	31.0	22.5	18.7
or						
Add 75% alcohol	61.6	57.7	45.5	39.5	25.4	20.3
Change to second 75% alcohol	72.6	71.0	63.4	58.2	42.2	35.1

Clearly, high water content of tissues and high specimen volume in containers result in dangerously low concentrations of alcohol.

Taylor also calculated alcohol contents resulting from a system of three successive changes of increasing concentrations of alcohol (35%, 55%, 75%), but in general this technique does not yield as high final percentages as the methods in the table above. The topping up of bottles in which evaporation has occurred is discussed below.

Acidity is a problem in alcohol-preserved specimens as well as in material fixed or held in formalin. The acidity causes brittleness, especially in specimens with high oil content (e.g., ommastrephids, gonatids). The oils dissolved out of the specimens in alcohol, primarily from the digestive gland or "liver", break down into fatty acids. Acidity also may be caused by the residual formalin that remains in the specimens after initial fixation breaking down into formic acid. For example, a test solution of 1% formalin in 50% isopropyl alcohol was nearly neutral (pH 7) at mixing but dropped to pH 5.5 within one month (Dingerkus, 1982). Therefore, formalin-fixed specimens must be drained and rinsed in freshwater, with all residual formalin and rinse water drained from the mantle cavity, prior to placement in the alcohol preservative.

To prevent or reduce acidity one or more periodic changes of alcohol may be necessary before the final preservation, but since dehydration of tissues also may be a problem with 70-75% ethanol, the addition of a nonclearing buffer (e.g., hexamine, not borax) might be preferable to several changes of alcohol. Alcohol should be diluted with distilled or deionized water to avoid initial acidification and precipitate formation.

Some workers suggest adding a small amount of glycerine (glycerol) to the alcohol to prevent hardening and brittleness in specimens. Also, propylene glycol (1-2 propanediol; $CH_3CH(OH)CH_{20}H$) can be used as an additive (2-5%) to formalin fixatives and preservatives and to alcohol preservatives to promote softening of fixed protein and to maintain flexibility of tissues (Steedman, 1976). It also appears to promote penetration of formalin as a fixative (in combination with propylene phenoxetol) and to inhibit growth of molds. We have no direct experience with these softening agents, but we encourage experimentation.

II.C.2. ISOPROPYL ALCOHOL. Isopropyl alcohol has become more frequently used as a preservative for cephalopods in the last decade or so. Isopropyl alcohol ($(CH_3)_2CHOH$, has a higher molecular weight (60.09), a higher boil-

ing point (82.4°C) and a higher flash point (21°C, open cup) than ethanol. Isopropyl alcohol also is known as dimethylcarbinol, as well as isopropanol.

The advantages of isopropanol over ethanol include: (1) it is relatively inexpensive both in initial purchase cost and because it is used in greater dilutions, (2) it is less volatile, therefore evaporates less, does not desiccate tissues as much, and is less of a fire hazard, (3) it is not regulated as stringently by Federal and State agencies so there are fewer records-keeping, storage, and security requirements, (4) tissues are softer and more pliable, thus easier to work with, (5) lower concentrations (to a point) are adequate for long-term preservation without inducing desiccation.

Disadvantages associated with isopropyl alcohol in comparison with ethyl alcohol include: (1) it is noxious and relatively unpleasant to work with; adequate ventilation is required (adverse symptoms are conjunctivitis, corneal ulceration, skin irritation, headache, and nausea from fumes and decreased blood pressure, nausea, vomiting, haematemesis, anuria, uremia, hepatomegaly, and anaemia from ingestion; excessive exposure in career-long industrial production may cause carcinogenic effects in respiratory tract, ethmoid sinus, lungs, larynx, and paranasal sinuses (Sax, 1981)), (2) concentrations below 45% may cause degradation of specimens including clearing and disintegration of tissues, possibly the result of reactivation of autolytic enzymes in the cells (refixation and restoration of 50% concentration should solve the problem), (3) it is not highly soluble in water, so unless great care is taken to thoroughly mix the solution, layering will occur in the container inducing degradation of tissue near the bottom and increased evaporation from the higher concentration at the top, (4) once mixed it is difficult to measure solution strength because the specific gravity of isopropanol is very close to that of water, (5) impurities in stock alcohol may induce clearing of tissues (i.e., some drums may have contained other chemicals prior to alcohol), (6) dilution with tap water ("hard" water) causes a precipitate to form on the specimen or in the bottom of the container; this is remedied by using distilled or deionized water, (7) specimens and tissues preserved in isopropanol are unsuitable for histological purposes.

