NOTES ON METHODS FOR THE NARCOTIZATION, KILLING, FIXATION, AND PRESERVATION OF MARINE ORGANISMS

Compiled by Henry D. Russell

April 1963

Systematics-Ecology Program

Marine Biological Laboratory, Woods Hole, Mass.

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FOREWORD

An important activity of the Systematics-Ecology Program is the preparation of a study collection of the marine organisms of the Cape Cod Region. Preparation of animal specimens requires careful narcotization, killing, fixation, and preservation. Two very useful manuals in print are:

Lo Bianco, Salvatore. 1899. The methods employed at the Naples Zoological Station for the preservation of marine animals. Translated from the original Italian by E. O. Hovey. U. S. Nat. Mus. Bull. No. 39, Part M: 3-42.

Wagstaffe, R., and J. H. Fidler. 1955. The preservation of natural history specimens. Vol. I. Invertebrates. London: H. F. & G. Witherby Ltd. 205 pp., 139 text figs.

Reprints of the Lo Bianco paper are no longer generally available and the paper lacks an index. Lo Bianco discusses his material by species whereas Wagstaffe and Fidler consider only major taxa. To simplify the work of narcotization and preservation of animals, the following sections have been compiled to serve as a preliminary working guide. Improved methods will be incorporated in these notes as they appear and are solicited.

Two additional references, not abstracted here, are also useful: (1) British Museum (Natural History), 1954. Instructions for collectors: Invertebrate animals other than insects. Great Britain: Adlard & Son, Ltd., 76 pp. (2) Guyer, M. F., 1953. Appendix D. Preparation of microscopic material, pp. 283-306, in Animal Micrology. Univ. Chicago Press.

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Gastropoda (Cont.)

THE METHODS EMPLOYED AT THE NAPLES ZOOLOGICAL STATION FOR THE PRESERVATION OF MARINE ANIMALS

By Dr. Salvatore Lo Bianco

(Translated from the original Italian by Edmund Otis Hovey)

Utensils

The laboratory of the station is provided with large tanks containing running and stationary sea water, a table covered with sheet lead and furnished with a drain, a great variety of glass and earthenware dishes, and tools of different kinds and materials.

Cylindrical glass jars, with glass stoppers ground to fit, are used for exhibition purposes and for storage. Those with necks are employed for the most part, but those without necks and with a flat top are preferred for elegant installation. Cylindrical jars are the most economical of fluid and are the cheapest to get.[1] Since glass jars are expensive, earthenware jars and basins are used for many laboratory manipulations. The small, globular vessels which have the bottom formed by a glass stopper, concave within, are recommended for small spherical animals. Round-bottomed glass tubes are very useful, but care must be exercised to see that the walls are too thin. The edge of the orifice should be smoothed in the Bunsen flame. When the tubes are more than 30 mm. (1.2 inches) in diameter, the lip should be flared out so that a piece of bladder can be readily tied over the opening.

Corks should be selected from the best stock, should be as compact as possible, and should be without cracks or other defects. In form they should be cylindrical, so as to make a good joint with the sides of the tube. The ends must be flat, with clean cut edges, so that no fragments can get into the alcohol. With large tubes it is desirable to put a plug of cotton inside the tube next to the cork, since the alcohol extracts the tannic acid from the cork and is stained brown thereby.

To preserve small, delicate animals, such as eggs and larvae, it is well to place the small tube containing the objects in alcohol, closed with a cotton plug, inside a larger vessel,

[1] For convenience in suspending objects in the liquid, those having a glass hook in the under side of the stopper should be obtained.

which is likewise filled with alcohol. This arrangement prevents danger from evaporation and minimizes the liability to breakage. One must see, however, that the cotton contains no acids and does not stain the alcohol. Absorbent cotton is the most suitable, of course, but the best quality of ordinary cotton will answer every purpose.

For large, flat objects, such as Asterids, Pleuronectids, and the like, rectangular jars with flat sides are recommended. These jars are made to be closed with a plate of glass, cemented on. Gutta-percha cement is generally used. Such receptacles have the great advantage that they do not distort the view of the object within them. For delicate forms, which are long and stiff, like Funiculina, glass tubing of proper size, cut off at the right length, is used, one end being closed in the Bunsen flame and the other with a cork.

For preliminary manipulations much use is made of glass crystallizing dishes with flat bases and perpendicular sides, in which many specimens can be placed in little liquid without touching or interfering with one another. They are especially advantageous for keeping animals alive in sea water, letting them remain at rest until thoroughly distended; for killing by different methods, either slowly or quickly, and for hardening objects in different solutions until they are transferred to permanent receptacles. These crystallizing dishes have ground edges so that they may be tightly covered with disks of glass, when desirable. For hardening worms and other elongated animals use may be made of long rectangular vessels covered with a sheet of glass, or of the zinc trays to be described later.

It is also necessary to have a number of ordinary beakers (or battery jars) of different sizes, which serve for the preserving of animals alive, tubes for the reception of small animals, pipettes for the extraction of minute forms from jars of water, glass rods, reagent bottles, graduated cylinders, etc.

For preserving animals, especially fish, of a size too great for such glass receptacles as have been mentioned, a rectangular case or box of zinc with a shallow trough around the margin is very useful. The cover, likewise of zinc, has its edge made to fit into the trough. To prevent evaporation the trough may be filled with water and a layer of oil. The cover has an opening in the middle to permit the escape of the air which is compressed under it by closing the box. This opening is provided with a cork. It must be acknowledged that these boxes have the disadvantage that, after a time, the zinc becomes corroded, probably by some acid formed in the alcohol through the action of dead animal matter. It is a good plan to protect the metal box by an exterior wooden case.

In place of the rectangular vessels of glass for hardening animals of elongated form, the station uses some made of zinc with a layer of wax in the bottom. The wax bottom is for the purpose of holding the wooden pins used in straightening out worms while they are hardening. Pins of orange or other hard wood are preferable to those of metal, because the fixing fluid attack metals. A convenient size for such a tray is 60 by 6 by 6 cm. (24 by 2 1/2 by 2 1/2 inches) with about 1 cm. (one-half inch) of wax in the bottom.

For the transfer of objects from one receptacle to another spatulas are largely used. These are made preferably of horn, because that material is not attacked by the reagents in use. They range in size from 6 mm. (one-fourth inch) to 10 cm. (4 inches) in width, and are of a convenient length, say 17.5 to 20 cm. (7 or 8 inches).

A pair of soft iron forceps 30 cm. (12 inches) long is very convenient for taking objects out of deep receptacles. Iron is both cheaper and just as good as brass for that purpose. Small forceps, wire cutters, syringes, and so on are used at times.

The apparatus for narcotizing certain Actinians is constructed as follows: The nose of a pair of bellows is provided with a metallic bowl which fits over the metal bowl of a tobacco pipe. The latter is provided with a peg which fits into a slot in the bowl on the bellows and fastens the two together. The tube of the tobacco pipe is continued with a piece of flexible rubber tubing which terminates in a U-shaped piece of glass tubing, the distal end of which has been drawn out to a point. With this apparatus one can easily force smoke into a receptacle.

Reagents

Alcohol.--Without doubt the most indispensable liquid is alcohol. For the preparation and preservation of delicate, transparent animals it is necessary to use purified spirit which has been filtered and diluted with distilled water. For coarser animals ordinary alcohol may be used, if desired, even that which has been obtained by redistilling what has once been used being available, care being exercised to see that acids and alkalies have been neutralized. The station always has on hand a quantity of alcohol of 70 per cent strength, which is what is ordinarily used for preserving animals, that of 90 per cent being used only in special cases. By mixing the alcohol and water somewhat in advance of actual need one avoids the innumerable bubbles of air which form on the surface of an animal when immersed in freshly diluted alcohol. Soft or gelatinous animals must be allowed to remain from two to

six hours in alcohol of 35 to 50 per cent, according to their consistency, and then be transferred to that of 60 per cent, and afterwards to that of 70 per cent. If the preparations are too delicate to bear handling, the transfer may be made by pouring off the liquid and adding the proper amount of alcohol to make a 35 per cent solution, continuing the process until the standard strength is attained. When necessary to avoid disturbing the animal at all a siphon may be used in effecting the transfer. Frequently it is necessary to change the alcohol after a few days, on account of discoloration. Some forms are immersed directly in 70 per cent alcohol, the liquid being changed after a few days. Changes should be made until the alcohol remains colorless. When an animal which has been in alcohol is transferred to that which is stronger, it is necessary to agitate the jar from time to time to avoid the formation of a layer of weaker alcohol on the bottom.

Many liquids have been tried at the station in search for a possible substitute for alcohol, but always with poor results. Some liquids, like those of Goadby and of Owen, when used on gelatinous forms, eventually produce contraction and consequent distortion. Wickersheimer's solution, which was highly praised when first brought out, distorts or macerates marine animals. Alcohol of 70 per cent is preferable for the permanent preservation of animals for the reason that it is sufficiently absorbed by the tissues after repeated changes. A stronger solution not only is unnecessary for good preservation in the majority of cases, but it is even harmful in some, because it eventually hardens the objects too much and renders them brittle. Alcohol is useful, furthermore, for narcotizing animals and for killing them slowly or quickly.

Formalin or formaldehyde.--Formalin is a very useful liquid for keeping animals temporarily, but not for preserving them permanently. Some pelagic animals--for example, certain Medusae, Pterotrachaidae, and Salpidae--may remain in it for even two or three years without serious detriment, but if they are not transferred to alcohol by that time they begin to disintegrate or decompose. Formalin therefore may be used on a voyage or a long journey when alcohol is scarce or not to be had. As a provisional fluid it is useful for many other animals which are not contractile, and especially for those which contain no lime spicules, skeleton, or shells. Shell-bearing mollusks, echinoderms, and such things, can not be preserved in formalin on account of the free acid[1] in the

[1] It is said by the advocates of the use of formalin that this free acid may be neutralized by sodium carbonate and many of the objections to the fluid thus removed.

fluid, which attacks the calcareous portions and causes them to lose form or brilliancy, or both. In the case of large animals, such as fish, one must make an injection through the anus of a solution of at least 5 per cent strength. With formalin, as with other preservatives, only one, or at any rate only a very few, objects should be put into the same receptacle at the same time, and there must be a good amount of fluid in proportion to the animal matter present. For gelatinous animals the solution should be of 1 to 4 per cent strength. Carmarina and similar things may be killed and hardened at the same time by the use of formalin of the right strength and chromic acid of 1 per cent in equal parts. For animals of some consistency, like ascidians and fish, one should use a 2 to 6 per cent formalin solution, the general rule being that the softer the animal the weaker the formalin. Either fresh or salt water may be used in making the solutions, as may be convenient. The sea water solution, indeed, preserves the transparency of gelatinous bodies better than the other. It is not necessary to wash objects which have been in formalin before transferring them to alcohol. For killing and hardening Rhizostoma, Tima, and some other animals formalin is excellent, and the objects may remain in the fluid a long time before they are transferred to alcohol. It is readily perceived that the contractile animals, when they have been narcotized by one of the usual methods, may be temporarily preserved in formalin in case alcohol is lacking. Colors certainly are preserved for a longer time in formalin than in alcohol, but in time those which are fugitive in one disappear in the other also. The preservative medium has not yet been discovered which will permanently preserve the colors which are due to a pigment in the skin or substance of an animal.

Chromic acid.--Next to alcohol an aqueous solution of chromic acid is the most useful reagent, and it serves especially for killing and hardening gelatinous and soft animals. Objects, however, should not remain in the fluid longer than is necessary, because they become too deeply tinged and are rendered fragile.

After treatment with the acid it is necessary to wash the animals with fresh water to avoid the formation of the precipitate when they are placed in alcohol. If they are not well washed, they will acquire in time a greenish hue. Chromic acid is used mixed with osmic, acetic, or picric acid, with corrosive sublimate ($HgCl_2$) and rarely with alcohol. The solutions are made in ordinary fresh water when possible, though occasionally salt water may be used. They will not keep long. That which has served once may be used again if it will not be too dilute when added to the water containing the animal and if too much time has not elapsed. When the solution has turned green after standing it is not fit to use.

Acetic acid.--This is a reagent which has the property of permeating tissues instantly and hardening them, and it is a very efficacious means of rapidly killing contractile animals, but it has the disadvantage of softening them again if they remain in it too long a time. Objects remain relatively transparent. In certain cases it is necessary to use a concentrated solution of the acid. It is often mixed with chromic acid for killing and hardening noncontractile transparent animals.

Osmic acid.--In general, osmic acid is not used as much now as formerly, because its use has several inconveniences. Efforts have been made at the station to substitute other reagents for it, and in many cases they have been successful. It hardens gelatinous forms well and preserves the transparency sufficiently, but its action is too great eventually. The preparations become dark-colored and are rendered fragile; consequently they should remain only until they have acquired a light brown tint.[1] Before they are transferred to alcohol they should be washed for some minutes in fresh or distilled water, as should be done with all animals which have been treated with any mixture containing osmium.

Kleinenberg's liquid[2] was one of the first adopted at the Zoological Station for the preservation of marine forms. Since it presents the disadvantage of staining the alcohol, even after repeated washings, and of not hardening the animals sufficiently, its use has been given up little by little, until now it is confined to the preparation of histological subjects, with the single exception of Balanoglossus, which is killed with this solution for exhibition as well as for study.

Lactic acid, in a solution of one in a thousand in sea water, serves well in treating larvae and small gelatinous organisms.

Hydrochloric, pyroligneous, and sulphuric acids are used rarely.

Corrosive sublimate, recommended first by A. Lang, is much used as a fixing agent, because it has the property of permeating tissues rapidly and hardening greatly. It is used

[1] Dr. Paul Mayer's method for bleaching objects which have been too much blackened is not practicable for soft animals, since it softens them too much. (Mitth. Zool. Stat. Neapel, II, 1880, p. 8.)

[2] Kleinenberg's liquid is made by mixing 100 c.c. of a saturated aqueous solution of picric acid with 2 c.c. of concentrated sulphuric acid. Filter and add three volumes of distilled water.

in concentrated solution in fresh or sea water, either cold or hot. In manipulations with sublimate, metallic implements must not be used, because they decompose the solution and stain the preparations. The solution is made, when possible, with hot water for economy of time, and in vessels of glass or porcelain. Care must be exercised to avoid boiling the sublimate in open vessels, and not to inhale the vapors. The hands must not be immersed in the solution if they have on them open cuts or sores.

