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PRACTICAL FREEZE-DRYING AND VACUUM DEHYDRATION OF CATERPILLARS

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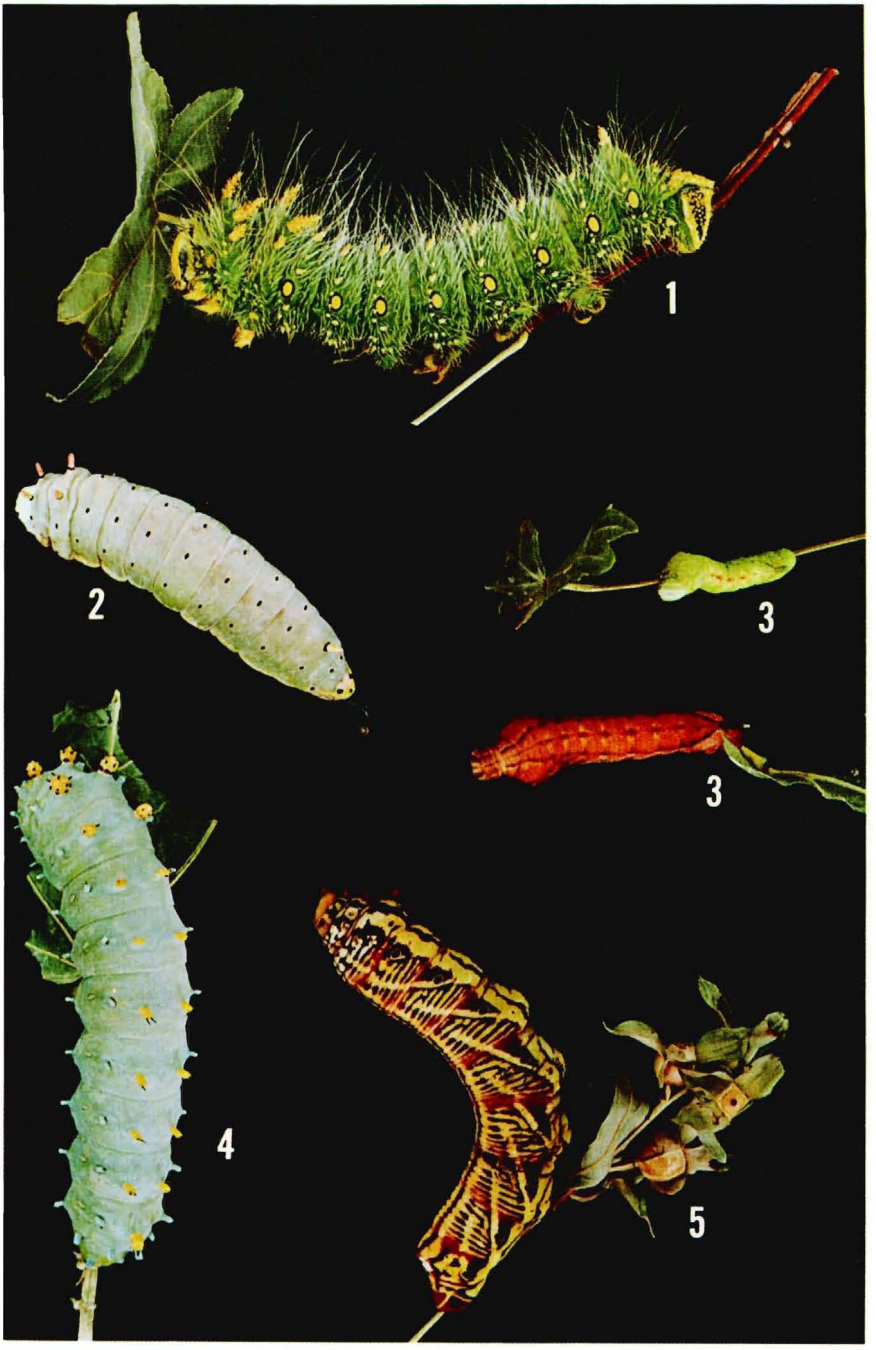
The purpose of this paper is to describe a method of preserving larvae of Lepidoptera and other soft-bodied insects whereby a lifelike appearance can be maintained, the process completed in a short time, the internal anatomy preserved, and the required equipment purchased within the budget of a small laboratory, even of the make-shift variety.