Cephalopods have been preserved in different concentrations of isopropanol: 40%, 45% and 50%. Recent observations, particularly in fishes (many of which have tissues of comparable consistency, thickness, and texture with cephalopods), indicate that a 50% solution gives superior preservation; lower concentrations will be diluted by the free water in the specimens to levels considered unsafe for preservation (Taylor, 1981). On the other hand, some workers have reported that tissues become stiff and brittle at isopropanol concentrations of 55% and above. Therefore, a 50% solution of isopropanol is recommended for preservation of cephalopods.

Information given in the section on ethanol concerning specimen to fluid volume ratio, acidity, changing fluids, etc. is applicable to isopropyl alcohol.

II.C.3. FORMALIN. The characteristics of formalin were discussed in detail in the section on fixation. While formalin is considered the best fixative for cephalopods, it is not regarded as a good long-term preservative. It has strong disadvantages: (1) it is noxious, allergenic, toxic, and possibly carcinogenic, so working on formalin-preserved specimens is very unpleasant, (2) it turns acidic through oxidation and through interaction with proteins from the specimens, causing decalcification of chitinous tissues, degradation and clearing of soft tissues, and eventual disintegration of the specimen.

Advantages of formalin as a preservative are difficult to find. However, it is inexpensive and standard dilutions in water yield osmotic pressures close to sea water so it can be used as a temporary preservative especially in the field where alcohol might be unavailable or undesirable for some reason. Since it can be made from powdered paraformaldehyde, its use greatly reduces the weight and bulk associated with solutions of formalin and alcohol (at least going into the field). It must be stressed very strongly that all formalin used as a preservative must be buffered, just as is

necessary when used as a fixitive. Calcium carbonate in the form of marble dust appears to be the best buffer for this application. Containers should be completely filled and tightly sealed so no air comes in contact with the formalin to induce oxidation and acidification.

Concentrations will vary according to specimen consistency, size, etc. (see section on Fixation) but should never exceed 10%; even for short-term preservation a maximum of 8% should be sufficient (a maximum of 6% for eggs, larvae and gelatinous forms). If specimens are to be retained in formalin for temporary preservation, they should be changed from the initial fixative solution into a fresh, generally more dilute, buffered solution.

II.C.4. FREEZING. Freezing is recognized as a good technique for keeping cephalopods under unusual circumstances (see section on Fixation). However, it generally cannot be recommended for permanent preservation, unless, again, unusual circumstances necessitate it and special caution is exercised. For example, formalin and alcohol might be unavailable, the specimen(s) might be too large or too numerous for any available containers. Freezing should be achieved as quickly and thoroughly as possible to prevent tissue degradation, e.g., by the "flash" technique, and the material should be retained at temperatures low enough to prevent the slightest possibility of thawing.

Specimens to be fixed and preserved by freezing must be sealed in polyethylene (plastic) bags if at all possible to prevent the dehydration and "freezer burn" inherent with unprotected frozen specimens.

The following section on procedures explains how to properly thaw and fix specimens initially fixed by freezing.

II.D. *Containers.* The kinds of containers in use to store cephalopods are as varied as the countries and institutions which house them. The storage container which has the longest shelf life and requires the least amount of curation is a clear glass jar with a rubber gasket and glass lid sealed with a wire clamp-top (bail top). Other types of containers have drawbacks, such as the "backing-off" of screw top lids caused by temperature fluctuations, rusting of metal lids, distortion of paper liner in some screw top lids and the eventual breakdown of some types of plastic jars and lids caused by the alcohol.

Any weakness in the type of container used eventually will lead to evaporation of the preservative and degradation of the specimen. If topping up of the preservative is required, the % alcohol and pH first should be determined to ensure that the strength of the preservative to be added will return the solution to its proper concentration and pH. Several techniques have been used with varying success to try to reduce the amount of evaporation from screw-top containers, such as parafin wax and sealing tape. These have the drawback of having to be redone every time the container is opened.

Currently the cephalopods at the National Museum of Natural History are preserved in the following range of sizes and types of containers: 2 and 4 ounce glass jars with plastic screw tops and conical plastic liners; 8, 12, 15, and 32 ounce glass jars and lids with metal, bail-top closure and rubber gaskets; 1, 2, 3, and 5 gallon glass buckets with plastic lids and plastic liners, $14 \times 16 \times 18$ inch and $14 \times 16 \times 36$ inch stainless steel tanks and lids with rubber gaskets and snap closures; and finally, $2 \times 3 \times 5$ foot marine plywood tanks lined with fibreglass (each contains three stackable PVC plastic trays constructed with stainless steel rivets).