All animals which have been prepared with this reagent can be used for histological researches. Corrosive sublimate is also used mixed with acetic or chromic acid or with sulphate of copper. Animals which have been treated with corrosive sublimate must be washed carefully and thoroughly in fresh water before they are placed in alcohol. Add a solution of iodine drop by drop until the alcohol remains permanently colored thereby; this insures the entire removal of crystals of corrosive sublimate from the substance of the animal. If this precaution is not taken, the mercury will be reduced from the corrosive sublimate and will stain the animal black, forming a black precipitate on the sides and bottom of the vessel. The amount of iodine to be used depends upon the size and character of animal to be treated.

Bichromate of potassium.--This is used as a 5 per cent solution for slowly hardening gelatinous animals without rendering them too fragile, when it is not possible to work with chromic acid. On account of the troublesome precipitate which forms when objects treated with bichromate are transferred to alcohol, the use of this reagent is not recommended. For bleaching the preparations before they are put into alcohol, use a few drops of concentrated sulphuric acid.

Sulphate of copper.--This is used only in solutions from 5 per cent to 10 per cent strength, which are made with hot fresh water, and used alone or mixed with corrosive sublimate for killing larvae and delicate animals. The objects which have been treated with this reagent must be washed repeatedly with water or else they will not remain perfectly clear, owing to the formation of crystals within the tissues, which render them opaque. If they afterwards prove to have been washed too little, the objects should be treated several times with an acid.

Chloral hydrate.--This is used in very weak solution, from 0.1 to 0.2 of 1 per cent made fresh in sea water, for narcotizing several forms before fixing them. This method has the advantage that, if the animal after a certain time does not remain in the condition desired for preservation, it can be replaced in sea water, where it will regain power of motion and continue to live. It is used for killing animals which live in the crevices of a

rock, in incrustations of calcareous algae, and among colonies of serpulas and of madrepores. Such must be allowed to remain in the solution from six to twelve hours. It is not necessary to use a fine quality of the drug.

Cocaine.--A solution of cocaine is made by dissolving 2 grams of the powder in 100 cubic centimeters of 50 per cent alcohol. This is a most excellent narcotizing medium, but its high cost prevents its extensive use. It is the best reagent thus far discovered for the treatment of gastropods. A few drops are carefully distributed over the surface of the water containing the animals, and the operation is repeated until the animals cease to respond to any stimulus.

Other reagents that are used occasionally are chloroform, ether, and tincture of iodine.

Mixtures frequently used.

Alcohol of 70 per cent and chromic acid of 1 per cent in equal parts.

Alcohol of 50 per cent	cubic centimeters		100
Hydrochloric acid (concentrated)	"	"	5
Alcohol of 35 per cent or of 70 per cent	"	"	100
Alcoholic tincture of iodine	"	"	2.5
Alcoholized sea water:			
Sea water	"	"	100
Absolute alcohol	"	"	5
Chrom-acetic mixture No. 1:			
Chromic acid of 1 per cent	"	"	100
Concentrated acetic acid	"	"	5
Chrom-acetic mixture No. 2:			
Chromic acid of 1 per cent	"	"	10
Concentrated acetic acid	"	"	100
Chrom-osmic mixture:			
Chromic acid of 1 per cent	"	"	100
Osmic acid of 1 per cent	"	"	2
Chrom-picric mixture:			
Chromic acid of 1 per cent	"	"	50
Kleinenberg's solution	"	"	50
Sulphate of copper, solution of 10 per cent strength	"	"	100
Corrosive sublimate, saturated solution	"	"	10
Potassium bichromate, 5 per cent solution	"	"	100
Osmic acid, 1 per cent solution	"	"	2
Corrosive sublimate, saturated solution	"	"	100
Acetic acid, concentrated	"	"	50

Corrosive sublimate, saturated solution ..	cubic centimeters		100
Chromic acid of 1 per cent	"	"	50
Kleinenberg's solution:			
Picric acid, saturated solution	"	"	100
Concentrated sulphuric acid	"	"	2
Filter and add three times the volume of distilled water.			
Flemming's solution:			
Chromic acid of 1 per cent	"	"	25
Osmic acid of 1 per cent	"	"	10
Acetic acid (glacial)	"	"	5
Distilled water	"	"	60
Perenyi's solution:			
Nitric acid of 10 per cent	"	"	40
Chromic acid of 1/2 per cent	"	"	30
Alcohol of 90 per cent	"	"	30
Muller's solution:			
Potassium bichromate	grams		2
Sodium sulphate	"		1
Distilled water	cubic centimeters		100
Formalin solution(standard):			
Commercial formalin 40 per cent	"	"	10
Water	"	"	90

Methods of Preparation and Preservation

Protozoa

Protozoa are so small for the most part as to be invisible to the unaided eye, and their preparation therefore comes within the field of the microscopist, for which reason only the larger species will be mentioned here. Certain Gregarinas which occur as parasites in the intestinal nucleus of Salpa maxima-africana are best preserved with Kleinenberg's solution, where they may remain for about an hour before they are transferred to weak alcohol.

Radiolaria

Thalassicolla is well fixed in chromic acid of 1/2 of 1 per cent, where it may remain about an hour and then be transferred gradually to alcohol of 70 per cent.

Aulacanthidae and Acanthometrae are placed directly in 50 per cent alcohol, and after a few hours are transferred to that of 70 per cent. Good preparations have also been made by dropping a few drops of 1 per cent osmic acid into the sea water containing the animals and then transferring them to alcohol after washing them in fresh water. Excellent microscopic preparations of some of these species of minute pelagic organisms

have been made by a treatment with a saturated solution of corroxive sublimate.

Sphaerozoidae.[1]--The different species of the genera Sphaerozoum and Collozoum, which have spherical or cylindrical form, are fixed in iodinized alcohol of 35 per cent, where they should remain from fifteen minutes to about an hour. The vessel containing them should be shaken from time to time, because the animals flatten out if they are allowed to remain too long on the bottom. If it is desired to prepare a large number at one time, it is necessary to put the fixing liquid into a crystallizing dish of ample size, for convenience in manipulation. After a sufficient time they are transferred to alcohol of 35 per cent, where they can remain a few hours. The change is effected by transporting the colonies with a spatula to another crystallizing dish of the same size without allowing the animals to be without liquid. In the same manner they are transferred to alcohol of 50 per cent, and after twelve hours to that of 70 per cent, and the last should be renewed after twenty-four hours. In this manner colorless preparations are obtained which can also serve for histological studies. Osmic acid is not recommended, because it darkens the preparations too much.

In colonies of Sphaerozoum with isosporic structure the shape is not fixed with iodinized alcohol, and it is necessary to use saturated sublimate. The genera Myxosphaera, Acrosphaera, and Collosphaera are killed in chromic acid of 1 per cent, to which a few drops of osmic acid have been added, using the same form of receptacle and the same precautions mentioned under Collozoum. After from half an hour to an hour the acid solution should be poured off and fresh water substituted for washing, but great care must be exercised not to break the colonies. Then the objects are gradually transferred to alcohol.

Acinetidae.--Trichophrya salparum has yielded beautiful microscopical preparations when treated with concentrated sublimate in sea water. With Acineta foetida, which usually lives among hydroids, better results can be obtained with osmic acid.

Vorticellidae.--The colonies of Zoöthamnium are best killed with boiling saturated sublimate.

Porifera

For sponges which are to be used for exhibition, it is

[1] These methods are described in full by K. Brandt on pp. 7-11 of his monograph: Die Koloniebildenden Radiolarien (Sphaerozoïen) des Golfes von Neapel, in Fauna Flora Golf Neapel, XIII, Monograph, 1885.

enough to immerse them directly in 70 per cent alcohol, renewing it when it becomes discolored. To avoid the contraction of Halisarcidae, they should be fixed in chromic acid of 1 per cent for about half an hour, or in saturated sublimate for fifteen minutes. Those sponges which are to serve for study, if they are not too large--that is, if they are not more than 10 cm. (4 inches) in diameter--are immersed in 90 per cent alcohol or in absolute alcohol, which should be renewed after three or four hours, and again after twenty-four to forty-eight hours. If the specimens are too large, small pieces may be cut off and treated in this manner.

If they are to be dried, they should be washed first in fresh water for a few hours, then they should lie for about a day in ordinary alcohol, and then be placed in the air or in the sun. If treated in this way, they will not have an offensive odor. If it is desired to retain the rosy color of certain sponges (Suberites, Axinella) for several days, it is enough to place them in 40 per cent alcohol and not change it.

Anthozoa

The first thing to be done when an Anthozoan has been caught is to place it in a receptacle with fresh sea water. It always happens that these animals, when disturbed by the fishing apparatus or transportation, contract or withdraw into themselves completely. To cause them to expand, it is enough to let them remain in a jar with pure sea water, although it may be necessary to keep them for a longer or shorter time in running water. Many times it has been noticed that the water soon becomes bad if it is not changed.

The following methods, especially that with the chrom-acetic mixture No. 2, are used for preserving animals for museums and to some extent for the study of gross anatomy.

Since all the Alcyonarians contain minute calcareous spicules which furnish the specific characters, they shoud remain in the acid mixture as short a time as possible so that the acid may not attack the spicules.

In those cases in which chrom-acetic mixture No. 2 has not given good results, a mixture of sublimate and acetic acid may be employed, but always for the killing alone. The animals should be transferred quickly to weak alcohol.

A method used by G. von Koch is quickly to immerse the distended animals in absolute alcohol or that of 90 per cent, making an injection of the same afterwards into the interior of the animal.

When the colonies of Cornularia, Clavularia, Rhizoxenia, and Sympodium have become expanded, siphon off the water in the

receptacle until only enough remains to cover them. This operation should be performed with great care to avoid any shock which could cause the retraction of the tentacles. Then pour rapidly into the jar a volume of chrom-acetic No. 2 double that of the water in which the animals remain, and immediately afterwards transfer them to alcohol of from 35 to 50 per cent, giving the preparation a few gentle shakes to free the tentacles and dispose them in natural manner. Another good method of killing is with hot saturated sublimate, using the same proportions as of the chrom-acetic mixture, and washing the animals when scarcely dead in fresh water before the transfer to weak alcohol.

The large Alcyonium is treated in the following manner: After the rapid bath in chrom-acetic No. 2 it is suspended, scarcely dead, in a jar containing weak alcohol in such a way that its tentacles do not touch the walls of the jar, and if the polyps remain well distended, the change to the different grades of alcohol then goes forward very gradually. In the weak alcohol minute bubbles of air frequently form and attach themselves to the tentacles, giving them a tendency to float and thus causing distortion. Striking the sides of the receptacle with light blows will rid the tentacles of the bubbles.

Pennatula phosphorea and Kophobelemnon, when they have become well distended, are taken by the naked base and very swiftly immersed in a tall, cylindrical jar containing the chrom-acetic mixture No. 2, and after a few seconds are placed in a crystallizing dish containing 50 per cent alcohol, where they are allowed to rest on their backs. Then with a small syringe with a very fine point inject alcohol into them through a minute hole made in the extremity of the base. In this manner the alcohol penetrates to all parts of the interior and distends the polyps. Tie a thread around the end of the base above the hole which was made so that the escape of the alcohol may be prevented. After some hours the animals should be transferred to 70 per cent alcohol, and in the final receptacle Kophobelemnon should be suspended upside down by means of a glass float with a hook in it.

Pennatula rubra, Pteroides spinulosus, Veretillum, and Funiculina are killed like the Pennatulids just mentioned, but no injection is made after the transfer to weak alcohol. Soft forms like Veretillum should be suspended in the final receptacle. Small forms of the Pennatulids may be killed without removing them from the vessel in which they have become distended, and they are then treated like the Cornularians.

Gorgonia, Gorgonella, Primnoa, Muricea, Isis, etc., should be killed with chrom-acetic mixture No. 2 in the same dish in which they have become distended on account of the great sensitiveness of their polyps. It is always advisable to have as little

water as possible in the dish at the time of killing these animals, and to pour over them a volume of the mixture twice as great as that of the water in which they are. Several times it has been noted at the station that the Gorgonidae which have expanded in sea water which has begun to turn bad are those which have given the better preparations. The small colonies, or pieces of colonies, remain with their polyps distended if they are killed with boiling saturated sublimate. Isis may be well preserved by using a mixture of sublimate and acetic acid.

Corallium rubrum, after it has been allowed to expand in running sea water, should be killed with boiling saturated sublimate solution (half as much as the water containing the coral), and quickly transferred to weak alcohol. By this method the color is almost perfectly preserved, while by the use of the chrom-acetic mixture it is very much injured. The alcohol which has been used for this coral can not well be used afterwards for the preparation of any delicate organism. A colony of Antipathes which was placed in such alcohol was dyed red within twenty-four hours.

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Zoantharia

All the species of Antipathes are fixed with saturated sublimate, and, on account of the slight contractility of the polyps, their preparation always succeeds. The saturated sublimate, which is used cold, should be of the same volume as the water containing the animals.

Actiniaria

The preparation of this group is very difficult, the great contractility and resistance of the muscular system of the majority of the species frequently constituting insurmountable obstacles to success. Many times when it is thought that the animal has been deprived of any sensitiveness, immersion in a reagent of rapid action is sufficient to show sudden and surprising contraction of the tentacles and of the whole body. When several specimens of certain forms are treated with the same method and under the same conditions, some die distended and the rest contracted. Good results depend, in some cases, on circumstances which, up to the present time, are wholly unknown. After all, however, there are many species with which perfect results can be attained, if great care be exercised in the manipulations.

Anemonia sulcata (Anthea cereus) is the easiest to prepare. When well distended in running water, the animals are killed with the chrom-picric solution, used in volume equal

to that of the water in which they are. This should be rapidly poured into the jar containing Actinian, after as much of the water therein has been poured off as may be and leave the animal immersed. A solution which is now much used instead of the chrom-picric mixture just mentioned is made of--

Chromic acid, of 1 per cent ..... 1 part
Saturated solution picric acid ..... 1 part
Formalin, of 4 per cent ..... 1 part

When the animals die, they will fall from the sides of the glass, and they should then be transferred to another jar containing chromic acid of one-half per cent, where they should be suspended upside down by means of a glass float, the hook of which has been passed through the lower rim of the body. The animals should be gently shaken to give the tentacles a natural position. After half an hour they are placed in weak alcohol, and then gradually transferred to that of 70 per cent. It is a good plan to suspend the animals upside down by means of a glass float, the hook of which has been passed through the lower rim of the body. The animals should be gently shaken to give the tentacles a natural position. After half an hour they are placed in weak alcohol, and then gradually transferred to that of 70 per cent. It is a good plan to suspend the animals upside down by means of a float in the final receptacle, though it is hardly worth while to do this for the smaller specimens.

