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EMBEDDING BIOLOGICAL OBJECTS IN PLASTIC

By

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Introduction

Various types of plastics for the preparation of biological materials have been tested in several institutions both in foreign countries and in Sweden. The present investigations were undertaken because conventional methods of preparing plants and animals do not permanently preserve the natural appearance of specimens. This lack was particularly felt in the Department of Forest Zoology, where instruction should be based on demonstration material having a natural appearance. Undesirable changes in demonstration materials demand yearly renewal of specimens, when dried preparations or material in solutions are used for study.

The method of preserving specimens in plastic has therefore been studied in this Department, and techniques facilitating routine embedding of demonstration material have been developed.

In the experimental derivation of the methods, we have had valuable assistance and continued interest from Docent ÅKE HOLM, Department of Zoology of Uppsala University. Collection of the large amount of research material needed was made possible in part through the cooperation of Professor V. BUTOVITSCH, the Swedish Forest Research Institute, Forest Officer L. BRAMMANIS, Dr. B. LEKANDER, Messrs. K. J. HEQVIST, P. BUTOVITSCH and B. ENSTRÖM. During the final experiments many problems were solved through the stimulating cooperation of Mr. T. ROSSBY. We wish to express our gratitude to the many who cooperated during all stages of this investigation.

We are much indebted to Dr. A. Kahn and Dr. A. Kemp for all their help with the translation of the manuscript.

Choice of suitable plastic

A plastic with the following properties appears to be desirable:
1. The monomer of the plastic should polymerise readily without strong heating and preferably at room temperature.
2. The heat of the polymerisation reaction should be small.
3. Shrinkage during polymerisation should not exceed 10 %.
4. The plastic should be practically colourless.
5. It should polymerise into a crystalline and completely oriented state. (Tendencies to build unordered molecular chains lead to undesirable optical properties of the preparation).
6. The plastic should be resistant to pressure and blows.
7. Transparency should be permanent.

Many commercially available transparent plastics have been tested. Some proved useful for processing special objects but were hard to work with. Others were unsatisfactory because of strong shrinkage during polymerisation or their tendency to undergo secondary changes. Only a few types could be considered useful on optical grounds; only one of the plastics tested could be employed through the whole range of materials needed in the Department.

Technical data on common plastics lead to the conclusion that acrylic plastics (butyl- or methylmethacrylate) would best suit our purpose. These plastics are preferable to all others because of their brilliance and good optical qualities. However, it was found that only a limited number of species and developmental stages could be prepared using these plastics.

For the preparation of the entire series of objects studied, the polyester Castolite could be used.

Polymerisation

The plastic is received from the factory in the form of a slightly viscous mixture of unsaturated polyester and monostyrol. After the addition of an initiator (called hardener) consisting of organic peroxide, the plastic polymerises.

This process can take place at room temperature, and some heat is produced from the reaction. If, during polymerisation the mass of plastic is so great that the heat of reaction is not readily conducted away, then the temperature can rise so high that internal strains occur which can lead to splitting or other technical defects. Consequently, for the production of large blocks, a cooling arrangement had to be used.

The peroxides should be mixed well with the plastic. Because of the viscous consistency of the unpolymerised plastic, bubbles can easily arise during stirring. Bubble formation can be reduced by using a stirrer with a small diameter, e.g. a piece of steel wire.

Polymerisation of the plastic proceeds more readily and is accelerated by slight heating at the beginning of the process. A work table warmed to about 40° C. from beneath by a set of filament lamps has proved suitable.

Laurylmercaptans are often used for afterhardening of the plastic. Our experience with these afterhardening agents has not always been satisfactory. They often cause heterogeneous polymerisation, which leads to undesirable optical refraction. It seems preferable to cause the afterhardening with ultra violet light, which somewhat extends the time of polymerisation. The resulting plastic is more homogeneous, on account of the ordering of macromolecular chains in a more parallel arrangement.

Afterhardening using high temperature is not necessary.

Preparation of objects for embedding

Dehydration

The part of the object which is brought into contact with the plastic must be water-free. Many objects need special methods of dehydration, in order to preserve their natural appearance.

A simple method of dehydration is air-drying. Intact insects are commonly prepared in this way. Most often mounting the insect with a needle through the thorax causes no harm, because the hole through the thorax is undetectable in the finished plastic block. An object treated in this manner can afterwards be kept indefinitely, provided that the insect needles were of good quality, and that the collection was maintained in a dust-free place.

If, however, air-drying damages the objects, e.g. many crustaceans and spiders, dehydration can be carried out with glycerin. Sensitive species having large areas of soft shell should be passed slowly through increasing concentrations of glycerin.

A third method which is particularly useful for certain soft-skinned animals is dehydration using a series of increasing alcohol concentrations (50, 70, 80, 96% and absolute alcohol). A drawback of this method is that many pigments dissolve in alcohol. Often a compromise between slow dehydration to prevent shrinkage and rapid dehydration to prevent loss of colour is necessary. The method demands a knowledge of the sensitivity of various objects, and recommendations concerning certain objects are given later.

The moment at which the water-free specimen can be immersed in the plastic depends upon different principles, which in turn depend upon the physical characteristics of the objects. It is desirable to transfer the object in such a state that the mechanical, optical and structural characteristics do not change in the polymerising plastic where strong tensions can arise through shrinkage. It is further necessary to prevent the object beeing covered with a thin layer of air resulting from these tensions when contact between the surface of the object and the plastic is lost during polymerisation. Many methods have been tried, and it appears that a general method can hardly be worked out, because of the great differences in the physical and optical characteristics of the objects. Therefore, the most important methods are reviewed, and are grouped according to the principal kinds of methods which have been worked out.

Treatment with ether

To replace the air in air-dried objects with unpolymerised plastic an intermediate step with ether has been found useful. Ether is appropriate because of its low surface tension and high volatility.

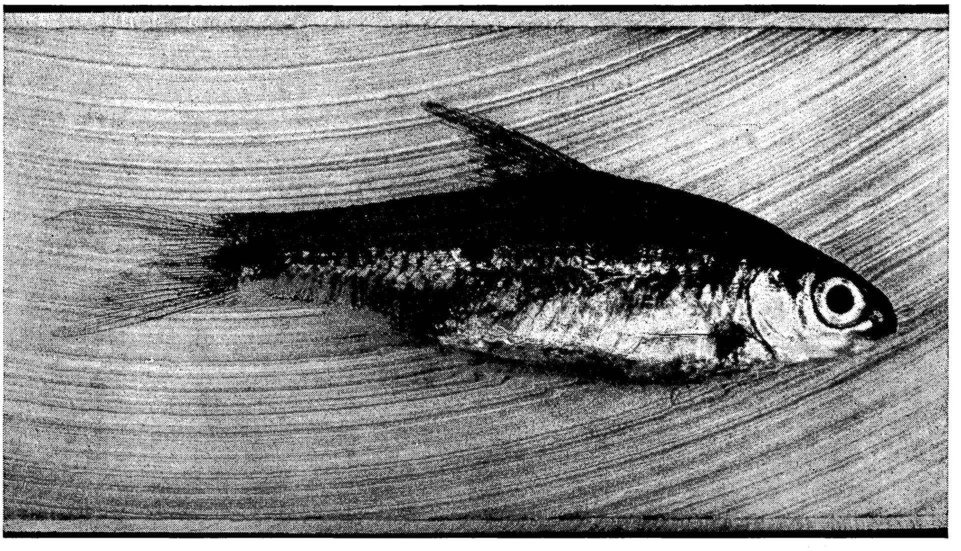
The dry object can be vacuum-infiltrated with ether. When the air has been satisfactorily removed, the object sinks to the bottom of the container. While still moist with ether, the specimen should be transferred directly to the polymerising plastic. The ether evaporates progressively during polymerisation. This method is useful for embedding parasitic insects, gall-inducing insects and wasps, small flies and mosquitos.