Papers on the subject have been published by Meryman (1960, 1961), Blum & Woodring (1963), Woodring & Blum (1963), and Harris (1964, 1968). But the equipment used by these workers is rather involved and expensive. Flaschka & Floyd (1969) developed a method that requires only a tightly stoppered jar with desiccant, and a home freezer. But the simplicity and low cost are countered by extremely long drying times. The method described here combines a minimum of drying time with an intermediate cost of a few hundred dollars or less. The description will be given in such a way that alternatives and modifications may be applied that allow further reduction of the expenses. For example, the two-stage vacuum pump rated at a vacuum of 0.1 micron is ideal, but a cheaper pump will be found satisfactory as long as it will pull down to about 100–200 microns. The degree of vacuum is one of the factors that has a direct bearing on drying time. The gas ballast recommended for museum systems is unnecessary here because of the relatively small amount of moisture encountered. In any case one must try to be sure that water vapor is not permitted to reach the pump oil, and the purpose of a gas ballast is to remove large quantities of water vapor without damage to the pump. By the same token, dirty oil should be changed.

Provided the specimens are in close enough proximity to the desiccant (or vapor trap) to lie within reasonable reach of the mean free path of the water vapor molecules, several factors influence the time required



Color Plate. Examples of freeze-dried larvae (natural size). 1. *Eacles imperialis imperialis* (Drury), on *Liquidambar stryaciflua*, L. 2. *Callosamia securifera* (Maassen). 3. *Darapsa myron* (Cramer), on *Vitis* sp. 4. *Hyalophora cecropia* (L.), on *Liquidambar stryaciflua*, L. 5. *Eumorpha fasciata* (Sulzer), on *Ludwigia leptocarpa* (Nuttall).

for, and the efficiency of, the drying. The two most important are temperature and degree of vacuum. Their appropriate choice allows manipulation of the process within wide ranges. The method of Flaschka & Floyd (1969), while simple, effective and inexpensive, cannot provide much leeway. The temperature of a home freezer is fixed and usually slightly lower than ideal, and no vacuum is applied. Consequently a drying time of about 100 days for a medium sized larva (e.g. *Malacosoma americanus*) results. Appropriate selection of temperature and vacuum can improve the situation to a remarkable degree. With a vacuum of 0.1 micron at 25° F., such a caterpillar will be adequately dry in 48 hours, finishing off for a few hours at room temperature. In general the specimen should be frozen before applying vacuum to prevent distortion in the freeze-dry process, and I have found the ideal temperature to be about 20–25° F. rather than the lower temperatures recommended in the literature. Such temperature guarantees that the frozen state is maintained while leaving leeway for opening the freezer door, but is not so low as to seriously curtail the rapidity of molecular motion. In principle there is a slight cooling effect due to the sublimation process itself (similar to the cooling experienced when water evaporates from one's skin), but the heat exchange involved is so small that the phenomenon can be disregarded. It becomes a factor of importance only when working with large museum specimens (mammals, reptiles, etc.) where it is one of the conditions that lead to prolonged drying time.

Some Theoretical Considerations

The following discussion is presented in the hope that it may give the reader enough understanding of the underlying processes to help him perform the job more efficiently and enable him to use his ingenuity when adapting the method to his own needs and budget.

Water in both liquid and solid form (ice) exerts a vapor pressure that is a function of the temperature. This means that if brought into a confined chamber, water or ice will evaporate and increase the total gas pressure by an amount equal to the vapor pressure at the prevailing temperature. In relation to the total gas pressure present, one refers to the partial pressure of water vapor. It is important to realize that this concept holds only as long as there is some water or ice still left. Then, when the required partial pressure is reached, a state of dynamic equilibrium is established; in other words, the number of water vapor molecules per unit time evaporating from the surface of the water or ice is exactly equal to the number of molecules condensing on that surface from the gaseous phase. If water vapor is removed from the chamber (e.g. by pumping out, freezing out, or binding on a drying agent) more water vapor will evaporate in order to restore the partial pressure to the level dictated by the temperature. The more molecules of other gasses present (air), the more difficult it is for the molecules evaporating from the water (or ice) to dissipate; then a state of quasi-saturation will be reached near the water (or ice) surface and further evaporation will cease. If, however, a vacuum is applied and the air molecules removed, the evaporation of water and dissipation of its vapor can proceed with less obstruction, and thus more efficiently. It may be noted in passing that no gasses other