The following Actinians may be killed with boiling saturated sublimate: Eloactis, Sagartia dohrni, Paranthus, Corynactis, and small specimens of Aiptasia. Before they are transferred to alcohol, the animals should be allowed to harden for some minutes in chromic acid of one-half per cent.

When Heliactis bellis, Bunodes gemmaceus, and B. rigidus are well distended, two-thirds of the sea water in the jar containing them should be removed and its place filled by a chloral hydrate solution 0.2 of 1 per cent. After a few minutes this liquid should be poured off until there is barely enough in the jar to cover the animals, which should then be killed with cold saturated sublimate.

Adamsia rondeleti is narcotized with tobacco smoke in the following manner: Remove the hermit crabs from the shells on which the actinias are growing and kill them in fresh water. Suspend the shells by threads in beakers of sea water in which the actinias will have ample room for expansion. The thread may be wound around the shell or passed through a hole in it and then tied over a stick of wood which rests on the edge of the

beaker. Place one or more of the beakers in a shallow tray (preferably with flat bottom and perpendicular sides) containing a little water and cover with a bell glass. Fill the bell glass by means of the bellows and pipe described on page 9, being careful at the same time to insert a U-shaped piece of glass tubing under the edge of the bell glass to permit the escape of the confined air as the smoke is forced in. Avoid jarring the glasses containing the actinias.

To regulate properly the duration of the whole operation it is necessary that the first fumigation should be made about 2 o'clock in the afternoon. Little by little the smoke clears up and the water begins to absorb the narcotizing substances contained therein and the animals for the most part distend the corona of tentacles. About 5 o'clock a second fumigation like the first should be made, and the objects are allowed then to remain overnight. The following morning carefully remove the bell jar and touch the tentacles with a needle to learn in what condition of sensibility they are. If they do not contract under this stimulus, place a small open beaker containing a few cubic centimeters of chloroform beside the jar containing the actinia and replace the bell jar, allowing the fumes of the chloroform to work for two or three hours. Lastly the animals are killed in the chrom-acetic mixture No. 2, hardened with chromic acid of one-half per cent and placed in weak alcohol and so on, where they are to remain suspended. If the tentacles give signs of sensitiveness, make a third fumigation and after a few hours test again. This is the only method which has proved successful in obtaining specimens with the column well distended and with the disk and tentacles in full expansion. Cold weather retards this and other narcotizing processes in a very marked degree.

Adamsia palliata can be prepared in the same manner without suspension. Good results have also been obtained by narcotizing the animal slowly with alcoholized sea water and then killing with the chrom-acetic mixture No. 2, or with hot saturated sublimate.

Cladactis, Cereactis, and the little Bunodeopsis strumosa are killed with the chrom-acetic mixture No. 2, and immediately afterwards hardened in chromic acid of 1 per cent. The first two should be suspended in the hardening and the preserving fluids by means of a glass float, the hook of which has been passed through the margin of the base. Before beginning operations, see that the specimens of Cladactis and Cereactis are perfectly sound and especially that they are not torn or cut, otherwise when they are placed in alcohol the liquid contents of the body will exude through the rents. Large specimens of Cerianthus are fixed with acetic acid and immediately afterwards are suspended

in weak alcohol by means of a thread fastened around the column near the base. A few gentle shakes may be needed to adjust the tentacles. It is not necessary to suspend the small specimens.

Actinia equina and A. cari are treated with boiling mixture of sublimate and acetic acid, followed by chromic acid of one-half of 1 per cent for hardening. Frequently success has been attained with the first of these species by lifting it gently with a spatula from the beaker in which it is expanded and immersing it in a saturated solution of sublimate.

Edwardsia is slowly narcotized by dropping from time to time a few drops of 70 per cent alcohol into the sea water in which it is. It is then killed with hot saturated sublimate. Success depends upon the complete loss of sensitiveness, which may be tested by touching the tentacles with a needle point.

Certain species of Polythoa are very difficult to prepare. Even with reagents of rapid action they will have the body well distended and often only a portion of the tentacles outside the disk. One species which lives in sponges and among calcareons algae (probably a variety of P. axinellae) is prepared very successfully with boiling saturated sublimate.

Larvae of the Actinians are killed with saturated sublimate or with chrom-acetic No. 2.

Madreporaria

Astroides calycularis is allowed to remain overnight in a beaker filled with clear sea water. The following morning usually shows the polyps in full distension. Then, after turning off a portion of the water (enough to leave the animals barely covered), kill them with a solution of boiling sublimate and acetic acid in volume equal to that of the sea water, and immediately afterwards transfer the colony to 35 per cent alcohol, making an injection of the alcohol into each polyp to keep it well distended. At each change of alcohol up to 70 per cent make a similar injection, and be sure to test the final solution with tincture of iodine to see that the sublimate has been eliminated.

Caryophyllia, Dendrophyllia, and Cladocora are fixed with boiling saturated sublimate, but it is very difficult to prepare them with the polyps in perfect expansion on account of their great contractility and also by reason of the extreme delicacy of their walls.

Hydromedusae

The Hydromedusae in general are very delicate forms, which are easily injured and which quickly decompose, hence it is

necessary to proceed with their preparation as soon as possible after they have been taken from the sea. Certain Companularidae in particular, such as Aglaophenia, Plumularia, Sertularia, and the like, which live in deep water, almost always arrive at the laboratory in a damaged condition or dead. They are more easily injured than other forms by the dredge, the bottom net, or other fishing apparatus. The best plan to follow with such specimens is to put them directly into alcohol, to preserve the perisarc at least. To treat the animals perfectly, they must be attended to on shipboard as soon as caught.

Other forms which live at less depth, and which can be fished by using every precaution against injuring them, must be handled with gread rapidity, otherwise, after a short time, the polyps become retracted, and it is very difficult to kill them in an expanded state. In general, these forms are more contractile than the Tubularidae.

All the Hydroidea--that is to say, the permanently polypoid forms, with very rare exceptions--are killed with hot saturated sublimate. If the polyps are not in complete expansion when received, the colonies should be allowed to expand in beakers of fresh sea water. As soon as the fixing fluid has been poured over the animals the whole should be turned into a crystalizing dish, containing fresh water, to cool; then the animals should be removed to another dish of fresh water for washing, and after five minutes to weak alcohol (50 per cent). If it is desired to avoid the heating of the liquid, cold sublimate can be used, but only for the Tubularidae.

Large colonies of Tubularia and Pennaria are killed with the mixture of sublimate and chromic acid in volume equal to that of the water in which they are, and after a few minutes they are washed and removed to alcohol. Antennularia may be killed in cold sublimate, washed in fresh water, and placed in 50 per cent alcohol, and so on.

Medusa Forms Of The Tubularidae

The small forms, Eleutheria (Clavatella), Cladonema, Podocoryne, and the like, are killed with the mixture of sublimate and acetic acid, used in large proportion. Eleutheria may be killed with Kleinenberg's solution.

Lizzia koellikeri and Oceania pileata, as soon as the tentacles have become well distended, are killed with concentrated acetic acid and immediately poured into a tube containing the mixture of alcohol and chromic acid. By gently agitating the tube the animal regains its normal form. After remaining in the mixture about fifteen minutes it is placed in 35 per cent alcohol, and then gradually transferred to that of 70 per cent. Another

and perhaps better way of handling these forms is to allow them to expand in a specimen tube less than half full of water, and when they are well distended to fill the tube with acetic acid. Transfer them at once by pouring into the tube containing the mixture of alcohol and chromic acid. A few minutes later pour out a small portion of the liquid in the tube and add chromic acid, because considerable water will have gone over with the animals. After fifteen minutes wash in fresh water and transfer to 35 per cent alcohol. Still another method is to use the chrom-osmic acid mixture as a hardening medium, but the animals do not remain as transparent and the tentacles are somewhat contracted.

During the hardening process, especially when many of the medusae are treated at the same time, the tube should be held in a horizontal position in such a way that the bells are down on the side of the tube and the animals not in contact. For the final preservation of certain forms (like Lizzia) place them in alcohol in a small tube, separating the individuals by wads of cotton, and put this into the exhibition jar.

Oceania conica and Tiara pileata are narcotized in alcoholized sea water (3 per cent) and then treated like the preceding.

Medusa Forms Of The Campanularidae

Eucope, Gastroblasta, and Obelia are fixed in the mixture of sulphate of copper and sublimate and after a few minutes are washed in fresh water until every trace of precipitate has vanished, and then placed in weak alcohol, and so on.

Mitrocoma and AEquorea are killed with acetic acid and immediately transferred to the chrom-osmic mixture, where they may lie from fifteen to thirty minutes, according to their size. Small specimens of AEquorea can be placed at first in the chrom-osmic mixture.

Tima flavilabris is best preserved by killing in formalin of 4 per cent, where it may remain from five to twenty days, if desired, before the transfer to alcohol begins. This transfer must be made very gradually. The older method of treatment is to kill the animal with chromic acid of 5 per cent in volume equaling that of the water in which it is, and after five minutes to transfer it to the chrom-osmic mixture. After half an hour in the latter mixture wash in fresh water and transfer to alcohol. This method stains the animal brown, while that with formalin leaves it colorless.

Olindias mülleri.--The old method is to kill with acetic acid and immediately transfer to chromic acid of 1 per cent, where the marginal tentacles are to be stretched out by means of a pair of forceps, and where the animals may remain about a quarter of an hour before they are removed to the weak alcohol. The new

and more satisfactory method is to place them bottom side up in a shallow tray partly filled with sea water and suddenly pour over them a volume of 6 per cent formalin equal to that of the water in the tray. In the 3 per cent solution of formalin thus made they are to remain at least a week before they are removed to 35 per cent alcohol, and gradually thereafter to 70 per cent alcohol.

Trachymedusae.--Rhopalonema, Cunina, AEgineta, AEginopsis, Liriope, and Carmarina are fixed in the chrom-osmic liquid for five to twenty minutes, according to their size, washed in fresh water, and gradually transferred to alcohol. Cunina is better when killed with concentrated acetic acid before being hardened with the chrom-osmic mixture. A simpler method for Carmarina is to use formalin of 4 per cnet and chromic acid in equal parts for killing and hardening. Allow the specimens to remain in this mixture from one to two hours; then wash in fresh water and transfer to alcohol.

To prevent the flattening of the bell of Carmarina, Tima, and other large forms place a watch glass in the bottom of the jar and rest the bell of the hydromedusa in its concave side.

Acalephae

Charybdaea should be killed with the chrom-acetic mixture No. 2 and immediately afterwards treated with chromic acid of one-half of 1 per cent. After a half hour transfer to alcohol, taking care for the proper suspension of the tentacles.

Nausithoe, the ephyra of Pelagia, and Rhizostoma are killed by pouring into the sea water containing them 3 per cent of a 1 per cent solution of osmic acid. When they have just begun to take on a brown tint they should be washed in fresh water and placed in 35 per cent alcohol. Formalin of 4 per cent may be used with excellent results with these animals, because it does not give the brown tint which is imparted by osmic acid. To avoid the flattening of the umbrella of Rhizostoma, the animal is killed in an exhibition jar with a somewhat narrow neck. After the transfer to weak alcohol the mouth of the jar should be covered with a piece of bladder and should stand upside down, with the convex part of the bell resting in the neck. The medusa should remain in this position until the alcohol has been changed to 70 per cent and the whole body has become permeated with the fluid. When formalin is used for killing and hardening, this inverted position should be maintained from the first.

Pelagia noctiluca should remain in the chrom-osmic liquid about an hour, and then be washed and placed in weak alcohol. In the alcohol the animal should be suspended by threads tied carefully to the extremity of each tentacle without tearing it. See

that the bell does not touch the bottom of the jar and let the animal remain thus only until completely hardened.

Cotylorhiza tuberculata (Cassiopeia).--Formalin of from to 3 per cent may be used to good advantage for killing and hardening this species. Another method is to treat with osmic acid, as was done with Rhizostoma, but when the brown tint begins to appear, a 5 per cent solution of bichromate of potassium should be substituted for the osmic acid and should be renewed after a few days. The animal ought to remain in this reagent for about two weeks, but it can not remain much longer than that without suffering injury. Then remove the object to 35 per cent alcohol. Numerous crystals of a salt are formed on the outside of the animal and a heavy precipitate falls to the bottom of the receptacle, making it necessary to change the alcohol frequently and to add a few drops of concentrated sulphuric acid thereto.

The larval forms of the Acalephs (Scyphistoma, Strobila) are killed with hot saturated sublimate. Strobila is also well fixed with a mixture consisting of 9 parts of concentrated acetic acid and 1 part of osmic acid of 1 per cent. From this it is quickly transferred to fresh water for washing and put into alcohol.

Siphonophora

As with the Hydromedusae, the preparation of the Siphonophora should be accomplished as soon as possible after capture, and only those specimens should be treated which are in good living condition. Particularly with the Physophoridae is it true that the whole colony will go to pieces if it remains for a few hours in the same receptacle in which the water has had a sudden change of temperature, though frequently the breaking up does not take place until the colony comes in contact with the fixing fluid. Much care must also be exercised not to shake roughly the vessel which contains the animals before they have been killed. It has often been observed that a trace of an acid or other reagent in the water is enough to destroy the colony. The receiving vessel must be perfectly clean.

Athorybia rosacea, the single representative of the family of Athorybiadae, which is found in the Bay of Naples, is very rare, and but one specimen has been prepared at the station. That was killed with the mixture of sulphate of copper and sublimate. The colony contracted somewhat, but remained entire. It was washed with fresh water and then placed in alcohol.

The very delicate species (Physophoridae, Agalmidae) must be transferred from the jar in which they were captured to the crystallizing dish in which they are to be killed by immersing both vessels in a tank of water and cautiously pouring the animals over. Leave water enough in the crystallizing dish to give the

colony free movement, and wait for the polyps and the nettle-filaments to become well distended and naturally arranged before going on with the treatment.

Physophora, Agalma, Halistemma, and Forskalia are killed with the mixture of sulphate of copper and sublimate in volume equal to or double that of the water in the crystallizing dish containing them. The mixture must be poured rapidly into the dish and not directly onto the animal. After a few minutes, as soon as dead, the colony should be transferred by means of a large horn spatula to the hardening solution, which is not the same for all the species.

(a) Physophora, Agalma, and Halistemma are put directly into 35 per cent alcohol, and after a few hours transferred to that of 70 per cent. As soon as Physophora has been put into the 35 per cent alcohol, its nettle-filaments should be stretched out as far as possible with a pair of light forceps before they become rigid. To change the liquid in the swimming bell it is necessary to make an injection with a pipette. Bubbles of air always form in the bells, which, through their tendency to float, tend to change the natural shape of the bells, or, raising the whole colony, flatten it at the surface of the liquid. To get rid of these bubbles it is necessary to compress the bells gently.