Alternatively, the ether saturated specimen can be placed into monomer plastic without initiator and the ether removed under vacuum; then, when normal air pressure is restored, the ether is replaced by monomer. Insects which have been succesfully treated in this way are *Acanthocinus spec.* and *Hylobius abietis.*

The pretreatment of butterflies with ether *in vacuo* constitutes another modification; the intent is then to prevent direct contact between the upper and lower plastic layer through the insect's wings. This method is described further on page 21.

Treatment with glycerin

Some species, whose pigments are leached by ether or alcohol, can be pretreated with glycerin, after which they can be transferred into monomer plastic. When the object has been kept in monomer for at least one day, the glycerin in the periphery of the object has been replaced with plastic to such an extent that the necessary contact between the outer layer



Silver shiny fresh water fish. Superficially prepared with stabilized monomer plastic. See page 10.

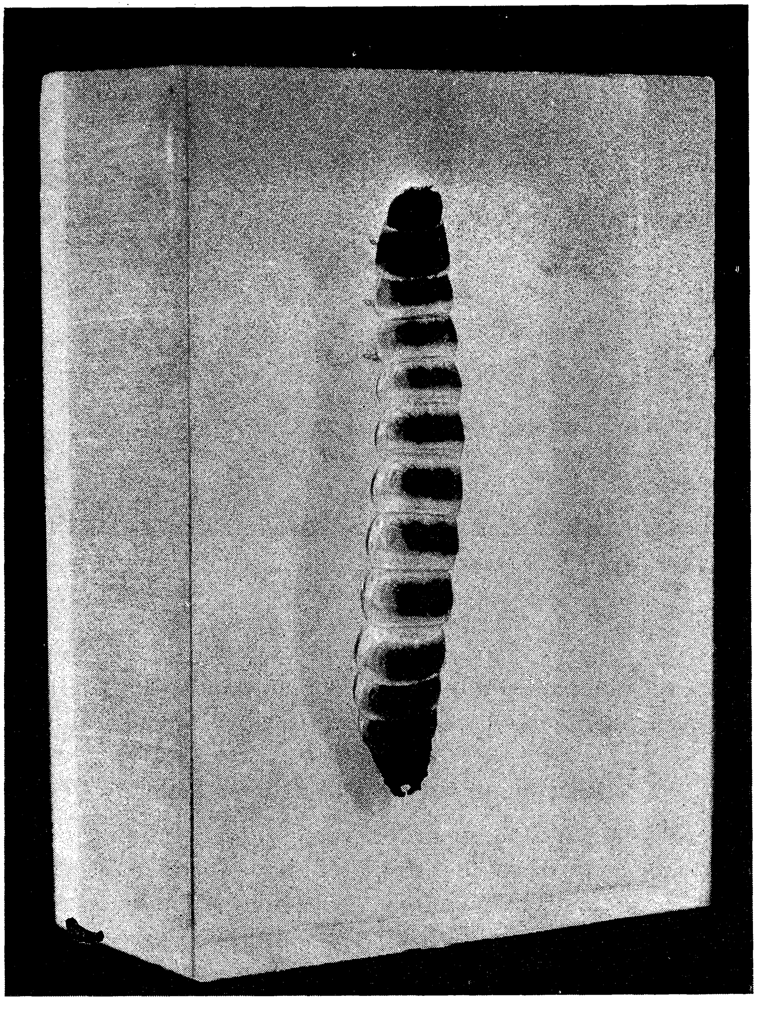
of the object and the embedding plastic is maintained. The method is useful for embedding spiders, small crustaceans, centipedes and millipedes.

Treatment with stabilized monomer

Often the object tends to separate from the surrounding plastic during polymerisation. A way of solving this problem is to treat the specimen with a substance, e.g. stabilized monomer, that prevents polymerisation of the plastic at the surface of the object. The idea is to embed the object in a layer of unpolymerised plastic, which is then surrounded by hardened plastic. In this way it is possible to avoid undesirable effects of the tensions which always arise during the polymerisation, and which appear especially in the contact region between the object and the plastic. These tensions are rapidly dissipated by the unpolymerised plastic. The monomer plastic must be stabilized to prevent its polymerisation through initiation from the surrounding plastic. As a stabilizer the monomer can contain 0.1 % hydroquinone. Higher concentrations may cause a yellowish colour of the plastic.

The dehydrated object is vacuum infiltrated with stabilized plastic during which the specimen must be weighted (e.g. with piece of metal screen wire) so that it sinks. After this pretreatment the object can be transferred directly into polymerising plastic.

Some hard and stiff-haired beetles, such as *Carabidae,* have been prepared satisfactorily in this way. Sometimes the stabilizer causes a weak yellow



Larva of *Athous rufus*. Treated with stabilized monomer plastic.

colour around the object. This can be minimized if the polymerising plastic contains a large quantity of initiator so that polymerisation is accelerated.

The stabilized plastic, described above, contains little monostyrol and is here called polyester type I. For many objects (e.g. fishes) plastic containing a larger amount of monostyrol (polyester type II) was found useful. Careful drainage of this stabilized plastic before embedding in the polymerising mixture is necessary to avoid undesirable border-zones around the embedded specimens.

The plastic types used for this method have such physical characteristics that they are particularly favourable for pretreatment of small fishes before embedding them in plastic. These objects are particularly difficult to conserve without loss of colour or the object being covered by a non-transparent layer of slime. With all fixing and conserving methods previously used, the general appearance of the fish is altered because of the greyish lifeless colour of the fixed proteins. Particularly great difficulties are met with in the attempt to preserve the natural appearance of fishes' eyes, which is usually lost because the lenses become opaque.

Through careful pretreatment in polyester II according to the soft-plastic method, it is possible to restore the natural colours of the fish, at least partially, before the final embedding in hard-plastic. This soft-plastic treatment must, however, be for a limited time only, otherwise the whole fish will be transparent and lose its natural colours.

The most satisfactory method to achieve this purpose is as follows: The anaesthetized fish is fixed in its natural position in Carl's solution (see page 13); it is then dehydrated as quickly as possible by using the standard alcohol xylene method. The treatment in xylene must not be so prolonged, otherwise the fish becomes transparent. After xylene the object is placed in a mixture of 80 % polyester II and 20 % methyl-methacrylate, to which 0.1 % hydroquinone has been added. By mixing polyester II with methyl-methacrylate, the viscosity of the mixture is reduced, and thus the air can be replaced more easily by the pretreatment solution during the following vacuum infiltration. At the same time, the ability of the solution to surround the object is maintained.

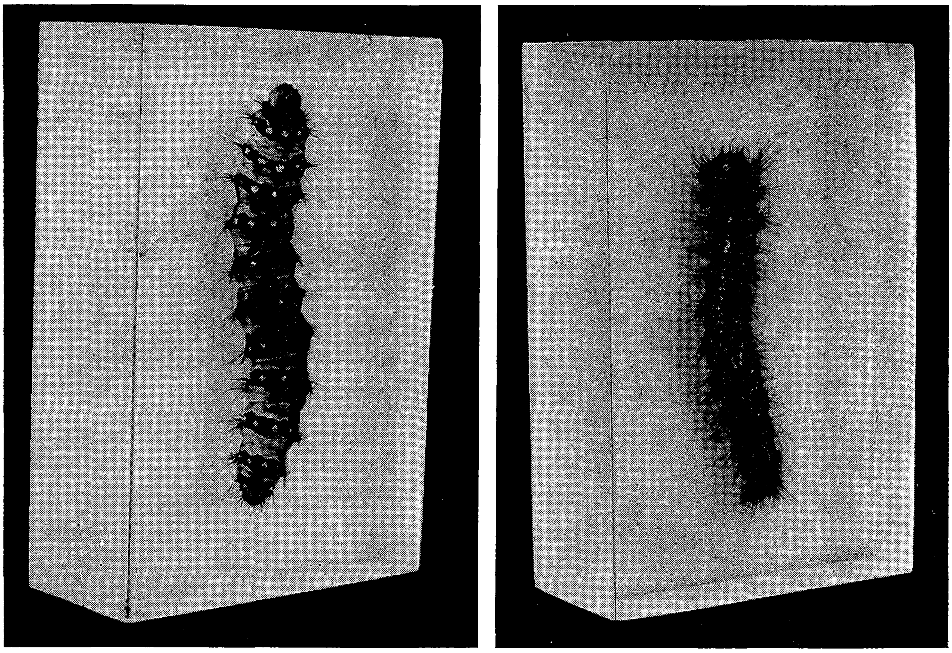
For certain objects a less viscous stabilized plastic has to be used. Reduced viscosity can be achieved either by including 20 to 25 % stabilized methyl-methacrylate or by warming the plastic.