II.E. *Labels.* Labels are an overlooked but critical component of curation. A rare or important specimen obtained through expensive ship-collecting techniques can be rendered almost useless by a disintegrated label. Formalin, and even alcohol, can completely digest some papers. Labels should be made of 100% rag paper with a high wet fibre strength. An alternative to this type of paper is currency paper which also has a high wear property. A permanent, waterproof India ink is recommended for writing. The high carbon, black inks which are indelible in formalin and alcohol provide the high contrast and permanency required for a museum or reference collection label. Ball point and fountain pen inks should never be used as the ink will dissolve in alcohol. Soft lead pencils are an acceptable alternative

for writing field labels but are not recommended for permanent labels. Typed labels cure the problem of illegible writing providing the ink is indelible in formalin and alcohol. When in doubt, always test the label paper and ink to be used in the fixative and preservative for several days to assure permanency.

Field labels should be made from the high quality paper described above, 100% rag. Very often the amount of time that elapses between the writing of the field label and the insertion of the permanent label is much longer than is anticipated. Also for this reason the data included on the label should be as complete as possible. Minimum data for field labels should include collector, station (or field or other identification) number and date collected. A preferable amount of collecting data for field labels would be collector, station number, date collected, geographic location or latitude and longitude (for oceanic localities). A log book always must be compiled with all pertinent collection data at the time the field labels are written.

Permanent labels should be generated once the material is transferred from the fixative to the preservative. These labels should include all known data pertaining to the specimen, including collector, station number, date collected, geographic location or latitude and longitude, depth collected, habitat, collecting gear (method), preservative and eventually identification of specimen(s), name of identifier, number and sex of specimens and some sort of catalogue or index number.

III. Procedures

III.A. *Prefixation Preparation of Specimens.* Before cephalopods can be fixed and preserved they require a certain amount of preparation to ensure maximum quality for systematic and morphological uses. Techniques vary with the size, structure, and number of specimens, the method of capture, and the state of the unfixed material (e.g., live, fresh-dead, long-dead, frozen). Of course, all specimens should be fixed as soon after capture as possible, because autolysis and decay processes commence at death.

III.A.1. DEAD SPECIMENS. Cephalopods captured in trawls or other moving collection devices (as opposed to traps and wiers, for example) generally are dead by the time they come on board, unless special techniques are employed to secure living material. Freshly dead specimens, regardless of how they were captured, must be handled very carefully because they are fragile; rough handling will tear integument and obliterate color patterns, rupture eyes, break off tentacles and arms, dislodge light organs, etc. Specimens should be placed into a tray, basin, or other container large enough to accommodate both the size (length) and numbers of specimens to be fixed. It is important to lay out the specimens so that the arms extend anteriorly and are not twisted and knotted, the tentacles, if longer than the arms are turned back along the head and body, the body and head are aligned, not bent and twisted; specimens should look as natural as possible.

III.A.2. LIVE SPECIMENS (NARCOTIZING). Specimens captured alive generally must be narcotized or killed prior to fixation to prevent contraction and distortion of arms, tentacles, mantles, which make the specimens very difficult to work on. Live specimens simply dropped into the fixative may contract so violently that their mantle openings are sealed and do not allow the fixative to bathe the mantle cavity and internal organs; heavily muscled forms may decay or autolyze internally before the formalin penetrates the mantle. Octopuses dropped into formalin alive will contract their arms so violently that they become rigid coil springs. Larval and juvenile cephalopods contract their heads into the mantle cavity, distorting the internal organs.

Narcotizing may be accomplished in several ways, but it must be emphasized that all techniques should be accomplished slowly so as not to disturb the specimen during narcotizing.

The objective of narcotizing is to get the specimen so relaxed and insensitized that it will not react by contracting at the introduction of the fixative. Since narcotics can lead to death (then rapid onset of decay), care and experience are required to determine when the animal is still alive, but sufficiently narcotized to be fixed; this can be done by probing the specimen with a

dissecting needle or glass rod. A great deal of experience and experimentation are required in using narcotizing reagents because different species and sizes (ages) react differently to different (or the same) reagents.