(b) The genus Forskalia is transferred from the mixture of copper sulphate and sublimate to Flemming's solution.[1] The animals remain in this from two to six hours, according to their size, and are then washed in fresh water and transferred to weak alcohol, and so on gradually. The hardening of large colonies succeeds better in the mixture of bichromate of potassium and osmic acid, where they can lie even twenty-four hours without hardening too much. To free the animal from the crystals which form in the tissues and render them opaque, a few drops of concentrated sulphuric acid should be added to the first alcohol into which the colony is put. After that, pure alcohol may be used.

For the permanent preservation of the Physophoridae, after they have remained for a few days in 70 per cent alcohol in crystallizing dishes for hardening, they are to be put into tubes, arranging them so that the anterior end of the colony is toward the mouth of the tube, by immersing the tube in the liquid and gently working the colony into it. Small specimens of Agalma and Halistemma can be taken by the posterior end with small forceps and gently forced into a tube filled with 70 per cent alcohol so that the bells point toward the opening. The tube should be small

[1] Chromic acid of 1 per cent, 25 c.c.; osmic acid of 1 per cent, 10 c.c.; glacial acetic acid, 5 c.c., and distilled water, 60 c.c.

enough to keep the colony in proper position within it. It should be plugged with cotton and placed within a larger tube filled with 70 per cent alcohol and closed with a cork. This double-tube system prevents movement of the liquid about the colony itself, even when the outer tube is not entirely filled with alcohol. It is likewise very useful for shipment of specimens, and especially so for purposes of demonstrations. It is recommended for all very delicate animals and those with appendages which can be injured easily.

Apolemia uvaria is killed as are the preceding species and hardened with 1 per cent chromic acid, which is substituted (in the same dish) for the sulphate of copper and sublimate mixture which has been drawn off through a siphon. After twenty minutes in the acid, wash in fresh water and transfer to alcohol, the change of fluids being effected by means of siphons.

Rhizophysa should be allowed to expand in a beaker with the least practicable amount of water and should then be killed with hot saturated sublimate. Wash at once and put into weak alcohol, rearranging as far as possible the nettle-filaments and tentacles which have become tangled during the handling.

Physalia caravella should be permitted to expand its appendages and polyps in a tall cylinder filled with clear sea water, taking care not to touch the pneumatophores on account of their severe stinging action. The preparation succeeds much better in a very high cylinder, on account of the great extensibility of the nettle-filaments. When the colony is well distended pour over it a volume of the sublimate and acetic acid mixture equal to one-fourth that of the sea water containing it. As soon as dead, the colony should be transferred in the same manner as at first, to a similar cylinder containing chromic acid of one-half of 1 per cent and after twenty minutes to 50 per cent alcohol, and finally to that of 70 per cent.

Hippopodius, Caleolaria, and Abyla.--Kill with the mixture of sulphate of copper and sublimate and put directly into weak alcohol. The bell of Abyla is also well prepared with the chrom-osmic liquid.

Praya is fixed like Hippopodius, but is hardened in the mixture of potassium bichromate and osmic acid, where it may remain one or two days.

Diphyes.-Use hot sublimate for killing, with the chain of the individuals distended.

Velella is killed with the chrom-picric mixture, or with that of sublimate and chromic acid, and after a few minutes is removed to weak alcohol.

Porpita is slowly killed by dropping with a pipette a few drops of Kleinenberg's solution into the beaker where it has become distended. When the beautiful blue color of the colony has

begun to turn red as an effect of the acid, it should be removed to the Kleinenberg solution, where it may remain fifteen minutes before it is put into weak alcohol.

Ctenophora

Beroe ovata, Hormiphora, Callianira, Lampetia, Euchlora, and young specimens of Cestus, Eucharis, and Bolina are killed in the chrom-osmic mixture, in which they remain from fifteen to sixty minutes, according to size, and are then transferred gradually to 70 per cent alcohol.

While Beroe ovata is hardening in the alcohol, insert a short glass tube of the proper size into its gastric cavity to keep it distended in natural shape. Fix the tube so that it acts like a float to keep the animal suspended in the liquid. This operation must be effected with great care to avoid injuring the longitudinal series of vibratile plates. After one or two days, when the animal is in the 70 per cent alcohol, the tube may be removed and the hardened animal will preserve its form.

Beroe forskalii, to be preserved in a state of expansion, must be immersed in the sulphate of copper and sublimate mixture, and as soon as dead must be placed in the chrom-osmic mixture to harden for an hour. Since this species is naturally flat, it is not necessary to introduce a tube into it.

Callianira may be treated like the last species, but another good method is to kill it in a solution composed of--

Concentrated pyroligneous acid	1 part.
Saturated sublimate	2 parts.
Chromic acid of one-half per cent	1 part

Cestus veneris.--Have the animal in a little water in the exhibition jar and rapidly pour over it enough of chrom-acetic mixture No. 1 to fill the jar three-quarters full, arranging the animal in a coil with the broad edge on the bottom by means of a slender glass rod. After ten minutes substitute chromic-acid solution for the chrom-acetic, and after fifteen minutes therein wash thoroughly in fresh water by decantation and place the animal in 35 per cent alcohol. The gradual transfer to 70 per cent alcohol must take several days, and the jar must not stand in direct sunlight or even strong diffused light. The specimens to be treated should be in perfect condition; otherwise they will go to pieces in the fixing fluid. Cestus veneris can be well prepared in the chrom-osmic mixture also, but many specimens are injured and colored too much, whereas by the method just described they remain white and nearly transparent.

Vexillum may be treated like Cestus veneris.

Crinoidea.--Antedon rosaces (Comatula) is put directly into 70 per cent alcohol, but A. phalangium, on account of its tendency to break in pieces of itself, must be killed in that of 90 per cent Shake the vessel violently to hasten death and prevent the animals from breaking off their arms. When in doubt about the species use 90 per cent alcohol.

The larval forms of the Pentacrinoids are narcotized with chloral hydrate of 0.1 of 1 per cent--a process requiring two to four hours. If they are then hardened in alcohol, they will remain with the arms perfectly distended. The more advanced stages are best killed with saturated sublimate, where, however, they should remain only a few moments to avoid injury to the membranes.

Asteroidea.--To prepare the Stellarids with the ambulacral feet in a state of distention, they are allowed to die in alcohol of 20 to 30 per cent, being placed in the vessel ambulacra uppermost.

Luidia.--Lay the animal on its back in a shallow tray in barely enough water to cover it. Then, when the ambulacral tentacles, which are very long, are well distended, pour over it the chrom-acetic mixture No. 2 and immediately transfer to 50 per cent alcohol and after two hours remove to 70 per cent alcohol. Small individuals may be killed with 55 per cent acetic acid, but greater care must be used to transfer them to alcohol as soon as dead.

Brisinga easily breaks off its arms, to avoid which it should be quickly immersed in absolute alcohol.

Bipinnaria makes excellent preparations when killed with the chrom-acetic mixture No. 1, and even better with the chrom-osmic mixture, in which it should remain only a few minutes. Other larval forms are treated with saturated sublimate solution.

Some Ophiuroids are allowed to die in fresh water because they thus remain distended and entire. Ophiothryx echinata is an example. Certain small forms (Amphiura, Ophiactis) can be fixed directly in weak alcohol, shaking the vessel violently to hasten death. Ophiomyxa pentagona, which has a soft body, is hardened in chromic acid of one-half per cent. Ophiopsila annulosa breaks in pieces of itself in fresh water, and is therefore killed in absolute alcohol.

Echinoidea.--To prepare these with the ambulacral feet well distended they are placed in just sea water enough to cover them and an equal volume of chrom-acetic mixture No. 2 is poured into the jar. They must be transferred at once to alcohol, so as not to give time to the acid to corrode the calcium carbonate of the animal. If desirable to preserve the soft parts of the animal for anatomical purposes, or even for form only, two small holes must be made in the shell opposite each other to discharge all

the fluid contained therein. After immersion in alcohol one must see that the liquid fills the internal cavity, and, on changing the animal to stronger alcohol, that the liquid contained within the shell is changed also.

If dry specimens of echini are wished, discharge the water within the shells and let the animals lie in ordinary 70 per cent alcohol for one or two days before placing them in the wind and sun to dry. Starfish also make better dried specimens if they are killed in 70 per cent alcohol and allowed to remain there two or three days before they are dried.

Holothurioidea.--Holothurians require more care than other echinoderms, because they have soft and very contractile bodies and all are furnished with tentacles which contract or retire within the body on contact with a reagent. Some species, furthermore, soon after they are immersed in the fixing fluid expel their viscera and become valueless as specimens--a thing which may happen in the sea water also, if that is changed too suddenly. All these inconveniences are avoided by treating the animals by the methods here described.

First of all, as with other animals which must become expanded, they are placed in clear sea water. Those species which are killed in acids should be allowed to remain in them only just long enough to cause death, so that the calcareons cutaneous spicules be not injured. Large specimens of Holothuria and Stichopus as soon as the tentacles are fully distended should be seized with two fingers or with a pair of forceps, a little below the tentacles, lifted from the sea water and be immersed as to the anterior portion only in a rather deep vessel containing concentrated acetic acid. At the same time another person should inject some 90 per cent alcohol into the animal through the anal aperture with a syringe, taking care not to exert too great pressure, lest the body be distended too much. Before the Holothurian is quite dead it is to be immersed in 70 per cent alcohol, closing the anal orifice with a small cork to prevent the excape of the liquid and the consequent flattening of the body. The injection should be repeated at each successive renewal of the alcohol.

With certain species, as, for example, Holothuria poli, the operations must be performed with much caution, because the skin is easily injured.

H. impatiens, which has a long, soft body, is to be seized by the neck, so that the tentacles can not contract, and by the posterior extremity, so that the body may not shorten, and in this manner the whole animal is immersed in concentrated acetic acid. When dead it is transferred at once to alcohol, without making an injection.

Thyone, Thyonidium, and Phyllorus are strangled without using much force, wholly immersed in acetic acid, and then removed

to alcohol as soon as dead. If the individuals are very small, the pressure on the neck should be made with forceps instead of the fingers.

Cucumaria plancii is treated like the large Holothurians, except that the injection of alcohol is made through the mouth, taking care to keep the tentacles distended, and it is not necessary to close the opening with a cork. The other species of Cucumaria are killed in the same manner. It is not necessary to inject the small specimens.

The large Synapta, in the preparation of which much difficulty has been experienced on account of its tendency to break to pieces, is killed by immersion in a tube containing sea water and ether in equal parts. In this mixture the animals usually die completely distended. When dead and well distended, they are washed in fresh water and put into weak alcohol. The transfer to 70 per cent alcohol should be very gradual, to avoid contraction. Chloroform may be used instead of ether. The hardening may also be done by putting 2 or 3 c.c. of 1 per cent chromic acid into the water in which they have been washed. After a few seconds remove to weak alcohol.

The rare Molpadia musculus and the little Chirodota venusta have been also prepared by this method. Auricularia is best killed in the mixture of sulphate of copper and sublimate or in sublimate alone.

Enteropneusta

Balanoglossus is killed with the Kleinenberg solution or in chromic acid of one-half of 1 per cent, but the former is much better. When narcotized in alcoholized sea water the animals remain well distended and straight. Tornaria is killed with the sulphate of copper and sublimate mixture. It is well preserved also with saturated sublimate or with the chrom-osmic mixture.

Vermes

The Cestodes are fixed with cold saturated sublimate, the Trematodes with the same solution hot. If it is desired to have flat preparations to mount for the microscope, the animals should be placed between two plates of glass, which are brought together by gradual pressure, and then placed in a crystallizing dish under moderate weights. When the animals are flattened enough and there is as little water as is practicable in the dish, pour over them boiling saturated sublimate and leave them therein until they show no signs of contraction. Then, the plates of glass being removed, the worms are allowed to harden thoroughly in cold saturated sublimate, since the boiling sublimate fixes only the margins

of the objects, not being able to penetrate well between the two plates. In this manner well-distended, flat preparations have been made of Tristomum, Acanthocotyle, Distomum, Calicotyle, and of many other Distomata and Polystomata.

Rhabdocoelia and Dendrocoelia.--When they are not quite thoroughly distended in a little water they are killed with boiling saturated sublimate and at once poured into a larger receptacle containing fresh water, where liquid and animals are allowed to cool. From this mixture they are transferred to fresh water, and after a few minutes to alcohol. For certain Polyclades (Eurylepta, Pseudoceros) it is necessary that the sublimate be warmed a little again, otherwise the bodies break up.

Müller's larvae may be killed with saturated sublimate, either cold or hot.

Nemertinae.--Great difficulty has been encountered in dealing with the nemertine worms, because before they are completely distended they contract again, twisting the body badly and breaking to pieces. For some time the effort was made to narcotize the different species by dropping alcohol little by little into the sea water containing the animals, so that as the two mixed the animal would gradually lose sensitiveness and would die. Although this operation was performed with the greatest care, and the worms showed no signs whatever of life, when transferred to the fixing liquid they contracted and became distorted. If the method just described be used with large specimens of Cerebratulus marginatus, one can not tell whether the animals are entirely dead or not. Good success, however, has been attained by rapidly plunging them head first into a mixture consisting of Müller's solution[1] 7 parts and concentrated hydrochloric acid 1 part. After a few minutes wash the animals and harden them in alcohol in a wax-bottomed tray.

After repeated experiments the Nemertines were at last successfully narcotized in a solution of chloral hydrate in sea water of 0.1 of 1 per cent strength, where they were allowed to remain from six to twelve hours and then hardened in alcohol in the long zinc box with wax in the bottom. When these animals have been narcotized for not too long a time in the chloral hydrate they will fully regain vitality and power of movement after a little if placed again in sea water.

By this method good preparations can be made of the genera Carinella, Cerebratulus, Drepanophorus, Nemertes, Polia, etc., in a state of perfect distention and with the proboscis protruded. The more resistant forms (Langia, Amphiporus, and also Drepano-

[1] Potassium bichromate, 2 grams; sodium sulphate, 1 gram; distilled water, 100 grams.

phorus), after they have been narcotized in a 0.1 of 1 per cent solution, may well be placed for some hours in a 0.2 of 1 per cent solution before they are killed.

The larval form, Pilidium, is killed either with the sulphate of copper and sublimate mixture or with saturated sublimate alone.