Sometimes the stabilized plastic rapidly runs off the surface of the specimen during transfer to polymerising plastic.

Adhesion of the stabilized monomer can be secured by a heat treatment. The specimen is placed in stabilized plastic, vacuum infiltrated, and heated in a water bath to 80° C. for at least 30 minutes, after which the insect is once more vacuum infiltrated while still warm. The mixture is allowed to cool, and the object remains in this solution some days before embedding. This method is suitable for species such as *Aromia, Monochamus* and *Spondylis*.

Modification of the stabilized monomer method

Preparations of hard beetles sometimes show small areas of splitting between the specimen and the plastic, which give the impression of a layer of air between the object and plastic. The defective contact between the object and the plastic is probably caused by the inflexibility of the specimens



Larvae of *Saturnia pavonia* (left) and *Dendrolimus pini* (right) prepared according to the dry method. See page 13.

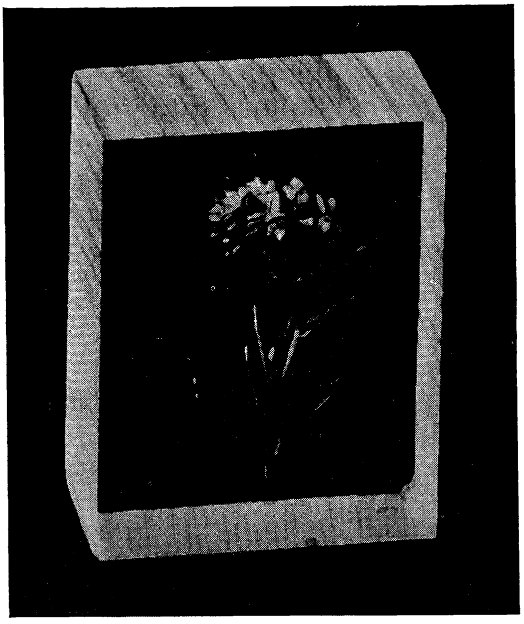
chitin surface. A way of avoiding these defects is to treat the chitin surface by carefully heating the specimen in a detergent solution with the aid of a water bath.

Too strong a heat treatment can cause the loss of colours, which are located in the chitin coat. Through cooking, the surface is freed from fat or wax-like layers, and the epicuticula is etched. In this way the capacity of the surface to maintain contact with the thin stabilized plastic layer during polymerisation can be increased.

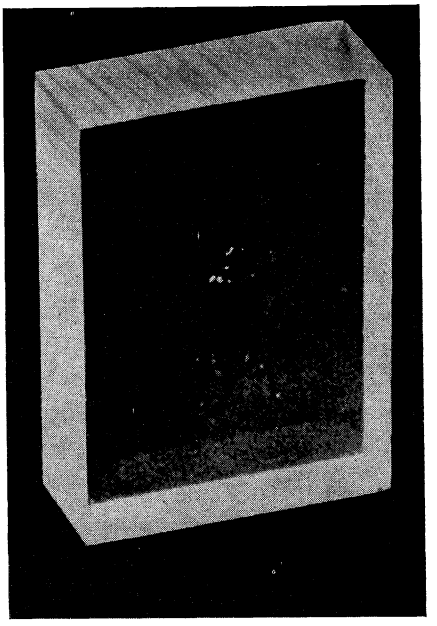
Dry preparation

Delicate and little sclerotized insects can be embedded while filled with air. The principle is to bring the plastic into such intimate and strong contact with the surface of the skin, that the surface follows the movements of the plastic during shrinkage rather than separating from it. This method is suitable only for insects with thin and flexible skin, which could not be satisfactorily embedded otherwise. The insects are prepared in the following way:

The dead insects are placed in cold water, which then is slowly brought to the boiling point. The proteins are coagulated by the heat, and the shape



Spruce with gall of *Chermes*. Treated with stabilized monomer plastic. See page 9.



Leptura rubra; the insect is air-dried and vacuum infiltrated with silicone-ether. See page 14.

of the specimens is fixed. After the insects have swollen somewhat above their normal volume, heating is discontinued. When the water has cooled, the animals are transferred to Carl's solution* or 70% alcohol, where they can be kept for a rather long time. The pretreatment for embedding is started by dehydrating the objects, using the standard alcohol xylene method. Then the insects are air-dried at room temperature, and can then either be stored further or embedded directly in rapidly polymerising plastic.

Use of this technique on larvae of beetles (especially wood-destroying insects), hairy or naked caterpillars of butterflies and thentredinids, have yielded some of our most successful preparations.

Modified treatment with ether

Colouration of butterfly wings disappears if direct contact between the plastic and the scales is not prevented. For this purpose ether combined with wet embedding and rapid polymerisation should be used.

The mounted and air-dried insect is placed in ether, and the air is evacu-

* Carl's solution is prepared as follows:

95% alcohol	— 170 cc	glacial acetic acid	— 20 cc
40% formalin	— 60 cc	distilled water	— 250 cc

ated. The ether-wet insect is then quickly placed into rapidly polymerising plastic, so that the plastic does not come into intimate contact with the wings. After polymerisation the wings are separated from the plastic by a subsequently described method, upon which the natural colours reappear.

Treatment with silicone

Shiny, metallic colours of insects can be preserved by an intermediate treatment with silicone, which facilitates the separation of the object from the surrounding plastic layer. A 1% solution of silicone in ether is prepared, filtered, and the insect is vacuum infiltrated with this solution. After this the insect is air-dried, whereupon the ether evaporates, leaving a very thin layer of silicone surrounding the insect. The embedding is done directly in rapidly polymerising plastic.

Modification of the silicone method

The colours of many beetles partially depend on the refractive index of the substance between the elytra and the abdomen and plastic in this area causes changes in colour. To exclude plastic from this area, the specimen should be treated with silicone-ether as above and embedded in rapidly polymerising plastic without allowing the ether to evaporate. Theoretically, the plastic should be in contact with the outer side of the wings and thorax, but because of the ether and the solved silicone it should not form a definite contact area on the underside of the chitin skin facing the body cavity. Thus during the polymerisation contact is maintained between the upper side of the chitin and the plastic, while the underside of the chitin is more or less free from plastic. In this way it is possible to preserve the colours, which depend upon the difference in refractive index between vacuum and chitin within the insect, and these colours are visible through the chitin. Objects for which this method is suitable are *Leptura,* Chrysomelids and Coccinellids.

Embedding procedure

1. Fabrication of moulds

It is important to adjust the dimensions of the moulds to the shape and size of the objects. For preparing a series of objects, intended for a demonstration collection it is important, furthermore, to design the moulds so that the finished preparations can be readily stored in simple standard boxes or drawers.

The moulds can be made from a material which can be removed leaving plain, clear and lustrous surfaces. Alternatively the blocks can be ground down to definite dimensions and polished.