1. Ethanol is an effective narcotizing agent on cephalopods. Pure 95% alcohol is added slowly to the sea water until a 0.5 to 1.0% solution is reached; narcosis is achieved at a rate proportional to the size of the animal, generally within 1-2 hours. Since ethanol in these concentrations is not toxic, narcotized specimens can be operated on and manipulated; recovery is induced by placing the specimen back in pure running sea water. Or, if fixation is desired, the specimen can be fixed when it no longer reacts to gentle stimulation with probe or glass rod.
2. Cold water serves well as a narcotizing agent, especially on tropical forms. Lower the temperature of the water that contains the specimen gradually to 4°C. If narcosis is not satisfactory, the temperature can be lowered to near the freezing point of sea water: −1.9°C.
3. Decreased salinity has proven effective in narcotizing octopuses. Salinity is reduced gradually over a period of several hours until it reaches ½ normal salinity (i.e., 17 to 18‰). By this time the specimen is so lethargic that its sensitivity can be tested by probing or handling. Special techniques still are required for fixation, however, to prevent the arms from becoming coiled and distorted (see following section).
4. Magnesium chloride isotonic with sea water, $MgC_{12}\cdot 6H_2O$, works well as a narcotizing agent on other molluscs, including adult bivalves, in the ratio of 7.5 mg $MgC_{12}\cdot 6H_2O$, 92.5 ml distilled water, 100 ml sea water. We are unaware of its use on cephalopods, but it would be worth experimenting with if the other techniques fail.
5. Urethane (Ethyl carbamate, $NH_2\cdot COOC_2HS$) has been used to anaesthetize octopods prior to surgery (Boycott & Young, 1955). Urethane is now suspected to be a carcinogen and should not be used.

III.A.3. PHOTOGRAPHY. The color and color patterns of cephalopods are important features that are not retained well in preservation, because the pigments in the chromatophores are denatured by the fixative and preservative. Therefore, notes on coloration and color photographs are especially desirable documentation obtainable only when the animal is still alive or very freshly dead. Chromatophores may remain active for some time after the rest of the animal is dead, but they show only color, not the full array of color patterns of the live specimen. Photographs should be taken of the dorsal and ventral surfaces of each fresh specimen that has been laid out in a "natural" position in a tray. Close-up shots should be taken of special features, such as the ocelli on octopuses and color patterns in squids. Specimens should be in water when photographs are taken to eliminate reflections from the smooth surfaces. A size scale in the photo is recommended. A log should be kept of each (series of) photo(s) listing the state of specimen (alive, narcotized, fresh-dead, etc.) and any pertinent data that will identify the photo with the specimen at a later date.

III.A.4. ORIENTATION OF SPECIMENS. Prior to fixation, dead or narcotized specimens must be oriented properly so that they will be fixed in a position that will be most useful to the researcher. Specimens are laid out in a plastic or enamel tray or basin so that the body and head are aligned with no bends or twists, the arms are extended anteriorly and parallel to each other, the tentacles, if longer than the arms, are turned posteriorly along the side of the head and mantle.

III.B. *Fixation.* The fixative, e.g., 8% formalin, is added carefully until the specimens are completely covered; volume of fixative to volume of tissue ratio should be 2:1 to 4:1. If larger specimens are fixed on a rolling vessel they can be kept in proper orientation with cheesecloth or towelling placed loosely around them, *not* tightly wrapped, as this will retard fixation and specimens will be fixed with any

pattern or wrinkles that is in the covering material.

Cephalopod eggs and eggs masses are fixed in 4% neutral formalin or in Bouin's solution. For this material 70% ethanol is a poor fixative. If the eggs are embedded in a tough coating or matrix they should be opened to ensure good penetration of the fixative.

Larval, juvenile and small adult specimens up to about 20-30 cm total length and gelatinous, soft bodied specimens of any size, can be fixed as they are, but larger specimens, especially those heavily muscled forms (e.g., ommastrephids, onychoteuthids, gonatids), should be cut open to allow penetration of fixative into the mantle cavity. Start the incision several centimetres posterior to the mantle opening (to prevent the mantle from being fixed as an open, flat slab) and continue along the ventral midline to a point several centimetres anterior to the tip of the tail. The incision should be made only through the thickness of the mantle to avoid damage to the internal organs, some of which press against the inner surface of the mantle. If any pattern of chromatophores or photophores occurs along the ventral midline, the incision should be made slightly lateral to the midline to prevent damage. On large specimens with necessarily long incisions it may be necesary to tie thread around the mantle to maintain its cylindrical shape as much as possible, while leaving the slit gaping enough to allow penetration of the fixative.

Very large specimens, e.g., large *Dosidicus*, *Ommastrephes*, *Moroteuthis*, *Architeuthis*, should be injected in the head and body with formalin to ensure fixation of tissues. Long, large diameter syringe needles are recommended.

Eggs, larvae, and soft bodied juveniles should be fixed as soon as possible in: (1) 4% neutral formalin for a period of 12 to 24 hours and then preserved in 70% ethanol. Note—it is very important to include the method of fixation and preservation on the lable (S. v. Boletzky, pers. comm.). Larger and more heavily muscled forms require proportionally more time in the fixative, up to (8% formalin) a maximum of two weeks for the largest, heavily-muscled specimens; acidity must be (2) rigorously controlled at neutrality, which will require periodic changes of buffered fixative.