The Nematodes, free and parasitic, are killed with saturated sublimate or with Kleinenberg's liquid.

Chaetognatha.--These are very well treated in the mixture of sulphate of copper and sublimate and in the chrom-osmic mixture.

Gephyrea--Sipunculus is killed with chromic acid of one-half of 1 per cent or even weaker, in which the tentacles usually, but not always, expand before death. The animal, after being narcotized with chloral hydrate of 0.1 of 1 per cent in sea water, dies with tentacles distended. Both methods are good, but sometimes a portion of the animal remains contracted, and sometimes during the process the skin in front breaks and allows all the perivisceral liquid to escape, with resulting distortion of the body.

Phascolosoma may be placed in alcoholized sea water and allowed to remain there until dead (three to six hours). Phoronis is allowed to remain half an hour in alcoholized sea water and then is killed with boiling saturated sublimate. With the large specimens of Bonellia it is best to wait until the proboscis has become well distended and then seize the body of the animal with one hand and the extremity of the proboscis with a pair of forceps so that it can be kept distended. Then quickly immerse the whole in Kleinenberg's liquid in the wax-bottomed tray, and, always keeping the animal stretched out to prevent contraction, wait until it dies. After lying for an hour in this solution the transfer to alcohol may begin. Small Bonellias are narcotized in alcoholized sea water and fixed in weak alcohol. The very small specimens of these Gephyreans are very well killed with hot sublimate. The pelagic larvae of Echiurus are well fixed by allowing them to lie for some minutes in the mixture of sulphate of copper and sublimate.

Hirudinei, Pontobdella, and Branchellion are killed in chromic acid of one-half of 1 per cent. If in doubt about worms of this family, hot saturated sublimate may be recommended for use. In any case long specimens must be straightened and hardened in the wax-bottomed tray.

Chaetopoda.--Many of these, if placed in a fixing fluid which is too energetic in action, contract greatly and twist out of shape, and many of them break to pieces, so that an idea of the natural form is lost. This trouble has been obviated by very gradually pouring over the surface of the sea water in the crystal-

lizing dish a stratum of a mixture of glycerin, 1 part, 70 per cent alcohol, 2 parts, and sea water, 2 parts. This stratum will slowly diffuse throughout the water, and after some hours the animals will be narcotized and will remain fully distended, if transferred to alcohol.

Experience at the station has shown that alcohol alone suffices for the treatment of these worms. Instead of the mixture just described, one may add to the sea water 5 per cent of absolute alcohol and let the animals remain therein until they have lost motion, an operation which varies from two to twelve hours for the different species. It is a good plan not to allow these worms to become entirely dead in the sea water. The hardening is done in the long wax-bottomed trays with 70 per cent alcohol, straightening out the animals and holding them in place when necessary by means of wooden pins. After a few hours in the tray, they should be put into tubes and allowed to rest in a horizontal position for a day or so. Since 70 per cent alcohol does not penetrate the tissues of these animals well enough to prevent maceration, 90 per cent alcohol must be used for permanent preservation. Large specimens should be suspended in the tube by means of a thread attached to a float.

The method just described has given good results with annelids belonging to the following families: Polygordiidae, Opheliadae, Capitellidae, Telethusidae, Maldanidae, Ariciidae, Cirratulidae, Spionidae, Terebellidae, with the exception of the genera Polymnia and Lanice, which are killed with the mixture of sublimate and chromic acid; among the Aphroditidae certain Polynoinae, and all the Sigalioninae; the Amphinomidae, which can also be well treated with saturated sublimate; among the Eunicidae, the Staurocephalinae, Lysaretinae, and Lumbriconereinae; all the Nereidae Glyceridae, Syllidae, Hesionidae, and Phyllodocidae.

In the family of the Chlorhaemidae the genera Stylarioides and Trophonia are narcotized with alcoholized sea water, hardened in chromic acid of 1 per cent, and transferred to alcohol. Siphonostomum diplochaitos of the same family is killed in a solution of chloral hydrate of 5 per cent, and after hardening for fifteen minutes in 1 per cent chromic acid is transferred to alcohol. Another good method is to use the sulphate of copper and sublimate mixture for killing, allowing the animals to remain five minutes in the solution. Animals of this species, when treated with the ordinary reagents, break to pieces with the greatest ease.

Hermionidae are immersed directly in 70 per cent alcohol (old solution will do), taking care that the animals do not die in a curved position.

Chaetopteridae, Sternaspidae, the large Spirographis and the large Serpulinas of the genus Protula are killed in 1 per

cent chromic acid, where they are allowed to rest at least half an hour. Then after thorough washing in fresh water, they are put into alcohol of 70 per cent, and afterwards into that of 90 per cent. Spirographis can not be well treated in its tube or be returned to it after treatment. Myxicola infundibulum is killed in saturated sublimate, and, after ten or fifteen minutes, thoroughly washed and put into 50 per cent alcohol for a few hours before it is put permanently into that of 70 per cent.

The following annelids are killed with cold saturated sublimate, in which they should not be allowed to remain more than fifteen minutes; all the Amphictenidae (which may be placed in alcoholized sea water until well out of their tubes), the Hermellidae, the Serpulidae (some of which should remain for some hours in a 0.1 per cent solution of chloral, so that they may come wholly or partly out of their tubes), of the Aphroditidae certain Polynoinae, Polyodontes maxillosus, of the Eunicidae all the group of the Eunicinae. Some of these, like Dioptara, are best fixed by narcotizing them in alcoholized sea water.

Alciopidae are very well prepared by letting them die in the sulphate of copper and sublimate mixture. They should remain in the solution not to exceed five minutes, and then be washed thoroughly in fresh water before they are placed in alcohol.

Tomopteridae are preserved in the way just described, or with cold saturated sublimate. Remove the last traces of sublimate with iodine.

Crustacea

The marine Cladocera (Podon, Evadne) are killed with saturated sublimate, or with a few drops of osmic acid of 1 per cent in the sea water containing them, removing them when they begin to turn brown. Wash and put into 70 per cent alcohol.

Ostracoda are put at once into 70 per cent alcohol.

Copepoda.--The free forms are killed in a saturated solution of sublimate in sea water, where they are allowed to stay from five to ten minutes. The parasitic forms may be killed in the same way or be put at once into weak alcohol.

Cirripedia.--To prepare Lepas, Conchoderma, etc. with the cirrhi distended, let them die in alcohol of 35 per cent. If the cirrhi of certain species contract they can easily be drawn out again by means of forceps.

Balanus and similar forms are immersed directly in alcohol of 70 per cent, taking care to change the liquid soon.

Rhizocephala (Sacculina, Peltogaster, etc.) are placed for fifteen minutes in a mixture of 90 per cent alcohol and sublimate

in equal parts, and then transferred to 70 per cent alcohol.

Amphipoda.--All the Laemodipodes, Crevettines and Iperines were formerly prepared by putting them at once into alcohol of 70 per cent, but now they are put into Perenyl's solution for fifteen minutes before they are immersed in alcohol. The transparent forms of the last division (Phronima, etc.) are killed in sublimate.

Ispoda.--These are put at once into 70 per cent alcohol, with the exception of the Bopyrides and Entoniscides, which are first placed in a mixture of 90 per cent alcohol and saturated sublimate in equal parts (like the Rhizocephala) or in a saturated sublimate alone.

Cumacea and Stomatopoda go directly into alcohol, though the transparent larval forms of the latter are first put into saturated sublimate for a few minutes.

Schizopoda go at once into alcohol or they may be treated first with saturated sublimate.

To avoid the breaking of the appendages of the Decapoda, these forms are allowed to die in fresh water before they are put into alcohol. They remain in fresh water only as long as is necessary, otherwise the membranous appendages are injured.

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With the Paguridae the alcohol must be changed often, and they are to be preserved permanently in 90 per cent alcohol, because the shell is permeable to only a small degree.

The larvae of the Decapods (Zoëa, Phyllosoma, etc.) are killed in sublimate or with a few drops of osmic acid of 1 per cent.

Pantopoda

The Pantopoda are killed in chromic acid of one-half per cent so that they will remain with the legs distended. As they are almost always covered with foreign bodies, it is necessary to let them live for several days in beakers of fresh sea water, so that they may clear themselves of these extraneous growths.

Mollusca

Lamellibranchs may be prepared with the valves open by narcotizing them in alcoholized sea water, where they may remain from six to twelve hours or even more, according to the species. The siphonate forms should not be transferred to alchol before they are thoroughly stupefied, otherwise the siphons will contract. After the animals have been narcotized, it is an excellent plan to place them in chromic acid of 1 per cent for about a half hour.

They are very likely to open their shells wider after they have been placed in the chromic acid. When chromic acid is not used, it is well to place a bit of wood between the valves to keep them apart when first put into alcohol. Cocaine may be used instead of alcoholized sea water for narcotizing the animals. The animals are preserved in a more distended condition, but its use is not as necessary for lamellibranchs as for gastropods, and the method will be described when the latter are discussed.

Lima, which has a large number of tentacular filaments around the edge of the mantle, which break off if alcoholized sea water be used, is killed with chromic acid of one-fourth of 1 per cent. Large specimens, however, yield better results if treated with the copper sulphate solution first.

Scaphopoda.--Dentalium is narcotized with chloral hydrate of 0.2 of 1 per cent, in which it remains from ten to twelve hours or more, and is then put into 70 per cent alcohol.

Gastropoda.--The use of cocaine for narcotizing all species of gastropods is strongly recommended. The solution consists of 2 grams of cocaine powder dissolved in 100 c.c. of 50 per cent alcohol. Place the animals in the least practicable amount of water. Drop in a few drops of the cocaine solution, and after two hours add a few more, and continue the operation until the animals are thoroughly insensible. The action is much slower in winter than in summer. To avoid the contraction into the shell, which is apt to take place with prosobranchs having a spiral shell, even when the narcotizing has seemed to be complete, draw the operculum as far out as possible with a pair of pincers and bind it to the shell.

As cocaine is not always available, the old methods for treating gastropods will be detailed. It is to be understood that when cocaine is used for narcotizing the subsequent treatment is that indicated below:

The Placophora and the families of the Patellidae, the Fissurellidae, and the Haliotidae may be prepared in a distended condition by narcotizing them with alcoholized sea water.

Natica josephinia may be fixed in complete distention by dropping 70 per cent alcohol little by little into the sea water until the animals no longer respond to any stimulus, an operation which often lasts two or three days. Then they are killed by rapidly pouring concentrated acetic acid over them, and they are transferred at once to weak alcohol. If one desires to get perfect specimens he must treat several at once, because out of every lot some are sure to remain more or less contracted.

Natica millepunctata and N. hebrea, when treated in the manner just described, remain entirely contracted. Good results may be obtained, however, by letting them remain for some days

in a mixture of sea water and fresh water in equal parts, and afterwards killing them with acetic acid. This may be used for preparing several species of Nassa, Columbella, Conus, and Trochus.

Heteropoda.--The Atlantidae may be narcotized in alcoholized sea water, where they are allowed to remain for from six to twelve hours, and are then placed directly in alcohol. Cocaine, however, is much better for narcotizing.

The Pterotrachaidae are killed by immersing them in chrom-acetic mixture No. 1 for from ten to thirty minutes according to their size. Wash thoroughly in fresh water and transfer gradually to the different grades of alcohol. These animals are well prepared also with the chrom-osmic mixture, and the little specimens of Carinaria are best treated with the mixture of sulphate of copper and sublimate. Large specimens should be suspended in the permanent receptacle by a thread tied around the end of the proboscis.

Opisthobranchiata.--The Bullas are slowly narcotized in the mixture of sea water and fresh water in equal parts or in alcoholized sea water and allowed to remain therein until thoroughly insensible. They are killed with concentrated acetic acid and transferred at once to alcohol.

Gastropteron meckeli is killed in Kleinenberg's solution, thereby retaining its natural red color very well. It looses its color in the ordinary liquids.

Doridium and Scaphander.--Narcotize in alcoholized sea water, kill in concentrated acetic acid, and quickly transfer to alcohol. If not hard enough, or if softened at all in the acetic acid, they may be placed in chromic-acid solution of 1 per cent for ten or fifteen minutes before they are put into alcohol.

Philine.--When the animal is well distended in a little sea water, suddenly pour over it concentrated acetic or pyroligneous acid and quickly transfer to alcohol.

Pleurophyllidia is narcotized with alcoholized sea water and then killed with concentrated acetic acid.

Aplysia limacina and A. punctata are fixed in 1 per cent chromic acid, where they are allowed to remain from fifteen to sixty minutes, according to their size. A. depilans is narcotized in chloral hydrate solution of 1 per cent (which may take twelve hours), killed with concentrated acetic acid, transferred at once to chromic acid of 1 per cent, and after half an hour put into 50 per cent alcohol, and so on.

Pleurobranchia meckeli is best treated with cocaine and then put into chromic acid of 1 per cent, where the animals may remain about an hour before they are washed and put into alcohol.

Pleurobranchus meckeli and P. testudinarius may be killed in chromic acid of 5 per cent. When scarcely dead the animals are transferred to that of 1 per cent, where they may remain from fifteen to sixty minutes, according to their size. The small specimens can be well prepared with chloral hydrate, also, afterwards fixing them with chromic acid of 1 per cent.

Umbrella is slowly killed in alcoholized sea water, after which it is put into weak alcohol.

The Elysiidae and the AEolidiidae are permitted to expand in the least practicable amount of sea water. They are then killed by rapidly pouring over them a volume of concentrated acetic acid double or equal to that of the sea water, and when scarcely dead they are transferred to weak alcohol.

Phyllirrhoe bucephalum is fixed in the chrom-osmic mixture for a few minutes or in the chrom-acetic mixture No. 2.

Doris, Chromodoris, etc.--The larger specimens of these animals may be narcotized by adding 70 per cent alcohol, a little at a time, to the water containing them, until touching the branchial appendages on the back produces no contraction. They should then be killed with concentrated acetic acid or boiling saturated sublimate. If cocaine is used for narcotizing the animals, they should be killed in concentrated acetic acid, placed in chromic acid of 1 per cent for ten minutes and then transferred to 50 per cent alcohol, and so on. The small specimens need not be narcotized.

Triopa, Idalia, and Polycera are treated like the Elysiidae,

The large specimens of Tritonia are immersed until dead in fresh water, to which a few drops of acetic acid have been added, when they are hardened in chromic acid of one-half of 1 per cent. By this method they remain well distended and the shape suffers no alteration. Small Tritonias are treated with cocaine and then hardened and placed in alcohol.

Marionia is narcotized in alcoholized sea water and killed in acetic acid.