than water vapor are produced from the specimen by the drying process in sufficient quantity to merit serious consideration. A further process is involved, namely that of bringing the water from within the specimen to the surface where it can evaporate. This process is called diffusion, and its rate is a function of molecular motion and thus also of temperature.

So one tries in freeze-drying to operate at as high a temperature as practical while still keeping at all times below freezing. In vacuum dehydration, one depends on the internal pressure of the specimen, the comparative toughness of the integument, and the rapidity of drying for the maintenance of the shape of the specimen, though in this latter process there is the inherent danger of overstretching.

For a closer look at this situation it is helpful to introduce the concept of the *mean free path length*, which is defined as the average distance a gas molecule can travel before colliding with another gas molecule (bumping into the wall does not count as a collision). The mean free path length depends, therefore, on the extent of evacuation (of air). For water vapor at a pressure of 10 mm Hg the mean free path is 0.0034 mm; at 10 microns, it is 3.4 mm; at 1 micron it is 34 mm (Meryman, 1961). It can thus be seen that in either system the advantage of operating in a vacuum is enormous. If the vacuum is adequate, water vapor molecules leaving the drying specimen can travel almost without obstruction to the desiccant where they are held.

It should be kept in mind that the sole function of the pump is to create a vacuum rapidly and efficiently. Once the vacuum is attained the stop-cock (G) is closed to keep the leak-proof desiccator evacuated, and the pump is shut off. It does *not* run continuously in order to pump out the water vapor as it forms, nor does it circulate an air stream which carries away the humidity. The drying of the specimen is achieved exclusively by the molecular motion of the water vapor passing from the specimen to the desiccant.

The Desiccant

While in theory the cold trap removes water vapor from the specimen more efficiently, the equipment and operation are considerably more complex. A desiccant is perfectly adequate for the purpose at hand. Silica gel (SiO_2) or calcium sulfate ($CaSO_4$) impregnated with an indicator such as cobalt chloride do very well. Both chemicals with the indicator are blue when able to adsorb water and pink when exhausted. Both can be regenerated by spreading in a pan and drying in an oven at about 350–400° F. for about 2 hours. Calcium sulfate is the slightly more effective drying agent but silica gel will be found equally satisfactory. It is advisable not to mix the two agents because much of the efficiency of the more effective of the two will be lost, since it will first dry out the less effective, until finally the higher vapor pressure dictated by the latter will prevail. It is preferable to use the desiccant in granules of about #8 mesh (roughly 3–5 mm in diameter). Smaller granules are difficult to manage cleanly, and a certain amount of fine dust is produced in any case which must be kept off the greased portion of the desiccator and its lid (a ring of paper cut to size and laid on the greased surface helps), otherwise the vacuum will not hold. The dust should also be wiped from the inside of the vessel to prevent the formation of a deposit on the specimen that may be difficult to remove.

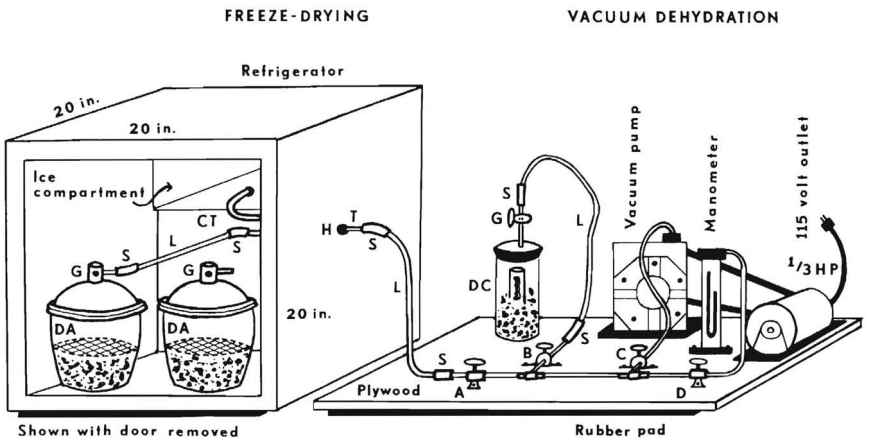


Fig. 1. Apparatus for freeze-drying and vacuum dehydration. Explanation in text.