How many specimens should be fixed? Frequently this is a question that poses no concern because only one of a few specimens of each species are available and all should be fixed. But in survey work samples may contain many hundreds of specimens usually of the same species. When samples (stations) are very close in space and time, a minimum of 7-10 specimens per species should ensure that at least both sexes are represented; a few more are necessary if a significant size range occurs. If samples are separated in space and time, 25-50 specimens should be fixed to ensure an accurate representation of sizes, sexes, species, etc.

Special problems. The arms of octopuses, especially, are prone to coil tightly during fixation if the specimen is alive and unnarcotized. It is extremely difficult to identify and study specimens with coiled, contracted arms. Therefore, it is recommended that octopuses be dead or heavily narcotized before fixation. Narcotization in gradually diluted sea water works well (see section on narcotizing). During late stages of narcosis the arms should be stroked and pulled out straight. During fixation the arm tips are first immersed in fixative then removed and stroked straight. This process is continued gradually until the whole length of the arms has been dipped in fixative. The specimen is laid in the tray with the arms arranged out straight. Further stroking might be necessary as the formalin penetrates the muscles of the arms or the arms can be rolled in paper to form a supporting tube. Although time-consuming, these procedures ensure high quality fixation. If many specimens are to be fixed, at least a few should be fixed in this manner.

III.C. *Transfer from fixative to preservative.* The method of transfer to preservative depends on whether the specimen has been fixed or frozen.

III.C.1. FIXED SPECIMENS. Transferral of cephalopods from fixative (formalin) to preservative (alcohol) is a relatively simple procedure, as stepping them up through a graded series of

alcohols generally has not been considered necessary. However, recent research indicates that one or two changes in alcohol will ensure removal of the free water in specimens that dilutes the alcohol. Specimens should not be transferred to alcohol if histological work is anticipated, but it is recommended to transfer specimens from the original formalin fixative to a fresh solution of strongly buffered (neutral, pH7) formalin; a slightly reduced concentration from that of the original fixative is recommended.

Specimens should be removed from the formalin and all fluid drained out of the mantle cavity. Specimens then should be gently rinsed off and the mantle cavity flushed out with fresh water to remove any free formalin, but cephalopods should *never* be soaked or washed over a period of time to remove most or all traces of formalin. The small amounts of bound or residual formalin in the tissues will enhance storage life so long as the alcohol does not become acidic. Most specimens can be transferred directly into 75% ETOH or 50% isopropyl alcohol, but especially gelatinous or "watery" material should be stepped up (several days at each step) through a graded series of alcohols, e.g., 35%, 55%, 70%, and 75% for ethanol and 20%, 30%, 45%, and 50% for isopropyl alcohol to eliminate or reduce the probability of shrinkage and distortion of the tissues. Especially important specimens of any consistency, e.g., types or those to be used for anatomical or morphological dissections, also should be run up through the alcohol series. Alcohols should be buffered to prevent acidification (see above).

Preserved collections must be periodically inspected to ensure that alcohol levels and concentrations are maintained. With proper bail-top closures or with soft plastic lids (polypropylene) and polyethylene disc liners on bottles (see above) an annual inspection should be adequate. But if metal, bakelite, or other plastic (phenolic or polystyrene) lids are used with cardboard or foil liners it will be necessary to check alcohol levels more frequently.

When evaporation has occurred the alcohol must be restored, but problems arise with simply "topping up" the bottle. During evaporation the more volatile alcohol goes off leaving a dilute alcohol solution or in severe cases only water, so topping up with the normal concentration (50% isopropanol or 75% ethanol) will not restore the solution to the correct shelf concentration. Topping up with a higher than standard concentration will help, but unless actual concentrations are measured, the strength will not be known. An alcohol hydrometer should be used to measure the concentrations to ascertain whether they are too low or high. Successive topping up over a period of years will lead to ever-increasing dilutions. So, if more than 10% of the solution in the bottle has evaporated, it is recommended that the fluid be replaced entirely. If topping up is the only recourse (because of the financial burden of replacing the alcohol, for example), it should be done with a stronger solution than the shelf concentration, say 80-85% ethanol or 55-60% isopropanol, depending on the amount of evaporation.

III.C.2. FROZEN SPECIMENS. Frozen specimens must be thawed before they are fixed and preserved. Specimens placed in fixative while completely frozen will be fixed in their frozen shape and configuration, usually quite distorted. Also, external tissues will be fixed as they thaw, while the retarded thawing of internal tissues may prevent their fixation, because penetration of fixative will be inhibited by already fixed outer tissues. Further, the effectiveness of formalin as a fixative is suppressed at low temperatures.