To prepare Tethys with the dorsal appendages in position, the animal is allowed to expand in a large, low crystallizing dish in the least amount of water possible necessary to cover it. It is killed by pouring over it a quantity of concentrated acetic acid at least as great as the water in the dish. Then the liquid is removed by means of a siphon and chromic acid of 1 per cent substituted therefore. Then carefully try to give the animal a life-like appearance by flattening the foot on the bottom of the receptacle and arranging the cephalic lobe so that it rests easily rolled up in conical shape. In this manner it should harden, and after half an hour the chromic acid should be siphoned off and weak alcohol introduced. The animal must be suspended in the final receptacle.

Pteropoda.--The Hyaleidae are placed in a little water and allowed to expand the two wings, when saturated sublimate solution is poured over them. After a few minutes they are washed and placed in weak alcohol, and so on. Criseis acicula is well prepared by killing it with alcoholized sea water.

Cymbuliidae are very well killed in Perenyi's solution, where they may remain fifteen minutes before they are transferred to 50 per cent alcohol. If they are prepared with the chrom-osmic mixture, their form is perfectly preserved, but the transparency is partly lost.

The Gymnosomata are placed in chloral hydrate solution of 0.1 of 1 per cent for from six to twelve hours and then are quickly killed with acetic acid or sublimate. Good preparations of Cliopsis have been made by letting the animals die in chromic acid of one-fourth of 1 per cent.

Cephalopoda.--The preparations are much better, of course, when the animals are immersed in the preserving fluid while still alive. If they have been dead for some time when received, they can be made to regain their shape in part by allowing them to lie in sea water for about an hour. Then they had better be hardened in 1 per cent chromic acid for fifteen to sixty minutes, according to their size.

Small octopods are narcotized in chloral hydrate of 0.2 of 1 per cent, and then immersed in alcohol, where they sometimes contract, twisting the arms about the body, but after the animals are dead it is easy to stretch out the arms and dispose them in a natural position. The larger animals (of a length of 15 cm. (6 inches) or more) are killed in 1 per cent chromic acid, where they are usually kept a half hour, though very large ones may remain even so much as two hours. After washing them in fresh water, transfer to 70 per cent alcohol, taking care to change the latter several times.

Ocythoe catenulata (Philonexisa).--Females of medium size are immersed directly in 70 per cent alcohol and after they are dead their arms are straightened out. Scaeurgus tetracirrhus (Octopus) is killed in the mixture of alcohol and chromic acid and after twenty minutes is transferred to alcohol.

The Decapods may be put at once into alcohol of 70 per cent, taking care, before they are quite dead, to pull out the two tentacular arms, which usually have contracted. The small species should be narcotized in chloral hydrate of 0.2 of 1 per cent or in alcoholized sea water before they are put into the alcohol. To facilitate the penetration of the alcohol into the visceral region of the largest specimens an incision should be made in the ventral part of the body.

The transparent pelagic forms (Loligopsis, Verania) are put first into Kleinenberg's solution and after an hour are

transferred to weak alcohol, from which they are gradually changed to that of 70 per cent. The forms which are contained in a common gelatinous substance are fixed with chromic-acid of one-half of 1 per cent and then transferred to 50 per cent alcohol, where they remain permanently.

Bryozoa

Bryozoa are best preserved when they are treated on board ship immediately after they have been caught.

Pedicellina and Loxosoma are left for an hour in chloral hydrate of 0.1 of 1 per cent and then killed with saturated sublimate, cold or hot. Wash immediately afterwards and place in alcohol. Certain species of Bugula (purpurotincta, turbinata), after the animals have become well distended in a little sea water, are killed quickly with hot sublimate. By pouring some 70 per cent alcohol slowly over the surface of the water in which they are, Flustra, Cellepora, Crisia, Bugula, and Zoobotrium have been prepared in a state of complete distention. The other species can be killed with the animals more or less out of their cells by using a weak solution of chloral hydrate or alcoholized sea water, but generally good results depend upon the skill of the preparator.

Brachiopoda

Brachiopods are narcotized by letting them lie in alcoholized sea water for some hours before they are put into alcohol. Put a bit of cork between the valves to keep them open. Small forms may be put at once into 70 per cent alcohol.

Tunicata

The Appendiculariae are killed by letting them rest for five minutes in the chrom-osmic mixture.

Ascidiae simplices.--To treat Clavellina and Perophora so that the orifices shall remain open, first allow them to expand in running sea water and then immerse them in chloral hydrate of 0.1 of 1 per cent, letting them remain from six to twelve hours; then kill them with chrom-acetic No. 2 and immediately afterwards transfer to chromic acid of 1 per cent, some of which should be injected into the interior of each individual. After a half hour transfer the animals to 35 per cent alcohol, repeating the injection with that fluid, and finally to alcohol of 70 per cent. Clavellina rissoana is usually killed in acetic acid; it is not necessary to inject each individual.

Ascidia (Phallusia), after from three to six hours in chloral hydrate of 0.1 of 1 per cent, is hardened for half an hour in chromic acid of 1 per cent, washed, and transferred to alcohol.

Ciona intestinalis is killed slowly by putting into the water in which the animals have expanded a few drops of the chrom-acetic mixture No. 2. When the animal has died, which happens in about a half hour, it is to be taken by the anterior orifice, to avoid the discharge of the water within, and put into chromic acid of 1 per cent, making an injection of the same into the body cavity. The transfer to the alcohol series should be made in the same way.

Certain ascidians (Ascidia and Rhopalea) are killed in the following manner, so as to keep the orifices open: They should be placed in beakers with from 4 to 5 cm. (about 2 inches) of sea water above them. Then slowly drop in 1 per cent chromic acid in such a manner as to form a stratum on the top of the water. Little by little the chromic acid will diffuse through the water, usually killing the animals in from twelve to twenty-four hours. If the animals are not dead by that time, add a little more chromic acid. Harden in chromic acid of 1 per cent, wash in fresh water, and put into alcohol. The animals should not rest against the sides of the beaker during narcotization. If the animal has not a good base upon which to stand, some clean sand may be placed in the bottom of the glass, in which it can be arranged in the desired position.

Molgula, Polycarpa, Rhopalea, and Chevreulius (Rhodosoma) must remain for twelve hours in chloral hydrate of 0.1 of 1 per cent. Then kill them in chrom acetic No. 2 and transfer at once to 1 per cent chromic acid for a little time to harden.

Cynthia and Styela are narcotized in 0.2 of 1 per cent chloral hydrate for twenty-four hours and then treated like the last-mentioned genera. Cynthia papillosa, however, sometimes contracts greatly when immersed in chloral hydrate of 0.2 of 1 per cent. When it does, put it back into running sea water to expand again, and then try treating it with 0.1 of 1 per cent chloral hydrate.

Ascidiae compositae.--The gelatinous forms--for example, the Botryllidae, Polycyclus, Circinalium, and Fragarium--are allowed to lie in chloral hydrate of 0.1 of 1 per cent for a few days and then are killed by pouring hot saturated sublimate over them. Immediately afterwards they are transferred to chromic acid of one-half of 1 per cent, where they are left for a half hour before they are washed and transferred to alcohol.

Distaplia, after it has been narcotized with 0.1 of 1 per cent chloral, is killed with chrom-acetic No. 2, washed, and put directly into weak alcohol.

Diazona violacea should remain twelve hours in chloral hydrate of 0.2 of 1 per cent, and then the killing and the hardening should be done as with the Botryllidae, except that the individual animal should be injected with the liquid. Small colonies may be killed in acetic acid and hardened in one-half per cent chromic acid.

Leptoclinum and other forms of a certain consistency are transferred from the chloral directly to the alcohol.

Pyrosoma is suspended by a thread in the mixture of alcohol and hydrochloric acid in a cylindrical jar, and, after a quarter of an hour, is transferred to 60 per cent alcohol and gradually to that which is stronger. Good preparations have been made by putting the colony directly into 50 per cent alcohol. Care must be exercised to get rid of the minute air bubbles which are apt to form on the surface of the colony, though they usually disappear of themselves.

The Salpidae include animals of very various consistency, from slimy to cartilaginous. Certain species, furthermore, although they have consistency when young, become soft in the adult stages and difficult to preserve. Sometimes the Salpas when immersed in the fixing fluid contract greatly, closing the orifices and dying in this condition. This may be remedied by introducing a closed glass tube into one of the openings, which, by allowing the entrance of the liquid, causes the animal to resume its natural shape.

The species with a hard body (Salpa bicaudata when solitary and young; S. tilesi, both chain and solitary forms; S. zonaria both chain and solitary forms), are immersed in a mixture of fresh water (100 c.c.) and concentrated acetic acid (10 c.c.) where they remain for fifteen minutes. Then they are washed in fresh water for ten minutes, and then transferred gradually to alcohol, where it is necessary to float the larger forms by means of pieces of cork attached to them with threads so that the gelatinous sac shall not flatten down upon the intestinal nucleus within. When treated in this manner, the animals remain very transparent, crystals of marine salts forming in the tissues much less than with the other liquids.

The forms of medium consistency (young chains and solitary individuals of Salpa maxima and S. pinnata, young chains of S. bicaudata, both the adult forms of S. fusiformis and S. democratica-mucronata) are placed in the chrom-acetic mixture No. 1 for ten minutes and then put directly into weak alcohol.

The very soft forms (large chains of Salpa bicaudata and S. punctata, both forms of S. maxima, S. pinnata, and S. virgola) are immersed in the chrom-osmic mixture for from fifteen to sixty minutes, according to their size. Then they are washed in fresh water and transferred to weak alcohol.

Very large specimens of Salpa maxima flatten out their own weight when put into weak alcohol. This may be obviated by blowing a few bubbles of air into the cavity of the animal or by putting therein a tube of thin glass closed at both ends to act as a float. The tube or the bubbles of air should be removed before the animal is entirely hardened.

Professor Todaro, to preserve Salpas for histological purposes, immerses them at first in Kleinenberg's solution, and after an hour transfers them to alcohol. When preserved in this manner, however, all but the hard species lose their form entirely.

One can easily inject the circulatory system of living Salpas with Prussian blue by placing the point of a fine syringe in the slender canal of the heart and operating it with a very gentle pressure. After this the animals can be treated by the methods already detailed, and the color will remain very well after they have been put into alcohol.

The Doliolids give good preparations when killed with the mixture of sulphate of copper and sublimate, saturated sublimate alone, or with the chrom-osmic mixture. After a few minutes wash the animals thoroughly with fresh water and transfer them gradually to 70 per cent alcohol.

Fish

In general, fish present no difficulties in their preparation. If possible, they should be alive when put into the fixing fluid, because thus only do they preserve the shape of the body well and keep the fins completely distended. Those which have been dead for some time and have been left to dry, having already lost much water, have the fins contracted and dried, and when placed in alcohol they contract still more. To preserve dead fish for anatomical purposes, inject them first through the anus with 90 per cent alcohol and then put them into that of 70 per cent.

To prepare Amphioxus with the mouth cirrhi distended, the animals are allowed to die in sea water alcoholized to 10 per cent, and after death, which usually occurs in a few minutes, they are transferred to alcohol of 50 per cent, and gradually to that of 70 per cent. Müller's solution[1] can also be used for killing, if suitable for the purpose for which the animals are intended; but they remain colored, and often swellings are formed in the sides of the body. Chromic acid of 1 per cent is sometimes

[1]Potassium bichromate, 2 grams; sodium sulphate, 1 gram; distilled water, 100 grams.

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used for the killing.

Cyclostomans, Selachians, and Ganoids.--Small specimens are put at once into 70 per cent alcohol. With large specimens it is difficult for the alcohol to penetrate the viscera, and it is necessary to make an incision in the belly, or else to inject 90 per cent alcohol through the anus repeatedly and at every change of fluid.

Certain species of soft consistency, like Torpedo, are better fixed if they are allowed to lie in 1 per cent chromic acid for a half hour before they are put into alcohol.

Embryos of Selachians (from 1 to 10 c. m. in length) are fixed in saturated sublimate, where they may remain from five to fifteen minutes. Be careful to wash well and to make use of the usual test with iodine. When prepared thus they are also good for histological studies. Fair success has attended the treatment of the embryos of Torpedo, with the entire yolk sac attached, by placing them for fifteen minutes, in a mixture consisting of 1 per cent chromic acid and saturated sublimate in equal parts, then washing them in fresh water and putting them into alcohol.

If it is desired to preserve moderately large Selachians for the future preparation of the skeleton as well as the skin, it suffices to open the belly, remove the intestines, and immerse the animal in a 10 per cent solution of common salt.

The Teleosts are treated like the Selachians, but the alcohol penetrates the tissues with still greater difficulty, and it is necessary, particularly with the larger forms, to make repeated injections of the liquid. The Teleosts with silvery skin (Trachypterus) are put into saturated sublimate for a few minutes before they are placed in alcohol. The transparent larval forms may be put directly into weak alcohol, or may be fixed first with saturated sublimate.

The transparent fertilized eggs can be preserved for purpose of demonstration by allowing them to remain for some minutes in the mixture of alcohol and hydrochloric acid and then transferring them to pure alcohol.

SOME NARCOTIZING, KILLING, FIXING, AND PRESERVING REAGENTS AND THEIR USES, COMPILED FROM BOTH LO BIANCO AND WAGSTAFFE-FIDLER

(A) LIST OF REAGENTS (*INDICATES CONSIDERABLE TO MUCH USE OF THE SUBSTANCE)

ACIDS

Acetic, glacial, 55% concentrated
Acetic, Osmic
*Chromic .25, .5, 1, 5%
Chromic & Osmic .25, 1%
Chrom-acetic #1 = 1% chromic 100 cc & concentrated acetic 5 cc
*Chrom-acetic #2 = 1% chromic 10 cc & concentrated acetic 10 cc
Chrom-formalin 4%
Chrom-osmic 1%
Chrom-picric solution
Chrom-alcohol
Hydrochloric-alcohol
Nitric
Osmic 1%
Picric, saturated
Pyroligneous, concentrated
Sulphuric

Albumen
Alcohols - 35, 50, 70, 95%
Iodized 35, 70%
Alcoholized seawater 3, 10%
B-Eucaine hydrochloride
Benzamine hydrochloride 2% aqueous solution
Bouins
Cellosolve, pure (ethylene glycol mono-ethyl ether)
Chloral hydrate .2, 5%; .1% in seawater
Chloroform
*Cocaine
Copper sulphate
*Corrosive sublimate
Corrosive sublimate - 90% alc.
*Corrosive sublimate - acetic acid
Corrosive sublimate - Chromic acid
Corrosive sublimate - osmic acid 1%
*Corrosive sublimate - copper sulphate
Corrosive sublimate - seawater
*Distilled water

Ether - seawater
Ethyl acetate fumes
Ethyl bromide
Euparal
Fleming's fluid (1% chromic acid 25 cc 1% osmic acid 10 cc glacial acetic 5 cc, distilled water, 60 cc)
Formalin 4, 6, 10%
Formol alcohol
Gilson's fluid
Glycerin
Glycerin 1 part, 70% alc. 2 parts, seawater 2 parts
*Kleinenberg's solution (picric acid, saturated 100 cc, sulphuric acid, concentrated 2 cc, filter and add 3 times the volume of distilled water)
Magnesium chloride
Magnesium sulphate crystals
Menthol crystals
Muller's solution (Potassium bichromate 2 g, sodium sulphate 1 g, distilled water 100 cc)
Perenyi's solution (40 cc of 10% nitric acid, 30 cc of 0.5% chromic acid, 30 cc of 90% alc.)
Potassium bichromate - osmic acid
Prussian blue
Schaudinn's fluid
*Seawater
Sodium chloride solution
Sodium hydroxide 10%
Stovaine
Tobacco smoke

(B) SOME NARCOTIZING REAGENTS AND THEIR USES

Most invertebrates are highly contractile and to be preserved in an extended condition must be narcotized slowly in water, either until dead or until they may be killed or fixed without contracting. The following reagents and methods are recommended.