The type of mould first mentioned is best made from cellulose acetate sheets (approximately 0.5 mm thick), which can be folded without breaking. The sheets are cut by a machine into strips of the same breadth as the desired thickness of the block. It is of great importance that the strips should be of exactly the same breadth. A strip is wrapped around a wooden block with the desired dimensions of the side walls of the mould, the ends cut square and fastened end to end with tape. This frame is glued on to a clean piece of glass by briefly dipping the edges to be glued into a solution of cellulose acetate in acetone. Then the strip is rapidly placed on a clean piece of glass 3—4 mm thick, whose dimensions exceed those of the form by a couple of centimeters on each side. Through light pressure the strip is brought into direct contact with the glass plate. After drying for a few minutes the form is ready. Leakage can be detected by adding a few drops of xylene before use.

The plastic mass can be poured into the mould and covered with a glass similar to that used for the bottom, and subsequent grinding and polishing is necessary only on the sides.

Another method is to make the moulds of cardboard or aluminium foil having a thickness of 0.1 mm. These materials can easily be shaped over a form of the desired size, will keep their shape under the influence of the polymerising plastic, and are easy to remove from the preparation. Grinding and careful polishing are required to finish the surfaces, but this can be avoided on the top and bottom surfaces by using bottom and cover plates of polyethene or plexiglass instead of ordinary glass. These plates should be cleaned and cut to the dimensions of the mould, because they become permanently attached to the plastic. Polishing is then necessary only on the sides of the block.

Contrast can be produced by using a milky white or dark black plastic as a bottom plate. These plates must be prepared on their underside with a rather thick layer of vaseline. The coloured plate is attached to the block thus replacing the ordinary bottom layer of transparent plastic (see later).

The parts of the moulds that are to be removed afterwards from the finished blocks, are coated before use with a thin layer of a substance that prevents the mould from sticking to the plastic. Suitable substances are vaseline, wax in the form of a special preparation called »mould release», silicone or lecithin.

2. Embedding

As soon as the pretreatment of the object is finished, it can be embedded in plastic. In many cases it is necessary to carry out the pretreatment and embedding as an uninterrupted process, as for instance with ether-pretreated adult butterflies, but in others the object can be kept for a long time after pretreament without damage to the material, e.g. xylene-pretreated larvae or stabilized monomer-pretreated black insects.

Experience has proved that direct embedding should be avoided; partly because of the difficulty of orientating the object in the polymerising plastic, partly because of the tendency of some objects to sink to the bottom of the mould, in which case the embedding is incomplete (as no plastic layer is formed underneath the object).

Embedding is done in three steps. First a bottom layer some millimeters thick is poured into the mould, then a middle layer for the object and finally a top layer, on to which the cover material (glass or plastic) is placed. The following standard technique can be used.

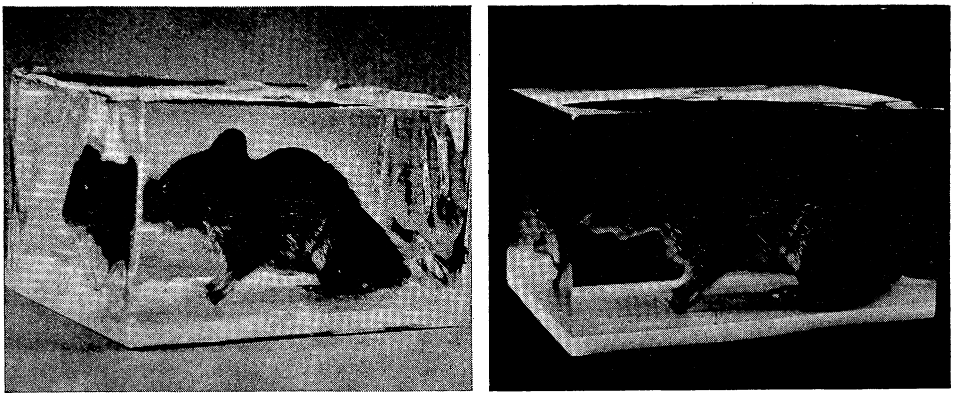
From a stock solution containing monomer plastic and initiator which is kept in a refrigerator at 8° C., a layer is poured so that the floor of the mould is covered completely out to the corners. This bottom layer is polymerised on a warming-table to a jelly-like consistency. The surface of the bottom layer should be viscous because if this polymerisation causes formation of a membranous cover at the surface, then bubbles may be formed when additional plastic is poured. Bubble formation can be inhibited by the addition of a few drops of styrene on to the bottom layer.

From the stock solution a middle layer is then poured to a height which depends on the object. The object is placed immediately into the middle of the mould before the polymerisation begins in the middle layer. Through its contact with the sticky bottom layer, the object is fixed in place.

Rather often small bubbles appear after the object is placed into the plastic. These bubbles must be removed quickly, this can be done with the aid of a preparative needle moistened with acetone. As soon as the bubbles are caught in the acetone, they rise to the surface, and can be removed without harming the object.

If possible, the object should be completely covered by the middle layer of plastic. When large objects are embedded, the middle layer must be poured in several steps, otherwise the heat produced by polymerisation can cause damage. The object is covered by monomer plastic without initiator, while the first part of the middle layer polymerises. Then another part of the layer can be poured and so on. The monomer plastic polymerises later through the influence of a hardener in the surrounding region.

After the middle layer has polymerised to a jelly-like state, the upper



Mus musculus. Prepared with stabilized monomer plastic and acetone. Left unfinished, right finished block. See page 18.

layer can be poured up to the rim of the mould. After polymerising at room temperature for 12 hours, the form is ready for »covering».

3. Covering

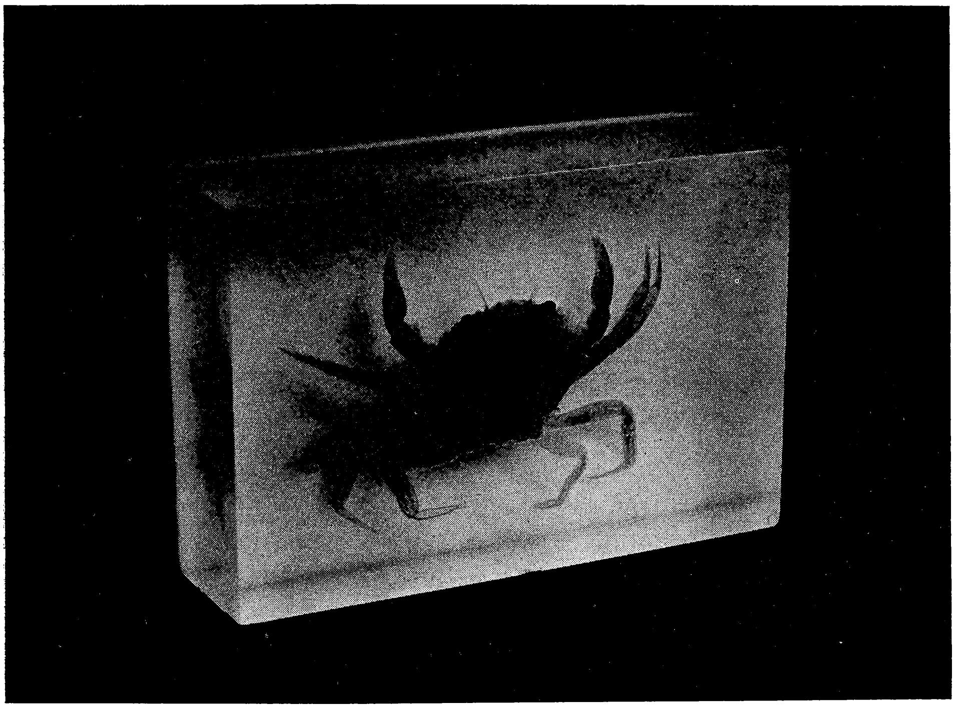
Aerobically polymerising Castolite maintains a soft and sticky surface for a long period. This surface can be ground away, and the new surface exposed, highly polished. In such a case it is not necessary to equip the form with a cover. Grinding and polishing, on the other hand, can be avoided by using cover plates, which can be permanent (cf. p. 15) or temporary glass plates. This is achieved as follows.