Frozen specimens should be thawed slowly either at room temperature or in a polyethylene bag in warm (never hot) water. When specimens are soft and pliable, but *not completely thawed*, they should be oriented properly, especially the arms and tentacles, and fixed according to standard procedures (see above). Completely thawed specimens, especially the internal organs, become very soft and quickly lose their structural integrity, so that even subsequently properly fixed they are poor specimens. Also, the sucker rings and/or hooks on the arms and tentacles are lost from the thawed, relaxed suckers. So, such specimens are of reduced value for taxonomic and anatomical purposes.

III.D. *Packing and Shipping.* The method of packing depends on the quantity (i.e., size and weight) of material and how it is to be shipped. Small specimens (e.g., most of those caught in nets the size of a 3 m Isaacs-Kidd midwater trawl) and lots with few specimens should be sealed in a polyethylene bag with a volume of fluid twice that of the volume of the specimens. The bag then should be sealed in a second bag to prevent desiccation of the specimen(s) in the event of leakage. If weight, thus cost of shipping, is a major concern, the specimens should be wrapped in cheesecloth, placed in the polybag, saturated with preservative and sealed in the two bags. The bagged specimens are carefully packed in a liqui-pack or other strong shipping container (preferably one that retains fluids) with appropriate cushioning materials. If a leak-proof container is not available, a large polybag (e.g., trashbag) should be used as a liner for the shipping carton or crate. Polybags should never be overcrowded in the shipping container so that those on the bottom become mishappen, crushed, or ruptured.

Large specimens must be thoroughly wrapped in cheesecloth and saturated in preservative. They may then be double bagged or if too large, placed directly into a liquid retaining container with additional preservative.

Very small or delicate specimens and important museum specimens (e.g., types) frequently are shipped in glass containers. These vials and jars must be individually wrapped in some type of cushioning material (e.g., "bubble-sheeting"), and packing material must be placed around all interior surfaces of the shipping container. It is also a good practice to cushion the specimen within the jar. When in doubt always use additional protection for the specimen and the strongest shipping container available. The roughest treatment specimens receive occurs during shipment from one location to another. Always be certain to include a proper label in each polybag, vial, or jar of specimens. An invoice listing all lots should be enclosed in the shipping container. If the specimens are to be shipped internationally, a label on the outside of the shipping container should state that the contents are "preserved biological specimens of no commercial value".

IV. Chitinous and Calcareous Structures

Several calcareous or chitinous anatomical structures must be removed from cephalopods for specific taxonomic purposes, and it is important that they be preserved properly for future reference. These features include the gladius of squids, cuttlebone of cuttlefishes, beaks, radulae, and statoliths. The techniques for removal or extraction of these parts and for their preservation are presented below.

IV.A. *Gladius.* The chitinous gladius or pen lies below or partially embedded within the mantle musculature along the dorsal midline of squids. Two procedures are used for extracting the gladus from fresh and preserved specimens, the first being recommended for most species (Toll, 1982).

1. Ventral. The mantle is cut open along the entire ventral midline. The left gill is cut from the mantle wall along the gill membrane and the stellate ganglion is severed either proximally or distally. The left lateral edge of the free rachis is freed anteriorly from the surrounding tissue; then, working posteriorly, the remainder of the gladius is freed from the shell sac with particular care being taken to prevent damage to the delicate posterior tip where a conus may be present. The viscera are reflected toward the right side away from the gladius and the gladius is lifted out.
2. Dorsal. In species that have incomplete mantle musculature across the dorsal midline (e.g., Onychoteuthidae) the midline of the gladius lies exposed beneath the dermal layers. An incision is made along the entire dorsal midline taking care not to cut into the gladius. The cut edges of the mantle are reflected laterally and the gladius is freed along its edges and lifted straight up out of the shell sac and mantle.

The removed gladius is best preserved with the specimen from which it was taken, either by replacing it into the shell sac and tying the mantle closed with thread or by placing it in a long vial or tube closed with a sponge plug. The

gladius should not be put in the bottle without protection as it might be damaged. The gladius also can be tied gently between glass slides or plates, with shims or spacers to prevent flattening. Gladii should never be dried as they distort, become very brittle, and break easily.

IV.B. *Cuttlebone.* The cuttlebone in cuttlefishes (Sepiidae) is the homolog of the gladius. It is calcareous and easily damaged, especially the posterior spine and conus, but because it has such important taxonomic value it frequently must be removed to identify the species. An incision is made with scissors along the entire length of the dorsal midline through the thick dermal layers (generally no mantle musculature extends across the cuttlebone), the edges of the incision are spread apart, and the chitinous edges of the cuttlebone are teased away from the shell sac. Particular care is required to free the conus/spine posteriorly. The cuttlebone then is lifted out of the sac.