ALCOHOL. This is advised for the larger invertebrates. A 50 or 70% solution is added drop by drop, to the surface of the water. Lo Bianco's mixture containing 40 parts of 70% alcohol, 20 parts of glycerin, and 40 parts of water is also excellent and is poured onto the surface of the water and allowed to diffuse slowly.

B-EUCAINE HYDROCHLORIDE. This is used as a 1% solution gradually introduced to the water containing the animals. It is especially valuable for Floscullariae, Vorticellidae, Rotatoria and many larval forms and dissolves in seawater to approximately 0.5%. In the British Collector's Handbook, the following is recommended;

Eucaine	1 g
Alcohol 90%	10 ml
Distilled water	10 ml

to be introduced slowly over a period of time. It is primarily for larger forms.

CARBONIC ACID GAS. This has been used for Coelenterata, Echinodermata, and Hirudinea. The gas is introduced by squirting the contents of a soda-water bottle into a considerable volume of water containing the animals so that the water is saturated by the gas.

CHLORAL HYDRATE. Very good rusults have been obtained with this chemical with Actinae, Annelida, Mollusca, freshwater Polyzoa and many larval forms. The crystals should be dropped directly into the water containing the animals, or the animals placed directly in a 5-10% solution. For more delicate work, the solution should be introduced very gradually over a period of several hours. It is used for killing animals in rock crevices, incrustations of calcareous algae, and colonies of serpulas and madrepores. The animals should remain in the solution for 4-6 hours.
ADVANTAGE: If animals do not narcotize as desired, they may be replaced in clean seawater, and will recover.

CHLOROFORM. This is useful for highly contractile animals and should be employed by squirting small quantities of it through a fine syringe every five or ten minutes onto the surface of the water containing the animals. It may also be placed in a separate container and enclosed under a belljar together with the animals to be treated. For some forms one or two drops should be added every 5 or 10 minutes to the vessell containing them.

CHLORETONE (Acetone Chloroform). This is recommended as a 1% solution for small Polyzoa, used in the manner described for chloroform and added slowly.

COCAINE. 2 g of powder in 100 cc of 5% alcohol is a very good narcotic.

ETHER. Oestergren (Zeit. Wiss., Mik., xix, 1903, p. 300) recommends a saturated solution (7-8%) in either sea-water or fresh water, used either concentrated or diluted to approximately 1%.

MAGNESIUM SULPHATE. Often used as a saturated solution into which the animals are plunged. More satisfactory results are obtained however if the crystals are added to the water containing the animals, or if it is introduced gradually over a period of some hours to the water in the form of a 20-30% solution. It is recommended for many marine forms, especially Actinae.

MENTHOL. One of the most useful narcotizing agents for many marine animals. It has been chiefly recommended for Mollusca, anemones (Zoantharia), sea cucumbers (Holothuroi-dea), and sea squirts (Tunicata). Animals are placed in a clean vessel containing clean seawater, and crystals are sprinkled on the surface.

ROUSSELET'S SOLUTION. A well known mixture widely used for narcotizing Rotifers and Polyzoa by many workers apparently with success. The original formula consisted of:

2% solution hydrochlorate of	Cocaine	3 parts
	Methylated spirit	1 part
	Water	6 parts

Stovaine provides a satisfactory substitute for Cocaine. The mixture is added drop by drop to the water containing the animals.

STOVAINE (Amyl Chlorohydrin). This is a most useful and power-ful narcotizing agent for small invertebrates. It now has largely replaced cocaine which is difficult to obtain. It is usually used as a 1% solution in distilled water which is gradually added, over a period of time to the water containing the expanded animals.

TOBACCO SMOKE. An effective narcotizing agent for many small organisms, such as Hydra and Infusoria. It should be slowly and carefully bubbled into water through a fine glass tube lying on the bottom of the container.

(C) SOME KILLING, FIXING, PRESERVING REAGENTS AND THEIR USES

ACIDS

Acetic

1) Permeates and hardens tissues instantly.
2) Rapidly kills contractile forms (tissues soften if left in too long).
3) Objects remain relatively transparent in it.
4) Used with chromic acid it is effective for killing and hardening non-contractile forms.

Chromic

1) Next to alcohol, in an aqueous solution, it is the most useful reagent for killing and hardening soft, gelatinous forms.
2) Caution - Objects become fragile and too deeply tinged if left in it longer than necessary.
3) After treatment, wash animals in fresh water to avoid a precipitate when they are later placed in alcohol.
4) When mixing chromic with osmic, acetic, picric or corrosive sublimate, use FRESHWATER.
5) Solutions do not keep long.
6) Solutions that have turned green are not fit to use. (Solutions may be used if they are not too dilute when mixed with the water containing the specimen).

Hydrochloric - USE RARE - Mixed with 50% alcohol.

Lactic

1) A solution of 1-1000 seawater effective for larval and small gelatinous forms.

Osmic - USE RARE TODAY.

1) Hardens gelatinous forms and preserves transparency well, but its action is too great.
2) Caution - Animals should remain in it only until they are light brown color.
3) It is used in
   a) Chrom-osmic mixture.
   b) Potassium bichromate-osmic acid.
   c) Flemming's solution.

Pyroligneus - USE RARE

Sulphuric - USE RARE. (Ingredient of Kleinenberg's solution).

Alcohol - Most indispensible liquid.

1) For preparation and preservation of transparent animals, filter and dilute with distilled water.
2) Caution -
   a) if reused, acids and alkalies must be neutralized.
   b) to avoid bubbles, mix previous to using.
3) Use 70% for preserving - 90% for special cases.
4) Soft and gelatinous animals must remain 2-6 hours in 35, 50, 60, 70% alcohol each.
5) Animals may be placed in alcohols or if too delicate siphon used alcohol and flood them with increasing strengths.
6) 70% used for permanent preservation.
7) Alcohol is effective for narcotizing and killing animals slowly or quickly.

Copper Sulphate

1) Use - killing larvae and delicate forms in a 5-10% solution of hot freshwater used alone or mixed with corrosive sublimate.
2) Caution - wash objects frequently with water or they will not remain clear. Treat with acid, if specimens become opaque.

Corrosive sublimate

1) Fixing agent. Permeates and hardens tissues quickly.
2) Use in concentrated solution in either fresh or seawater - hot or cold.
3) Caution -
   a) do not use metal tools; they decompose and stain specimens,
   b) make with hot water,
   c) do not boil in open vessel - vapors are very poisonous,
   d) keep hands out of solution especially if they are cut.

Formalin

1) Used to preserve animals temporarily only, because animals eventually decompose in it.

2) Can be used for animals that are
   a) non-contractile,
   b) have no lime, spicules, skeleton or shells.
3) Fish - use 5% injection through anus.
4) Place only a few animals in the same jar at the same time and have much fluid in proportion to animals.
5) Gelatinous animals - use 1-4%. Kill and harden at the same time with formalin plus 1% chromic acid in a ratio of 1:1.
6) Ascidians and Tunicates - use 2-6% formalin (the softer the animal the weaker the solution).
7) To make solutions, use fresh, or best, seawater.
8) Transfer animals directly from formalin to alcohol.
9) Effective for killing and hardening certain forms. Advantage - Colors preserved longer in formalin than in alcohol.

Potassium bichromate

1) 5% solution used for slowly hardening gelatinous forms especially when it is not possible to use chromic acid.
2) Caution - A troublesome precipitate is formed when specimens are transferred to alcohol so its use is not recommended.
3) To bleach forms before placing them in alcohol, use a few drops of concentrated sulphuric acid.

Tincture of Iodine - USE RARE.

1) Used in a 35 or 70% alcoholic solution after animals have been killed in corrosive sublimate.

METHODS FOR NARCOTIZING AND PRESERVING MARINE INVERTEBRATES, ABSTRACTED FROM WAGSTAFFE AND FIDLER, ARRANGED BY MAJOR TAXA

PROTOZOA, FORAMINIFERA, RADIOLARIA

Not narcotized, but killed, fixed and stained by various methods.

PORIFERA

Not narcotized.

(a) Wash in clean salt or fresh water.
(b) Immerse in 95% alcohol if small; if large, use only small piece for preserving.
(c) Store in 95% alcohol.
(d) Spicules
    1) Siliceous
        a) Boil in nitric acid to get rid of soft parts.
        b) Pour off acid and wash several times with distilled water, filter, dry in an oven and mount on a slide in euparal.
    2) Calcareous
        a) Macerate in 10% caustic soda.
        b) Wash as above, filter, dry and mount on a slide in euparal.
(e) Larvae
    1) Killing and fixing. Place in little seawater and flood for 2 or 3 minutes with Flemming's fluid (see page 52)

CNIDARIA (Coelenterata)

A) Hydroids
    1) Place in seawater and allow to extend.
    2) Use menthol, magnesium sulphate or chloral hydrate crystals in a 5-10% solution: Add over a period of 3 + hours until animals are unresponsive to touch.

B) Medusae
    1) Place in a shallow dish with a little seawater and allow to expand.
    2) Use menthol, magnesium sulphate or chloral hydrate crystals in a 5-10% solution, stovaine or chloroform.
    3) <u>Caution</u> - Do not let them die before preserving them.

C) Siphonophora
1) Caliconectae
a) Kill with saturated picric acid.
b) Store in 10% formalin.
2) Physonectae
a) Narcotize with menthol.
b) Kill with 10% formalin, or a saturated solution of corrosive sublimate.
c) Add Flemming's fluid (see page 52) or Kleinenberg's fluid (see page 52) and leave specimens in it 24 hours.
d) Store in 70% alcohol.
3) Cystonectae
a) Kill with corrosive sublimate and glacial acetic acid (95 ml corrosive sublimate saturated solution in distilled water and 5 ml glacial acetic acid). When dead, place in 1/2% for 1 1/2 hours, 30% alcohol for 1/2 hour, 50% alcohol for 1 hour.
b) Store in 70% alcohol.
4) Disconectae
a) Kill with saturated picric acid or corrosive sublimate solution. When dead add a little chromic acid and leave for 1/2 hour. If killed with picric acid, place in 30% alcohol for 2 hours, 50% alcohol for 1 hour. If killed with corrosive sublimate, wash well with running water and pass through alcohols as above.
b) Store in 70% alcohol.

D) Scyphomedusae
1) Kill with a 5% solution of formalin in seawater. In 10 minutes add enough 5% solution of picric acid to color the formalin. Stir contents over a 24 hour period and increase the formalin to 10%.

E) Actinozoa
1) Alcionaria
a) Narcotize with menthol crystals.
b) Store in 70% alcohol.
2) Zoantharia
a) Allow specimens to expand in seawater. Sprinkle in a few crystals of menthol or chloral hydrate, 5-10%, use 3% alcoholized seawater or bubble tobacco smoke through specimen container. Leave over night.

b) Killing. Draw off water but do not uncover animals and flood with 10% formalin.
c) Store in 5-10% formalin or 70% alcohol.

3) Other Methods
a) Add magnesium sulphate crystals gradually or place them in a muslin bag with the specimens.
b) Poison by adding a few drops at a time of dilute formalin. Double the quantity each time.
c) Freeze in block of ice, chip off excess ice. Allow to melt in strong formalin.
d) Draw water down without uncovering animals and kill suddenly with boiling formalin.
e) Caution. DO UNDER FUME HOOD.

4) Cnidae
a) Scrape off ectoderm. Place it in a drop of seawater on a slide. Tease apart with needles. Drop over it a cover slip and press up and down to help break up the tissue. With a camel's hair brush transfer the dissociated tissue to clean seawater. Smear a cover slip with albumen. Place a little of the dissociated tissue on it and air-dry until tacky. Fix with Schaudinn's fluid for 5-10 minutes. Wash with 50, 70% alcohol; then 70% iodized alcohol. Put in 90% alcohol for 1 hour and stain in light green (0.5% solution in 95% alcohol) for several minutes. Rinse in clean 95% alcohol. Mount in euparal.

CTENOPHORA

A) Allow to expand in standing or running seawater. Transfer from water to chromo-osmic (chromic acid 1%, 100 ml, osmic acid 1%, 2 ml) for 15-60 minutes, copper sulphate and corrosive sublimate. Pass through 30, 50, 70% alcohol.
B) Store in 70% alcohol.
C) Caution - DO NOT STORE IN FORMALIN.

PLATYHELMINTHES

A) Turbellaria
1) Narcotize by placing specimens in salt or fresh water depending on the species and add small amounts of menthol or chloral hydrate crystals to the

water for 2-3 hours or until movement is barely perceptible.
2) Kill by passing animals through 35, 50% alcohol.
3) Store in 70% alcohol.

B) Trematoda
1) Clean by shaking specimens in a 1% salt solution.
2) Kill by adding an equal (equal to the amount of salt solution) amount of hot (about 60°C) saturated aqueous solution of corrosive sublimate.
3) Fix in this solution for 1-2 days.
4) Wash in running water for 4-5 hours and pass through 50% alcohol.
5) Store in 70% alcohol.

C) Cestoda
1) Clean by washing in a 1% salt solution.
2) Kill by immersion in a 1% aqueous solution of chromic acid after wrapping around a glass bottle or dish. When dead, place in formol-acetic alcohol for 24 hours. Wash in 50% alcohol.
3) Store in 70% alcohol.