As soon as the upper layer has set, enough plastic is added to make the surface convex. A clean glass plate, at least 3 mm thick, and coated with a thin layer of vaseline or silicone, is slipped on to the form from one side. The addition of a drop of styrene can prevent the formation of air bubbles. When the glass-plate has been brought into place, the form is inverted, and the excess plastic builds a seal around the form which prevents the entrance of air when shrinkage occurs during polymerisation.

After at least 12 hours at room temperature, the glass plate can be removed. First the excess plastic around the rim of the form is loosened, and then the glass plate is pried off with a knife.

4. Grinding and polishing

The grinding is done by using carborundum canvas in a grinding machine with a vertical disc and horizontal work-table. For producing a large series of blocks, it is convenient to work with a block-holder, which can be slid



Carsinus menas. Treated with glycerin.

along a track arranged at right angles to the disc. The movement of the holder can be controlled by stop-pins, making it possible to rapidly grind the blocks to desired dimensions. The first side is oriented parallel to the carborundum disc. The other sides will then automatically be fixed by the block-holder. At least 6—7 mm. of plastic must be left surrounding the object.

Plastic grinding dust can be irritating and injurious to health if inhaled. Therefore special arrangements for removing the dust are necessary when many blocks are to be ground. A suction device with ordinary filters can be used. Alternatively the grinding area can be continuously washed with water. This removes dust effectively, and at the same time cools the block, which allows the use of very fine carborundum material.

For the polishing of the preparation, the rough carborundum canvas is then replaced by an ultra-fine diamantine-disc, during which washing is necessary. The final polishing is done with a soft woolen disc using moderate pressure to prevent excessive heating by friction and rounding of corners and edges. Wax can be used during the final polishing to enhance the lustre of the block.

Methods for different types of objects

1. Crustaceans

As mentinoned above, most of the animals which are suitable for embedding in plastic demand the application of particular pretreatments. In many cases these pretreatments are individual ones according to the characteristics of the objects. The methods evolved by experience in preparing common demonstration material will therefore be reviewed in the following.

Fixation is carried out with Carl's solution or formalin. During fixation the object must be arranged in the position it shall ultimately retain in the block. It is advantageous to carry out the fixation without excessive solution on a piece of paper or cardboard, so that the legs and antennae can be arranged in a proper position. As soon as the legs and antennae are fixed in this way, the object can be completely covered with the solution. Crustaceans can be kept in the fixation solution for longer periods without proceeding further in the treatment.

Dehydration of the specimens is performed with glycerin after which the object can be embedded according to the standard technique (p. 16). The covering is done with a vaseline-treated glass plate.

Some crustaceans, however, become decolourised during the above-mentioned procedure. Decolourisation can be reduced by keeping the animals in a sugar solution (1 kg. sugar per 1 liter of water with 1% formalin) after fixation in Carl's solution. After this, the object is transferred through a water bath to glycerin and treated according to the above scheme.

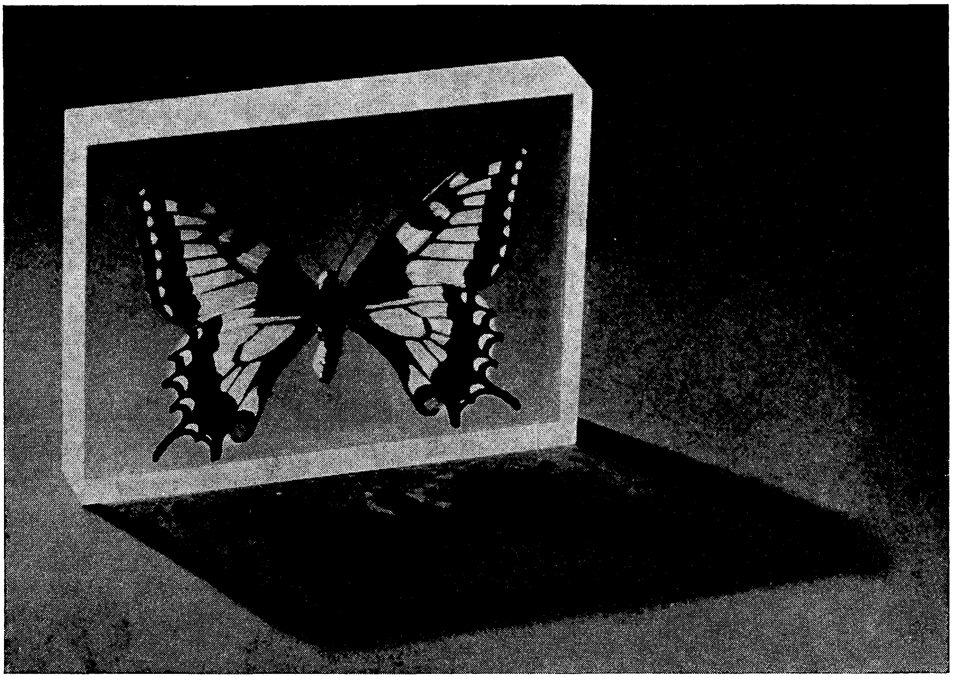
Partly transparent crustaceans become opaque during fixation. The stabilized monomer treatment (p. 11) with a methylmethacrylate-polyester type II mixture produces transparency. The degree of transparency can be controlled by varying the length of treatment.

2. Centipedes

These animals offer particular difficulties, because of self-mutilation if anaesthesia is applied without care. After the animal is killed with ether, it is fixed in its natural attitude in Carl's solution, after which it is kept in 70% alcohol. If the animal shrinks, its natural proportions can be restored by cooking in water. To minimize discolouration the time of storage in alcohol should be as brief as possible.

Dehydration is done with an alcohol series followed by acetone or water-free ether. Embedding is performed via stabilized plastic (p. 9), polyester II with methylmethacrylate. During and after dehydration, the animal is very fragile and must be handled carefully.

Embedding in plastic is done according to the standard technique (p. 16).



Papilio machaon. Air-dried and prepared with ether. See page 21.

3. Spiders

The smaller species are fixed in formalin after anaesthesising; the larger species are warmed up carefully in water containing approximately 10% Carl's solution, until the legs assume a natural position. After the solution has cooled, the animals are transferred to 70% alcohol where they can be stored.

Pretreatment is carried out with glycerin; after three changes of glycerin and cleaning in alcohol and acetone the spiders are ready for embedding on the bottom layer of plastic in the mould.

4. Lepidoptera

The most satisfactory method for embedding butterflies depends to a great extent on the effect of the plastic on their colours. Butterflies with normal body size and pigment colouration, e.g. *Vanessa urticae* and *Papilio machaon,* can be prepared using the simple ether method, but butterflies with irridescent, metallic colours must be prepared according to a completely different method. For this reason treatment methods for different types of butterflies will be reviewed.

a. Lepidoptera of butterfly-type

The butterflies are mounted in the usual way with needles that do not rust or oxidize. The dried butterflies are loosened from the needles and vacuum infiltrated with ether. Pressure is reduced until the ether begins to boil, then normal air pressure is restored. If the butterfly sinks in the ether, then the air has been replaced to a satisfactory degree by ether.

The middle layer is poured on to a thick prepolymerised bottom layer, and the wet butterfly is placed as quickly as possible into position so that the apices of the wings and body remain considerably removed from the edges of the mould. Transfer of the butterfly from the ether to the plastic should be done so rapidly that little ether evaporates.

If some part of the butterfly happens to dry during the procedure, it is remoistened with an ether-soaked brush. The entire butterfly is rapidly covered with plastic containing initiator, and subsequently the top layer is applied.

The wings of the butterfly become more or less transparent by this procedure. This is caused by the fact that the plastic permeates the wings. The original colours can however be restored by applying mechanical pressure to the polymerised plastic, thereby breaking the contact between the wings and the plastic. The pressure should not be too strong since the hard plastic can split in the plane of the wings, and should be applied before the polymerisation is complete.