The calcium carbonate composition of cuttlebones makes them very susceptible to dissolution at reduced pH. So every effort must be made to maintain neutrality and prevent acidity through addition of a buffer to the fixative and preservative (see above). Cuttlebones can be preserved with the specimens from which they were removed, but if any doubt exists about maintaining neutrality of the preservative, they should be preserved dry. The cuttlebone should be rinsed thoroughly in fresh water, drained and air dried. Some cracking of the chitinous edges will occur. It should be stored in a covered specimen box or tray, in a capped bottle, or in a "zip-lock" plastic bag properly labelled with complete data so it can be identified with the animal from which it was taken. Cotton should be placed at the posterior tip to prevent damage to the spine and/or conus in the box, or placed in the bottom of the bottle, where the anterior end of the cuttlebone is placed; the upward-pointing posterior end also should be protected with cotton.

IV.C. *Beaks.* Several techniques exist for extracting the chitinous beaks (jaws, mandibles) of cephalopods.

1. Beaks are easily removed from freshly dead, thawed, or stomach-content specimens by gently teasing them out of the muscular buccal mass. The lower beak should be removed first. If any resistance is felt when the beaks are pulled with forceps or fingers the muscles should be cut carefully from the edges of the wings, hood and lateral walls.
2. Beaks are much more difficult to remove from preserved specimens, so they must be dissected from the buccal mass by careful cutting along the surfaces of the wings, hoods, walls, etc. Soaking in fresh water for several days will help soften the muscular tissue. Excess tissue can be carefully scraped from the extracted beaks.
3. Chemical techniques frequently are used to extract beaks from the buccal mass, but they often are so traumatic that they distort or damage the beaks of some species. We suggest that chemical methods be tried on one specimen before subjecting others to possible damage. The results of chemical cleaning may vary depending upon the species and age of the specimens.
   a. Trypsin. Trypsin is a naturally occurring proteolytic enzyme. To make a saturated solution of Trypsin in distilled water (allow 1-2 days to insure complete saturation), mix 3 parts Borax and 7 parts distilled water with 1 teaspoon trypsin. Trypsin must be stored dry in a sealed container and refrigerated. Very little of the powdered trypsin will go into solution, so large amounts will not increase the masceration of tissue. Place the extracted buccal mass in the solution for 1-2 days until the tissue is softened or dissolved so the beaks can be extracted easily. With large buccal masses one or two changes of solution may be necessary to complete the process.
   b. Potassium hydroxide. Potassium hydroxide is a strong base. The whole buccal mass is placed into a 3-5% (weight/volume) solution of KOH and boiled for 20-30 minutes. Tissues will then fall off the beaks or can be pulled

off. Beaks are then washed in running tap water for 2-3 hours. This method works well on large octopus buccal masses and beaks but is too traumatic for the more delicate squid beaks and larval or juvenile octopuses. These should be boiled for only 5-10 minutes or placed in a 5-8% (weight/volume) KOH solution for 12-24 hours without heating. Extracted beaks are washed thoroughly in running tap water for 2 hours (techniques of M. Nixon, 1969 and pers. comm.). Note—care must be taken when working with heated KOH and adequate protective clothing and goggles should be worn.

c. Sodium hydroxide. Sodium hydroxide, also a strong base, provides a much safer medium for beak extraction because it does not have to be heated or boiled. The buccal mass is placed in a 10% (weight/volume) solution of NaOH at room temperature for up to 24 hours. Frequent checking is recommended so that beaks can be extracted as soon as possible to avoid damage by the chemical. Wash the extracted beaks as noted above.

d. Chlorox. Chlorox (hypochlorite) will mascerate buccal mass muscles, especially from small specimens. The small buccal mass is placed in full strength chlorox for a few seconds then removed to fresh water to inspect the progress of masceration. Several immersions may be necessary depending on the size and consistency of the buccal mass. A less traumatic extraction can be achieved by using a dilute solution of chlorox; dilutions and durations should be determined experimentally. Extracted beaks should be thoroughly washed.

Beaks are preserved in the same alcohol preservatives as the specimens from which they are extracted. They should be returned to the specimen jar in a small, sponge-stoppered vial (each vial should be labelled if beaks from more than one specimen per lot are present). Drying is not recommended, as the desiccated chitin becomes very brittle and strongly distorted. Dried beaks can be protected from further breakage in a solution of 25% glycerine and 75% alcohol.