NEMERTEA

A) Allow to expand in running or standing seawater. Narcotize in a 5-10% solution of menthol or chloral hydrate crystals.
B) Caution - Watch carefully that specimens do not vomit proboscides.
C) Killing.
a) After resting in standing seawater for 10-15 minutes, draw off most of water and flood with an aqueous solution of corrosive sublimate and leave specimens in this for 15-20 minutes.
b) Use almost boiling water and transfer to corrosive sublimate for 10 minutes. Wash in several changes of 50% iodized alcohol. Pass to 70% alcohol.
D) Store in 70% alcohol.

ROTIFERA

A) Slow Narcotization. Place specimens in 8 ml water in a watch-glass. Add 2 drops of a 2% aqueous solution of benzamine hydrochloride. Mix with a coarse pipette. After 20-30 minutes, add 1 or 2 drops of benzamine hydrochloride (as much as 6 drops may be needed if water is acid). Total time, 1-3 hours.

B) Rapid Narcotization. Place specimens in less water than above. For every ml of water, add 3-10 drops of:
2% aqueous solution of benzamine hydrochloride .... 3 parts
water ..... 6 parts
Pure cellosolve (ethylene glycol mono-ethyl ether)..1 part
Total time, 3 + minutes.

C) Killing.
a) with 4 ml of 10% formalin.
b) with very hot water. Allow specimens to expand in cold water in a watch-glass; then flood with hot water and transfer to 10% formalin.

NEMATODA

A) Extraction. Wash out gut with a 1% aqueous salt solution.
B) Cleaning. Place washings in a glass jar of 1% aqueous salt solution and shake well. When nematodes sink to the bottom, decant saline and replace with new saline. Continue this process until specimens are clean.
C) Killing. Pour off saline and add 70% alcohol for 1-2 minutes.
D) Store in fresh 70% alcohol.

ANNELIDA

A) Polychaeta
1) Place in clean seawater and add 70% alcohol drop by drop until specimens no longer respond to touch. Tubicolous forms will leave tubes if menthol crystals are sprinkled on their water surface and left over night.
2) Killing. Arrange parts on a dry glass plate and press behind the head to force the extension of the proboscis. Flood with formol-alcohol for 20 minutes. Place in a flat dish with formol-alcohol for 24 hours.
3) Store in fresh formol-alcohol.

B) Hirudinea
1) Allow to expand in water and from time to time add crystals of chloral hydrate or magnesium sulphate.
2) Killing. When specimens are unresponsive to touch, straighten out on a glass plate and flood with warm 10% formalin.
3) Store in 70% formol-alcohol.

C) Gephyra
1) Add a few crystals of chloral hydrate or magnesium sulphate from time to time.

2) Killing. Place on a glass plate. Extend probuscis and flood with 70% formol-alcohol.
3) Store in 70% formol-alcohol.
4) Larvae. Killing and fixing. Draw off most of the water and flood with Flemming's fluid (see page 52) for 2-3 minutes.

CRUSTACEA

A) Branchiopoda, Ostracoda, Copepoda.
1) Kill with 5-10% formalin for 10 minutes. Transfer to 50% alcohol for 10 minutes.
2) Store in 70% alcohol or 5-10% formalin.

B) Branchiura
1) Kill by dropping into caustic soda (NaOH). Transfer to 50% alcohol.
2) Store in 70% alcohol.

C) Cirripedia
1) Allow to expand in water and narcotize with menthol crystals.
2) Kill in 10% formalin or when no longer responsive to touch use hot (60°C) saturated solution of corrosive sublimate for 10-20 minutes and wash in 70% iodized alcohol.
3) Store in 70% alcohol.

D) Malacostraca
1) Decapoda
a) Kill by placing specimens in cold freshwater with or without chloroform. Drop small forms into 70% alcohol.
b) Store in 70% alcohol with a little glycerin added.
2) Larval Stages
a) Kill by placing animals in Bouin's fluid for 3-6 hours. Wash in several changes of 30% alcohol for 30 minutes; 50% alcohol for 30 minutes.
b) Store in 70% alcohol.
3) Isopoda
a) Kill by dropping specimens into 100 ml alcohol + 4 ml glycerin. They may remain in this for 10-14 days.
b) Transfer and store in 70% alcohol.

MOLLUSCA

A) Narcotizing substances and groups
1) Acetic acid and seawater.
a) Tritonia

2) Alcohol, 70%
   a) Chromodoris
   b) Doris
   c) Natica josephinia
3) Alcoholized seawater, 3% or alcohol added slowly.
   a) Amphineura
   b) Decapoda
   c) Doridium
   d) Fissurellidae
   e) Gastropods
   f) Haliotidae
   g) Lamellibranchia
   h) Marionia
   i) Opisthobranichia
   j) Patellidae
   k) Placophora
   l) Pleurophyllidia
   m) Scaphander
   n) Scaphoda
4) Chloral Hydrate, 0.1-0.2%.
   a) Aplysia depilans
   b) Aplysia limacina
   c) Aplysia punctata
   d) Amphinenra
   e) Decapods
   f) Gymnosomata
   g) Octopoda
   h) Scaphopoda
5) Chloroform added slowly.
   a) Lamellibranchiata
6) Cocaine.
   a) Chromodoris
   b) Doris
   c) Gastropoda
   d) Heteropoda
   e) Lamellibranchia
   f) Pleurobranchia Meckeli
7) Freezing in a block of seawater and thawing in 10% formalin.
   a) Nudibranchiata
8) Formalin, dilute. Add a few drops and double the quantity each time they are added.
   a) Nudibranchiata
9) Glycerin, 20 parts; 70% alcohol, 40 parts; seawater, 40 parts and place animals in as little seawater as possible.
   a) Techtibranchiata

10) Magnesium sulphate crystals, allowed to dissolve in a bag in with the animals, or free in their container.
    a) Nudibranchiata
11) Menthol crystals.
    a) Amphineura
    b) Lamellibranchia
    c) Scaphopoda
12) Seawater, standing.
    a) Aeolidiidae
    b) Atlantidae
    c) Elysiidae
    d) Heteropoda
    e) Hyaleidae
    f) Idalia
    g) Philine
    h) Pteropoda
    i) Polycera
    j) Tethys
    k) Triopa
13) Killing and or preserving.
    a) Alcohol. Place specimens in 50% alcohol for 48 hours and transfer to 70%.
        1) Amphineura
        2) Lamellibranchiata
        3) Scaphopoda
    b) Chloroform, ether or ethyl bromide. Inject into mantle cavity.
        1) Cephalopoda
            a) Preserve by placing specimen on board and pin down arms. Inject 5% formalin into mantle cavity and place animal in 10% formalin to harden. Wash in running water. Remove from board.
            b) Store in 70% alcohol or 10% formalin.

POLYZOA (Bryozoa)

A) Narcotizing. Place specimens in salt or fresh-water depending on the species. Use menthol, chloral hydrate 5-10%, stovaine or B-eucaine hydrochloride (the last, especially for freshwater forms). When polyps no longer respond to touch, draw off water and replace with 5% formalin.
B) Store in 5% formalin.
C) Cleaning. Transfer specimens to distilled water. Remove foreign matter with a soft camel's hair brush and wash several times in clean, distilled water.

D) Larvae
   1) Narcotize as above and fix in hot (60°C) Bouin's fluid. Wash in water.
   2) Store in 70% alcohol.

BRANCHIOPODA

A) Narcotize by adding alcohol to specimen's seawater to make a 5% solution.
B) Kill by leaving specimens in this seawater for 30 minutes.
C) Store in 70% alcohol.

CHAETOGNATHA

A) Narcotize by adding menthol or chloral hydrate crystals to small amount of seawater containing specimens.
B) Killing. Straighten specimens out on a piece of glass and flood with Bouin's or Gilson's fluid.
C) Storage. If fixed in Gilson's fluid, wash in 2-3 changes of 50% iodized alcohol. If fixed in Bouin's fluid wash in 2-3 changes of 50% alcohol and in both cases store in 5% formalin.

ECHINODERMATA

A) Asteroidea
   1) Narcotize by adding crystals of magnesium sulphate to their seawater (narcotize with tube feet uppermost).
   2) Kill by placing specimens in 70% alcohol.
   3) Store in 70% alcohol.
B) Ophiuroidea
   1) Narcotize specimens in seawater to which weak alcohol is gradually added.
C) Echinoidea
   1) Kill by placing specimens quickly in 70% alcohol or strong acetic acid. If acetic acid is used, wash out well in running freshwater.
D) Holothuroidea
   1) Narcotize in fresh water to which crystals of menthol or magnesium sulphate have been added.
   2) Kill after narcotized by immersing in 15% formalin for a few minutes and immediately inject 70% alcohol into body cavity.
E) Crinoidea
   1) Killing. Place animals head down in a dish of seawater and hold in position by pressing down. Flood with 90% alcohol for 3-4 minutes.

F) Larvae
   1) Narcotizing. Place in seawater and add a few chloral hydrate crystals or stovaine.
   2) Killing. Draw off most of seawater and flood with Bouin's fluid. Leave for 2-3 hours and wash with water.

TUNICATA

A) Narcotize in clean seawater to which crystals of menthol or chloral hydrate have been slowly added or 3% alcoholized seawater.
B) Killing. Draw off most of seawater containing animals and flood with Bouin's fluid or a saturated solution of corrosive sublimate and leave for 1-24 hours.
C) Storage. If fixed in Bouin's fluid, wash in several changes of 50% alcohol. If fixed in corrosive sublimate, wash in several changes of 50% iodized alcohol. In either case store in 70% alcohol.

SOME RECENT METHODS FOR NARCOTIZATION, KILLING, FIXATION, AND PRESERVATION OF MARINE ORGANISMS.

Appendix I. (1) Propylene Phenoxetol.
(2) Sevin and Rapid Freezing.....M. R. Carriker

(1) Propylene phenoxetol: for many bivalves, particularly those in which the valves do not close tightly; for gastropods in which the shell is wanting; and for some tunicates. It is important that the narcotic have access to the soft parts; in the case of tightly-closing bivalves this can be facilitated by pegging the valves open with slivers of wood while the animals are siphoning actively.

Narcotization may be accomplished by either of the following two ways: (a) Shake 5 ml of propylene phenoxetol in 15-20 ml of seawater to produce a fine emulsion and add this to a small quantity of still seawater containing the actively siphoning specimens. Within a half hour or so animals should be completely relaxed and may be fixed in this condition without causing any contraction of the tissues. (b) Add the phenoxetol (a quantity not to exceed 1% of the volume of seawater present) gently to the vessel of still seawater containing the animals, so that it collects as a globule on the bottom of the container. Organisms may be left in the solution overnight and should be well relaxed at the end of this period. Histological sections of animals treated in this way show no deterioration of the tissues and no loss of staining properties; and narcotized animals if washed and placed in fresh seawater, recover and appear to function normally (Owen 1955).

Killing, fixation, and preservation: Kill and fix narcotized animals in 10% neutralized formalin (200 g of hexamine per liter of commercial formalin: Smith, 1947) for 4 to 24 hours, depending upon the size of the specimens and inject the formalin into the body cavity of larger animals to insure good fixation and to help distend them. Wash preserved animals thoroughly in running tap water for 6 to 12 hours, depending on size and preserve them permanently in 1% propylene phenoxetol and 5-10% glycerol. Particular advantages of propylene phenoxetol as a preservative: is colorless, practically odorless, color retention in specimens is good, and tissues remain soft and flexible and suitable for dissection (Owen & Steedman, 1958). The method may also be useful for other invertebrates lacking an exoskeleton and is worth attempting.

References: (1) Owen, G. 1955. Use of propylene phenoxetol as a relaxing agent. Nature 175: 434. (2) Owen, G., H. F. Steedman 1958. Preservation of Molluscs. Proc. Malacol. Soc. London 33: 101-103. (3) Smith, J. L. B. 1947. A neutral solution of formaldahyde for biological purposes. Trans. Roy. Soc. S. Africa 31: 279-82.

(2) Sevin and rapid freezing: For full narcotization and killing of muricid and naticid gastropods. This is the only known method available to date which makes possible killing and preservation of fully relaxed muricids.

Prepare a stock solution of Sevin (1-naphthyl N-methyl carbamate, available from Union Carbide Chemicals Co.) as follows and store in refrigerator in tightly stoppered glass bottle. Add 0.1 g Sevin crystals to 15 ml (11.6g) acetone. To prepare a solution of 10 ppm. of Sevin add 1.16 ml of stock solution of Sevin to one liter of seawater and mix thoroughly. This must be prepared daily before use as it does not keep.

Narcotization: Place animals in the Sevin solution, 10 ppm. at room temperature for 1 hour, preferably keeping animals out of touch with each other and in the case of gastropods, upside down, and in a depth of fluid at least thrice the height of the specimens. After 1 hour transfer the animals to a fresh solution of Sevin and leave them in it for 3 hours. Employ a salinity of seawater in which the animals normally live. Slightly better narcotization is sometimes obtained if the second change of Sevin is held in 1 atmosphere of CO_2, and if a salinity some 30/00 below the environmental salinity is employed.

Killing: Remove relaxed animals from the Sevin solution one at a time and place the extended soft parts (if possess exoskeleton), or the maximum flat surface of non-shelled forms, against the surface of a block of dry ice held in a deep freezer or insulated chamber. Insulate the preparation and leave for 24 hours or more. Adding chipped dry ice around specimens accelerates freezing, although this is not always necessary. Many animals have to be frozen for at least 24 hours to entirely eliminate reaction to the preservative. Inject larger specimens for fuller distension. Specimens may be held in the frozen state for long periods provided they are not allowed to desiccate.

Freshly thawed, relaxed, unpreserved snails are ideal for detailed anatomical study since the organs retain color, texture and pliability characteristic of living relaxed tissues and take

aqueous stains readily. Prior to freezing, specimens relaxed in Sevin recover from the treatment with either Sevin and Sevin CO_2 when returned to running seawater. Deep freezing is required to kill the animals in a relaxed condition as treatment with Sevin does not insure complete insensibility. This method of narcotization and killing, as well as slow and/or rapid deep freezing alone, may be useful for other highly refractory organisms and should be tested on them.

Fixation and preservation: Animals may be fixed in formalin, washed and stored in 1% proplene phenoxetol-5% glycerol, or may be fixed and preserved in 70% alcohol.

Reference: (1) Carriker, M. R., J. W. Blake 1959. A method for full relaxation of muricids. Nautilus 73: 16-21.