A simple and satisfactory arrangement for applying pressure is a book-press with a spiral screw. The pressure is applied evenly over the whole block using a wooden cube covered with a piece of rubber.

During pressing it is often possible to notice how the plastic separates from the wings, and the colours »develop». If the polymerisation has gone far, this displacement occurs abruptly; if the plastic is soft, it appears slowly and locally, but one gets the same optical effect.

Sometimes no displacement is possible with evenly applied pressure. In such cases local pressure with a convex-faced cube, mounted instead of the plane-faced wooden cube, can give the desired result. This arrangement, however, must be used with caution and under continuous control during the procedure, which is facilitated by making the cube so small that it does not cover the whole block.

Common errors:

Butterflies lose legs or antennae: This can happen if the ether boils too violently during the vacuum infiltration. Every butterfly needs to be treated individually in its own glass container; boiling should not take place for too long a period, or else the butterfly can be torn apart by the rapid movement of the solvent.

Colours become dull: This is usually caused by pigment extraction during ether treatment, which therefore should be short.

The application of pressure totally or partly ineffective: In this case the plastic is too hard or too soft, or the pretreatment was defective.
Pressure gives the wrong effect or the preparation splits: Splitting of the block can be caused by uneven support during the application of pressure, or by too little plastic around the object. If pressure does not cause total regaining of the colours, this can be caused by the butterflies having dried during the embedding. If the application of pressure remains completely without result, the reason can be either that the plastic did not polymerise enough (the mass exhibits relative softness) or that polymerisation has gone too far.

b. Lepidoptera with irridescent colours

These are difficult to preserve with maintenance of their natural colour effect, since the colours are in part so-called refraction colours. Optical relationships are so changed during normal embedding that the original, beautiful colours disappear. Here the scales of the wings and the body have to be isolated particularly well from the plastic, which can be done by treatment with a filtered 1 % silicone solution in ether. After the treated insect dries, it can be laid directly on to the plastic of the bottom layer. Then the procedure is continued according to the standard technique.

Alternatively, the insect can be embedded while wet with silicone-ether solution.

Comparative experiments using both methods have proved that both can be used in most cases, but that the last mentioned method is simpler, more advantageous and above all quicker.

Common errors:
The silicone treatment causes a white powder on the butterflies, particularly in the outer chitin folds and between the hairs on the thorax and legs. This powder can be washed away by a quick rinse in pure ether.

c. Moths

The males of the smaller species can be embedded according to the standard technique. However, specimens with voluminous bodies require a special procedure.

Pretreatment in ether or silicone-ether is done in the usual manner. When the moth is placed in the middle layer, the upper parts of the thorax and abdomen are not covered with plastic. This allows the ether in the thicker parts to evaporate. Otherwise bubble formation and cloudiness around these portions can occur. Plastic without initiator is added dropwise on to the exposed parts, and the procedure is then completed as described for butterflies. Restoration of colours to the wings by application of pressure to the block is difficult with species like *Lymantria dispar, L. monacha, Dendrolimus pini,* and *Lasiocampa quercus.*

For these a stronger silicone solution should be used for pretreatment.

d. Caterpillars

The ether-killed larvae are embedded following dry preparation, as described on p. 12. The larvae should be perfectly dry and undamaged.

Lightly sclerotized as well as hairy larvae, such as *Arctia, Lymantria, Dasychira, Dendrolimus* and *Orgyia* are suitable for preparation by this technique.

Naked, highly sclerotized larvae, for instance *Cossus,* are pretreated and embedded like alcohol-fixed pupae.

Common errors:

1. The larvae present *transparent parts,* which seem to be dark. These are caused by cracks or other mechanical defects, through which the plastic has entered the cavity. Damaged areas can be detected by discolouration, when larvae are immersed in ether before embedding, and these larvae should be discarded.
2. The larvae show *darkening around mouth-and-anal openings and around the spiracles.* These defects are generally caused by entrance of too slowly polymerising plastic into the larvae.
3. *Air bubbles around the mouth-and-anal region* are caused by exit of air from the larvae into the plastic. The air bubbles can be removed before the plastic becomes hard.

e. Pupae of Lepidoptera

Pupae can either be air-dried or fixed in 70 % alcohol. In the first case the pupae are later vacuum infiltrated with warm stabilized monomer and embedded. In the latter case the objects are passed through a series of increasing concentrations of alcohol, followed by acetone or styrene and finally stabilized monomer. In each of these steps the object should remain for at least one day before final embedding is done by the standard technique.

5. Beetles

The beetles present such great differences that several different methods had to be worked out for embedding them in plastic. In the following some of these methods for preparing specimens most commonly used for instruction are reviewed.

a. Soft-beetles

Air-dried specimens are vacuum-infiltrated with monomer plastic without initiator. In this way the air is replaced by plastic. Before the infiltration the dry beetles are placed directly into the monomer plastic, after being weighted for instance with a piece of iron screen of sufficient weight, so that the beetles are pressed below the surface of the fluid. After the treatment the insects remain on the bottom of the container; if they float, the vacuum infiltration has to be continued.

For small objects, for instance tiny Carabids, monomer plastic with reduced viscosity — obtained by warming up the plastic to approximately 40°C in a water bath (p. 11) — should be used in order to avoid damaging the specimens.

Embedding by the standard technique can then be applied for *Cantharis, Clytus, Acanthocinus,* a few *Leptura* and others. However, it might happen that »silvering» caused by the separation of the object from the plastic, appears after several months, and the following procedure has then to be used.

b. Hard, highly sclerotized beetles

For reasons mentioned previously, a progressive deterioration of the object occurs easily when the object is so inflexible that it cannot follow the movements of the plastic during shrinkage while polymerising. In this case a thin layer of air or vacuum forms around the hard parts of the insect when tensions arise between the chitin and the polymerising plastic. To avoid the destructive effect of these tensions, we have worked out a new method, the so-called soft-plastic method, whereby the plastic in and around the insect is kept in a monomeric and flexible state.

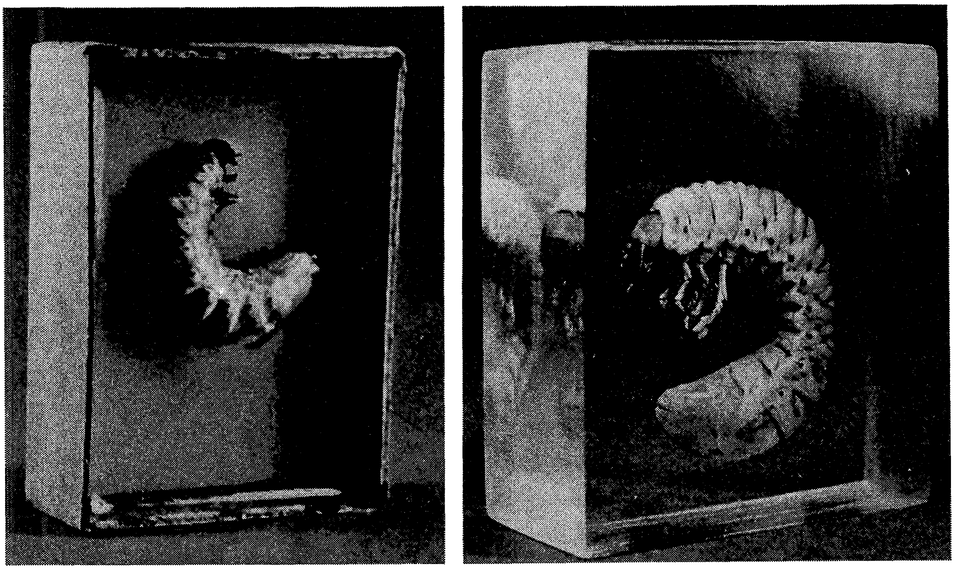
Air-dried specimens are placed directly into a mixture of 75 % polyester type II and 25 % methylmethacrylate with 0.1 % hydroquinone. The reduced viscosity simplifies vacuum infiltration during which the object must be kept under the surface of the solution. After the object has lain in this mixture for several days, it can be drained and placed into the polymerising plastic.