Replenishment of the desiccant may be necessary before the caterpillars are dry if they are large or numerous. A freshly charged desiccator should be on hand in the freezer and the transfer of specimens accomplished with reasonable rapidity so that no thawing occurs. It is generally best not to put too much material in the works at one time. Meryman forsees in an unpublished paper the difficulties likely to beset the overenthusiastic student who might be tempted to freeze-dry specimens the size of a physics professor, leading to excessive drying time and inferior results.

Basic Equipment

My equipment (Fig. 1) consists of a small ($20 \times 20 \times 20$ inch outside measurement) standard refrigerator with its normal ice-making compartment. By dint of wrapping a bit of tape around the capillary tube (CT) of the thermostat in the ice-cube compartment, the temperature of the whole refrigerator can be lowered to about 20° F. A hole (H) is drilled through the wall of the refrigerator (but *not* leading into the ice-cube compartment nor any other place where damage to the refrigeration coils might result). Through this hole is passed a length of copper tubing (T) of ¼ or ⅜ inch outside diameter, which is then firmly fixed (e.g. epoxy glue or bracket) to the refrigerator wall. The outside diameter of the tubing must closely approximate that of the glass tubing of the stop-cocks (G) in order to achieve air-tight connections. This length of tubing (T) is extended about an inch inside the refrigerator, sufficient to attach a short sleeve (S) about 2 inches long of tightly fitting heavy flexible plastic tubing. A piece of plywood is placed alongside the refrigerator, and

securely and permanently fastened thereto are the vacuum pump and manometer, the vacuum-tight brass line valves, and the permanent connecting copper tubing and fittings; these include such additional hookups as may be wanted to perform the vacuum dehydration (B). A rubber mat under the plywood will absorb any vibration from the pump and cushion any screws or bolts appearing on the under surface of the plywood.

It is worth emphasizing that all joints and valves, stop-cocks and desiccator lids—in other words the entire system—must be as absolutely leakproof as possible at high vacuum. Silicone grease, or even vaseline, on meticulously cleaned stop-cocks, desiccator lids, and around the joints connected by the plastic sleeves (S), will help ensure tight fits. The permanent copper joints depend on proper flaring for sealing; but Duco cement or similar sealer will help after the joints are made. Teflon sealer on any screw-in joints (as with the manometer) likewise is of advantage. Not shown in the figure, but easily incorporated if desired, are filters (glass wool is a good material or vacuum filters may be purchased) to prevent foreign material from entering valves or pump.

Various vacuum gauges are available which give accurate absolute readings, such as the McLeod or Pirani gauges. A simple double column U-shaped mercury manometer is cheaper and fully adequate since an absolute reading is unnecessary. When the two columns of mercury become stabilized in relation to one another, the system is evacuated as far as it will go. One can judge the degree of vacuum achieved by assuming an approximation of the manufacturer's rating of the pump employed. Valve (C) leading directly to the pump is then closed, and any leak will be registered in degree according to the rapidity with which the mercury column moves. It is a good practice in any case to check the vacuum in the desiccators periodically to be on the safe side. A well-sealed desiccator, of course, can be evacuated and after closing stop-cock (G) be removed from the system to be replaced by another desiccator containing another lot of specimens.