IV.D. *Radula.* The radula is extracted using the same techniques as applied to beaks. Once the beaks have been removed from the buccal mass the radula can be removed by gently pulling with forceps; occasional teasing or careful dissection might be necessary to loosen the radula from especially hardened masses. Wash the radula thoroughly in distilled water. Radulae can be examined temporarily on a glass slide with a cover slip. Preservation is the same as for beaks—the radula is placed in a vial, preferably with the beaks, and returned to the jar in which the animal is preserved. Permanent preservation also can be accomplished on slides as it is with other molluscan radulae. The cephalopod radula can be stored in 70% ETOH prior to mounting for optical microscopy. If it is to be examined by scanning electron microscopy (SEM) it must be run through a graded series of alcohols (70%, 85%, 100%) to dehydrate it and prevent shrinkage when it is dried. For optical microscopy, place the radula in a small amount of stained mounting medium (e.g., Turtox CMCP-9) until it is sufficiently stained. Then place unstained mounting medium (e.g., Turtox CMCP-10) on the final glass slide (flat or depression, depending on size of radula) and transfer the stained radula into it. It may be desirable to break the radula in several places with micro dissecting needles to allow easier examination of the teeth. Cover immediately with a cover slip and allow to set at least 12 hours; ring cover slip with clear nail polish, and label the slide with sufficient information to ensure its identity. Sealed slides can be stored in standard slide drawers or boxes.

To mount a radula for SEM examination, it should be carefully cleaned in an ultrasonic cleaner, then transferred from the absolute alcohol to a glass slide for orientation and drying (it may be necessary to break the radula). Remove the completely dried radula to a carefully cleaned SEM stub and mount with white glue (e.g., Elmer's) diluted 2:1 with

distilled water. Proceed with coating technique. (Above techniques courtesy of C. Hickman, pers. comm.)

IV.E. *Statoliths.* While the statoliths of cephalopods have not been extensively studied (with the exception of Ishikawa, 1924, 1929), a rapidly growing interest in them centres around their potential use in ageing studies. Also, statoliths seem to be specific enough in some groups to allow identification to the generic level which would be very helpful in fossil and stomach content studies (Clarke, 1978; Clarke & Fitch, 1979; Clarke *et al.*, 1980). Statoliths are small (usually less than 2 mm in length) calcareous stones composed of aragonite, and, as such, are very susceptible to dissolution in fixatives and preservatives that are in the least degree acidic. Consequently, they rarely are found in preserved specimens. Clarke (1978) gives the technique for extraction from fresh, unpreserved specimens which is summarized here. The funnel is removed, the head is flexed dorsoposteriorly, then the skin and the cartilage are sliced horizontally near the midline of the cranium between the posterior ends of the eyes until the statocysts are penetrated. The statoliths lie in the anterior end of the statocysts as small opaque to white stones. They should be removed with a micro probe, featherweight forceps, or microspatula. Normal forceps, even lightly squeezed, may crush them.

Statoliths can be preserved in small ampules or vials in buffered alcohol, mounted and sealed on slides, or desiccated and placed in covered depression slides.

IV.F. *Dehydrated specimens.* Specimens that have become hardened and dried through evaporation of preservative and subsequent dehydration may be partially rehydrated with one of the following solutions:

1. A solution of glycerine (glycerol) and ethanol is most effective on specimens that have not been completely desiccated and hardened. Begin with a ratio of 1 part glycerine by volume to 3 parts 75% ETOH, but experimentation with ratios might be necessary.
2. Tribasic sodium phosphate ($Na_3PO_4 \cdot 12H_2O$) in a 0.1% aqueous solution for 24 hours or longer has been effective on small specimens (larvae, juveniles).
3. Commercial wetting agent. We have had the greatest success with rehydration of desiccated specimens using the commercial wetting agent Areosol O.T. Solution, 10% aqueous (Fischer Scientific Company, Fair Lawn, New Jersey, USA. (Product endorsement is not implied by use of trade name or company name)).
4. Alconox or household detergents in strong concentrations in water are wetting agents that are useful as rehydration solutions.

Specimens should be kept in the rehydration solution, or in successive changes, until no additional improvement in the condition is noted (up to several weeks or months in the most severe cases). We emphasize that experimentation with concentrations of the solution and duration of exposure is encouraged, as no specific protocols have been established. Most dehydrated desiccated specimens never will regain their earlier state of preservation, but rehydration does improve material so that it can be worked with to some degree. Once rehydration is accomplished, specimens are preserved in the standard manner. Addition of glycerine (glycol) or propylene glycol to a 1-2% solution with the alcohol will help retain softness and pliability.

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