It is also important that the specimen is allowed to sink slowly into the plastic of the middle layer; if it is forced into position, defects can occur because part of the soft-plastic around the chitin surface is removed.

Examples of specimens that can be embedded by this method, are *Hylobius,* some *Carabidae, Rhagium, Coccinellidae, Cetonia, Pissodes, Saperda* and other *Cerambycidae.*

c. Beetles, which offer special difficulties

Beetles, which cannot be prepared satisfactorily by any of the treatments just discussed, can often be embedded after cooking in detergent solution or by infiltration with hot stabilized monomer, as described on p. 9—12. These two procedures have given good results in preparing *Spondylis buprestoides,* the cerambycids *Monochamus, Aromia, Cerambyx,* some shiny, large Carabids and the smaller Lamellicornia. The hot plastic can sometimes excessively darken the natural colours, and the method is therefore most suitable for uniformly coloured, dark species.

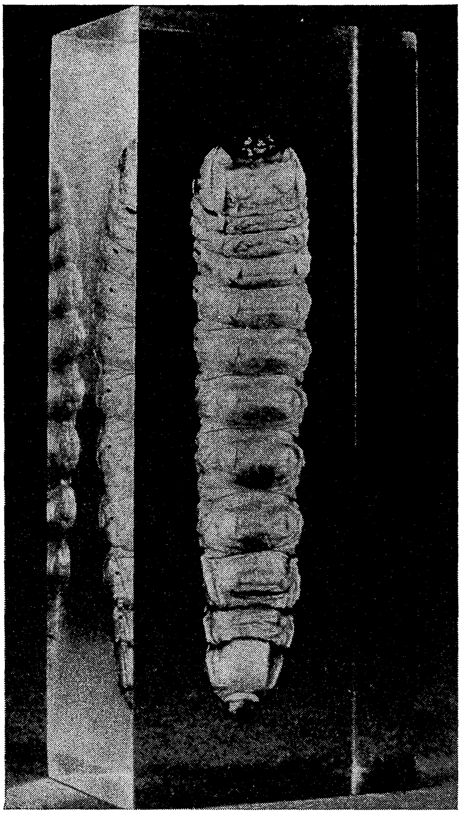
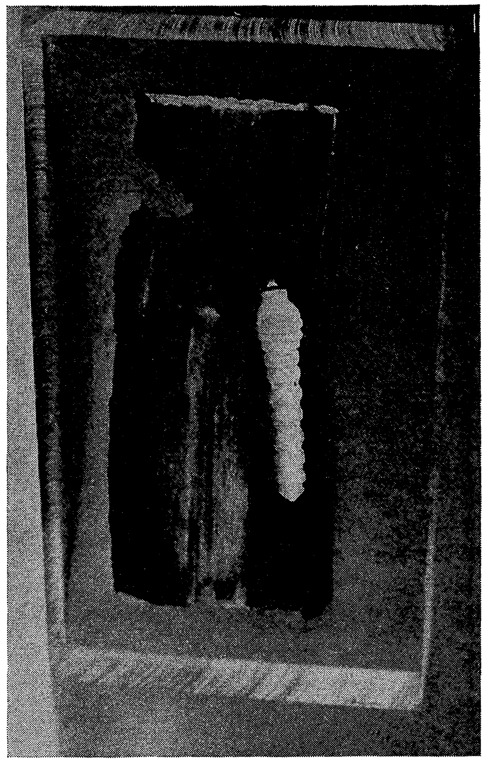


Larvae of left: *Potosia cuprea;* still in the mold. Right: *Melolontha* sp.; finished block. Dry method. See page 26.

6. Beetles and other insects with colours requiring an underlying zone of vacuum

The methods of treatment that have been discussed above lead to more or less complete injection of the beetles with plastic, either partly or wholly polymerised. In some light-coloured or spotted species, the above methods lead to reduced intensity of the pigment colours, or, in extreme cases, to disappearance of the colours (cf. p. 14), as a result of the altered refraction relations incurred by the plastic in intimate contact with those parts of the insect which have the most characteristic colours. A prerequisite for maintaining these colour effects is that the contact is broken approximately in the same way as by the treatment of butterflies. In the case of the insects mentioned above, the contact between the outer chitin of the insect and the plastic must be maintained; the goal of the treatment being to prepare the objects so that upon polymerisation the plastic separates within the body of the insect from the underside of the chitin covering, through which many species retain the typical colour characteristics.

To maintain the natural colouration the insect can be treated in the following way. The air-dried insect is vacuum-infiltrated with silicone-ether, and then washed rapidly with pure, water-free ether, to remove all of the external silicone. While still wet the insect is transferred to monomer plastic without initiator, sunk by the weight of a piece of metal screen and treated *in vacuo* until the ether boils away, and is substituted with plastic. Then

Left: Larva of *Ergates faber,* treated according to the dry method. See page 12. Right: Combined preparation, *Saperda perforata.* The damaged wooden block prepared with stabilized monomer plastic. The larva dry embedded. See page 27.

it is dipped into stabilized monomer, thoroughly drained, and finally placed directly into the middle layer of embedding plastic.

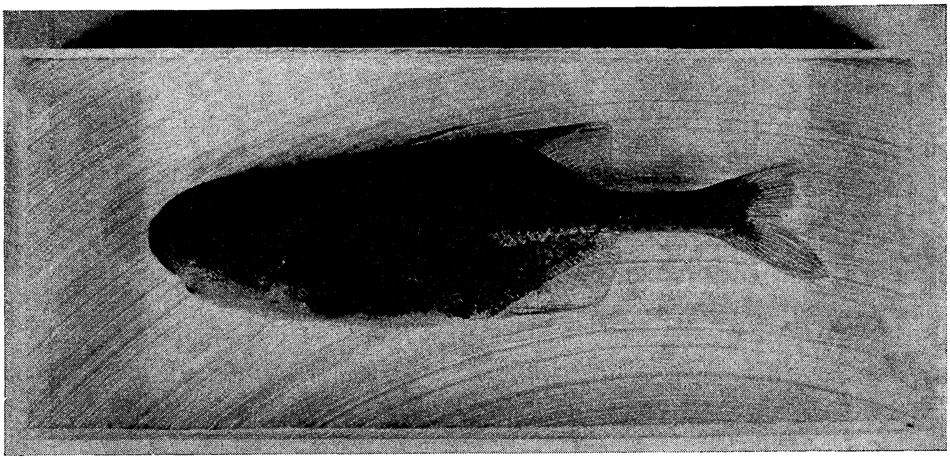
This procedure is useful for embedding Coccinellids, colorado-beetles, *Strangalia, Leptura rubra* and some *Hemiptera.*

7. Larvae of beetles

Most sparcely pigmented beetle larvae can be embedded like caterpillars (p. 12). For all objects the method must be combined with rapidly polymerising plastic, which can be obtained either through a higher dose of catalysts (which can readily induce unsuitable optical qualities in the plastic), or higher temperature during the polymerisation.

8. Combined preparations

The various procedures can be combined in such a way that the same block can demonstrate an insect's entire development and the characteristic



Mormurid. Prepared with stabilized monomer plastic. See page 27.

damage to the host plant. For example it is possible to embed in the same block: a beetle which has been treated with stabilized monomer, dry-prepared larvae, and a pupa treated in warm stabilized monomer. The dose of initiator must then be chosen so that the plastic polymerises at the rate demanded by the most sensitive object. In the example given rapid polymerisation is dictated by the dry-prepared larva.