I have used four types of desiccators, all of which hold both specimens and desiccant. The first (Fig. 1) is a sleeve-top desiccator made of heavy "Pyrex" glass (DA) whose sleeve-top incorporates a stop-cock (G) with hose connection. They come in different sizes and the small refrigerator shown will easily accommodate two of the 8-inch diameter vessels. The bottom is filled with desiccant, and a disc (or preferably a shallow basket) of fine screen wire cut to size is placed over the chemical. The lid and stop-cock of this desiccator require greasing. A second type of container is made of polycarbonate plastic with a rubber gasket beneath its

lid. The lid is made of opaque plastic and contains the outlet for the stop-cock. It is slightly taller than the first, and the gasket, being rubber, must not be greased. It is available from the Nagle Sybron Corporation in Rochester, New York. Both these containers are very satisfactory, being large enough to contain foodplant as well as larvae, and holding the vacuum well. A third type of desiccator is also commercially available and is the drying unit for "Drierite," a trade name for calcium sulfate. It is sold containing a charge of the desiccant labelled as approximately 1¼ lbs. of #8 mesh calcium sulfate with a capacity for 50 grams of water. It is made of "Plexiglas" and contains its own filter. After slightly widening the spring coil attached to the metal screw-on top, one can insert one of the plastic pill vials readily available at any pharmacy. The vial contains the larva, and its open end is covered with a bit of cheesecloth fastened on by a rubber band. The vial is inserted into the desiccator so that the open end is in almost direct contact with the desiccant. The metal top with its rubber gasket (ungreased) is tightly replaced, and a stop-cock is added to the lower tubing connection while the upper tubing connection remains sealed, and the unit attached to the vacuum system as before. The purpose of the plastic vial, as well as of the screen wire in the previous examples, is to prevent the specimens from becoming indented by loose granules of desiccant or other matter, a phenomenon which can occur even when the larva is thoroughly frozen. Still another desiccator (DC) can be home-made from any thick-walled wide mouth jar, say about 8–10 inches high. It is a good idea to wrap it carefully with transparent tape to prevent fragments of glass from flying around in case of an implosion. The mouth of the jar is fitted with a heavy bevelled rubber stopper into which a hole has been bored for the snug fit of one arm of a glass stop-cock (G). Again, the jar is partly filled with desiccant and the specimen(s) within the vial inserted as before and the vacuum applied. Desiccators must, of course, be transparent so that it is possible to watch both the specimens and the color of the desiccant.

Whichever desiccator is used, it is a good idea to keep two in the freezer ready for immediate use. In operation, the outlet from the stop-cock (G) receives a short tight sleeve (S) of the flexible plastic tubing, in turn connected by a suitable length (L) of copper tubing to the fixed end of the tube (T) that runs into the freezer. The other end of tube (T), of course, ends up at the pump. Attaching any desiccator to the vacuum system is reduced to simplicity itself by the use of an intermediate length of copper tubing (L) of any length and curvature desired, connecting it by the sleeves (S). Any desiccator must be able to withstand an outside pressure of up to one atmosphere, and some may be

ordered that can be fitted with a steel wire screen that acts as a guard against flying fragments in the event of an implosion.

Outside the freezer, a small number of line valves are added. One (A) is located near the end of the copper tubing leading to the freezer. Another (B) is located at such point as a vacuum dehydration line is desired. It may be found advantageous to have two or more of these. Valve (C) shuts off the pump alone, for the pump, when not in operation, will gradually bleed air back into the system and this valve allows the pump to be segregated from the entire system while leaving the manometer connected. The last valve (D) shuts off the manometer alone. Its purpose is to protect the manometer. This valve should be left either just cracked open, or closed and used only when actually measuring the vacuum. Otherwise a sudden loss of vacuum in the system (as may easily happen if one forgets to shut off the appropriate valve when removing a desiccator) may cause the mercury to blow through its glass top.

Procedure

Let us now proceed through a freeze-dry operation step by step. First one must kill the larva. While this can be done in boiling water, a cyanide jar or by other methods, I prefer to place it in some suitable container and simply let it freeze for two hours or so. One can let the caterpillar crawl along its foodplant, and with luck and if the animal is not too active, it *may* remain grasping the leaf or stem in a natural posture. Should it freeze in an undesirable position, let it thaw sufficiently (including its interior) before attempting to manipulate it, or the specimen will crack. Then refreeze it in such position as you choose. The desiccator is assumed to have been prepared and below freezing. The weight of the frozen larva is quickly recorded on a balance that will weigh to about $\frac{1}{10}$ of a gm. One can thus later determine the point at which a weight loss of 75% is reached. This represents approximately 90% of the total water content, and at this point the process is sufficiently advanced to allow removal and permanent storage in the collection. Since the ambient air always contains some moisture, the desiccator should not be opened until it has warmed to room temperature. Otherwise contact of the frozen larval surface with the warm moist air will be apt to ruin the appearance of the specimen. I make it a practice to leave it thus at room temperature, i.e. in a state of vacuum dehydration, for 24 hrs. One can hasten this process by use of a heat lamp provided the heat is administered with restraint. Of passing interest is the fact that about 5% of the water content of the larva is chemically bound, and therefore unavailable for evaporation. After a bit of practice it will be possible to judge with reasonable accu-

racy when the specimen is ready without having to bother with weighing every individual.

The frozen specimen is placed in the desiccator after weighing, the lid tightly sealed, and the container returned to the freezer, connected to the vacuum line, and the vacuum applied. The weighing and transfer of the larva should of course be accomplished as rapidly as possible so as not to allow thawing. When the vacuum pull-down is complete, close Valve (A) and the stop-cock (G), disconnect the desiccator from the system and move it to another part of the freezer in order to be ready for the next desiccator. During the drying process keep a check on the vacuum, and on the color of the desiccant, replacing the latter if necessary. If the foodplant is to be included as well, it too is preserved by the process. Contrary to what one might expect, dried specimens will not resorb water even in a humid climate, but it is important to remember that they are attractive to pests such as dermestids—and even to squirrels, as one collector discovered to his distress.

Another possibility is illustrated by the case of a caterpillar that has been attacked by a stink bug or other sucking creature. One can hasten to place a flaccid carcass of this sort, if fresh, in a desiccator without prior freezing and apply the vacuum. The empty skin will expand just like a toy balloon. A larva that has sickened and become flabby can sometimes be preserved in a similar manner. If there is danger of overstretching, the vacuum may be held at any desired level by closing the stop-cock and letting the specimen freeze at that level of vacuum. Then, when thoroughly frozen, full vacuum may be applied.

If a spreading board is cut into sections short enough to fit into a desiccator, the occasional mounted butterfly or moth can be rapidly dried. Often their abdomens may be soft and tender, so to avoid unsightly expansion freeze-drying is the method of choice. I have successfully applied this method on occasion to fresh specimens during the course of photographing plates for *Moths of America North of Mexico*, when a freshly caught specimen happened along that could be used to better advantage than the museum specimen originally chosen. There is scope for numerous experiments of this sort.

Meryman (1960) recommends that freezing be done at modest temperatures as in a home freezer, rather than at very low temperatures. Such relatively high temperatures cause the water content of the larva to freeze in large extracellular crystals that easily sublimate. As they grow, water is removed from both intracellular and extracellular locations without damage to the tissues or cells. Sudden very deep freezing will cause the formation of small intracellular crystals as well, causing possible

histological damage and increasing the drying time due to a slower rate of sublimation. However, the quick-freeze method provides the outstanding advantage of ensuring a lifelike position on the foodplant as shown on the color plate. Liquid nitrogen (used by cattle breeders among others) may be tried (temperature –320° F.) provided one is familiar with the proper procedures and precautions. Or one can use dry ice (temperature –110° F.) in alcohol or other compatible liquid, or a low temperature freezing unit. Another good agent is a quick-freeze aerosol such as ethyl chloride, or "Cryokwik" (a trade name for an aerosol mixture of fluorinated hydrocarbons).

Some of the larger larvae have comparatively thick integuments, and as the integument dries it presents an ever-increasing barrier to the passage of water vapor molecules from within the specimen to without. However, with a fresh larva the increase in drying time is negligible, and such large caterpillars as *Hyalophora cecropia* or *Citheronia regalis* will be well dried in 72 hours. It has been reported by Blum & Woodring (1963) that larvae frozen for weeks or months without attention to drying tend to distort and toughen, thus making the process less satisfactory. In such cases, as with the tough covering of pupae, holes punched through the integument will shorten the drying time. The same authors state that some, but not all, greens eventually tend to fade or change color due to chemical differences in the chromatophores. So far in the year and a half I have used this process, no noticeable change has occurred, but only time will tell.

Vacuum dehydration, which is carried out at room temperature, will be found satisfactory at times, and here the selection of appropriate specimens is of importance, as thin-skinned insects will stretch or even burst. The two advantages of this method are the greater speed of drying and the fact that no freezer is required. As a practical matter one can discount the latter since the majority of specimens will turn out more satisfactorily if freeze-dried at least in the initial stages of the procedure. With vacuum dehydration, of course, the vacuum is essential, since the drying process must proceed more rapidly than the enzymatic activity which at room temperature promotes rapid putrefaction.

As for pinning the specimens, an anal wire wrapped around a pin may be used as is usually done with inflated larvae; or a pin may be inserted directly through the dorsum after drying. A drop of cement is generally required for stability. As a rule, specimens should not be pinned before drying, as chemical action in some cases will tend to cause a dark spot around the pin. On the other hand, some very small larvae will have to be pinned while only semi-frozen since they may break when dry.

Larvae prepared by either process can be reconstituted for dissection or histological study (Van Cleave & Ross, 1947; Harris, 1964). To quote from a personal communication to the author from Mr. Harris: "We used from 0.5% to around 2% tribasic sodium phosphate and usually incubate at about 37° C. Be careful not to overdo things. I would point out the following: As far as histology is concerned it should not be necessary to use the reconstituting agent as no shrinkage should have taken place, and all that is necessary is to place the specimen in a warm preservative for the usual process of dehydration and embedding, etc. However, there are times when one is not quite sure and it may be safer to use the agent before further treatment. If you are able to look at a copy of *Man*, an article by A. Sanderson on *The Study of Mummified and Dried Human Tissues* published about 1959, you will find many interesting points that could be applied to entomology, etc." I myself have not yet attempted reconstitution.

I shall close by wishing the reader many happy hours with flaring tools and wrenches, and success in his endeavours.

SUMMARY

The principles involved in both freeze-drying and vacuum dehydration lie in the permanent removal of water vapor that evaporates from the tissues of the specimen. A method is presented of setting up a reasonably flexible apparatus on the basis of equipment at a cost that should be within the reach of most small laboratories and many private collectors. I have confined myself to the more practical matters wth enough theory to enable the reader to gain a reasonable understanding of what he is doing in order to prepare natural and lifelike specimens of larvae and similar insects, and to enable him to use his own ingenuity in improving or varying the apparatus according to his inclination, needs and budget. One or two theoretical matters are mentioned that are not applicable to the system described, because they will be found in the literature and might cause confusion when so encountered.

ACKNOWLEDGMENTS

I should like to extend my warm thanks to Dr. Hermann A. Flaschka, who has given much enthusiastic help and has steered me safely through some of the more dreadful pitfalls of physical chemistry. He has also taken considerable time, effort and care in reading the manuscript and has offered much by way of constructive criticism. The helpful cooperation of my colleague, Charles R. Edwards, is also appreciated. He shares in the daily work in the laboratory and grins over my shoulder and comes to the rescue when my own machinations with valves, wrenches and

plumbing end up in disaster. I should also like to extend grateful thanks to Mr. R. H. Harris, of the Experimental Laboratory of The British Museum (Natural History) in London, for the cheerful, stimulating and willing help and advice he has shared from his store of expert knowledge concerning the processes described.

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A NEW SPECIES OF THE GENUS *PYROMORPHA* HERRICH-SCHAEFFER (PYROMORPHIDAE)

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Some male specimens of this species have been in my collection for over five years. I have delayed describing it, hoping that I would take at least one female. In this I have been disappointed. As I have a sizeable series of males I am offering this description of it.

Pyromorpha caelebs A. Blanchard, **new species**

Male (Fig. 1): Head black, closely scaled, except on vertex where some long scales project forward between antennae or lean against their scapes. Tongue strong. Labial palpus short, filiform. Maxillary palpus vestigial. Antenna bipectinate, of about 35 segments, black, closely scaled above; each pectination slightly swollen near apex, tapering to base, bearing two rows of cilia. Collar, thorax, patagiae and abdomen black. Legs slender, closely scaled, black except yellow inner side of foreleg, some yellow scales distally on midfemur; one pair of terminal, rudimentary spurs on mid