9. Fishes

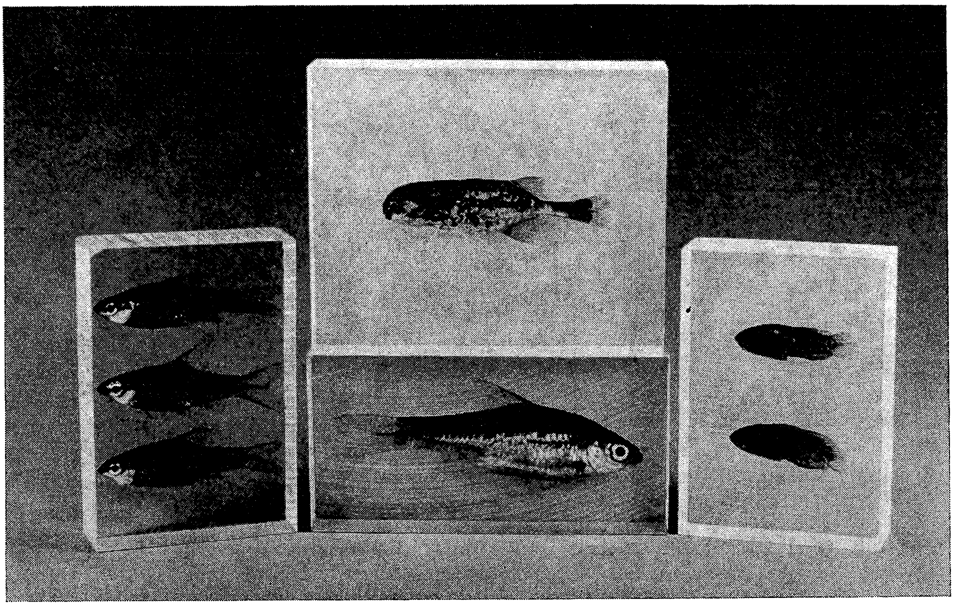
All previously known methods of conserving fishes for demonstration material bear the obvious disadventage that the natural colours are completely disturbed by the fixing solution, and at the same time eyes of the fish become cloudy and the body surrounded with a whitish covering of fixed slime.

For this reason it was proposed to test whether plastic methods offer better possibilities for maintaining the colours.

Of the methods tested, the following has given the best results.

The living fish is placed in a cuvette and killed with ether, which is added dropwise to the water. As soon as the fish dies, it is laid on to a glass plate, and Carl's solution is dropped over it. The fins and gill-plates are arranged in a natural position. After a short time the tissues become fixed, and the fins remain in a rigid position. The fish can then be transferred to a tube of Carl's solution, where it can be kept for a long time.

The pretreatment for embedding consists of a relatively rapid processing of the object through an alcohol series, where the time of treatment in absolute alcohol must be as short as possible to protect the sensitive colours. From alcohol the object is transferred to an alcohol-acetone or -xylene



Different fresh water fishes. Treated with stabilized monomer plastic.

mixture, and then to pure acetone or xylene. This fish is then placed into a mixture of stabilized monomer and acetone or xylene, and is then vacuum infiltrated with stabilized monomer, consisting of 80 % polyester type I and 20 % methylmethacrylate containing 0.1 % hydroquinone. In the stabilized monomer the fish clears and the cloudiness of the eyes disappears. Treatment in stabilized monomer is continued until the fish regains the degree of transparency that is characteristic of the living animal. Because of the occurence of alcohol in the inner parts of the fishes, the object never becomes completely transparent since the alcohol does not mix with softplastic. The fish can then be embedded by the standard technique.

The method has been used on some 30 African freshwater species fixed when caught, and some aquarium fishes fixed in this laboratory. Species such as cichlides, barbs and characids have given satisfactory preparations for instruction and demonstration.

10. Reptiles

For embedding small specimens of lizards and snakes, as well as organs or parts of these animals, the above described treatment with stabilized monomer (p. 9) can be used. Before the treatment complete removal of air by vacuum infiltration with ether is necessary.

11. Smaller mammals

Treatment with stabilized monomer can also be used before embedding smaller mammals. The object's size often necessitates several middle layers, which are poured one upon another. The middle layers of plastic should be poured from the same stock solution, which is kept at low temperature to avoid visible layering in the finished block.

Whole embryos are fixed in formalin, washed with water until the formalin is removed, and then stained with aceto-carmine. After differentiating to the desired colour tone (the subsequent treatment in plastic leads to intensification of the colour), the object is passed through an alcohol series to absolute alcohol and then to water-free ether. From ether it is transferred to monomer plastic without initiator, and pressure is slowly produced. After all ether has evaporated, the object is placed for some minutes in stabilized monomer, drained, and embedded by the standard technique (p. 16).

12. Anatomical preparations

The procedure described below gives satisfactory results with larger anatomical preparations such as sections of heart muscle, brain, lung, liver and kidney. It is based on a technique published by R. N. Haviland and O. T. Kampmeier (Anat.Rec.Vol.100).

The formalin-fixed objects are stained, differentiated and washed with water. The necessary dehydration can be done by rapid and peripheral treatment with alcohol and acetone or by air-drying. Only the outer layer, to a depth of a few millimeters, should be dehydrated, so that opacity of the object is retained.

Following dehydration, the object is placed into a mixture of monomer plastic without initiator and acetone. Air bubbles and loose material are brushed away. Then the object is embedded on a coloured bottom plate, which increases the contrast of the preparations.

Summary

Various methods for pretreating and embedding biological objects in plastic are described. The following objects are considered: Lepidoptera, Coleoptera, Hymenoptera and Diptera, spiders, fishes, crustaceans, centipedes, reptiles, small mammals and anatomical preparations.

The principal methods used are shown in the following table.

Table 1: General survey of the treatment methods.

Object	Killing	Fixation	Pre-treatment	Transfer to polymerising plastic	Post-treatment	Remarks
ECHINODERMA		Carl's solution	Glycerin—abs. alc.—acetone	Direct		Only short fixing
CRUSTACEA:						
Carididae		Glycerin	Glycerin—abs. alc.—acetone	Direct		
Isopoda		Carl's solution + ether	Glycerin—abs. alc.—acetone	Direct		Only draining in acetone
Other types		Carl's solution	Glycerin—abs. alc.—acetone	Direct		
Myriapoda	Ether	Carl's solution	Alc.—acetone or ether—soft plastic	After draining		
Chilopoda	Ether	Alcohol	Alc.—acetone or ether—soft plastic	After draining		
INSECTA:						
Lepidoptera: larvae	Ether	Heating in water—Carl's solution	Alc.—xylene—drying	Dry		Rapid polymerisation
pupae	Ether	Air drying or 70% alc.	Alc.—acetone—styrene—soft plastic in heat	After draining		
Butterflies with pigment	Ether	Mounting—air drying	Ether in vacuo	Wet	Slight pressure	
Butterflies irridescent	Ether	Mounting—air drying	Ether+silicone in vacuo	Wet	Slight pressure	
Moths	Ether	Mounting—air drying	Ether in vacuo	Wet, thin top layer	Slight pressure	
Coleoptera »soft» larvae	Ether	Heating in water—Carl's solution	Alc.—xylene—drying	Dry		

Object	Killing	Fixation	Pre-treatment	Transfer to polymerising plastic	Post-treatment	Remarks
»hard» larvae	Ether	Carl's solution	Alc.—acetone or ether—soft plastic in heat	After draining		
pupae	Ether	Carl's solution	Alc.—xylene—drying	Dry		
»hard» beetles	Ether	Mounting—air drying	Soft plastic in vacuo	After draining		
»soft» beetles	Ether	Mounting—air drying	Monomer plastic in vacuo	Direct		
»difficult» beetles	Ether	Mounting—air drying	Soft plastic in vacuo and heat (sometimes cooking in a cleaner+ water solution)	After slight draining		
Coccinellidae *Chrysomelidae* Coloured *Cerambycidae*	Ether	Mounting—air drying	Silicone+ether in vacuo—pure ether—monomer plastic in vacuo—soft plastic	After draining		
HYMENOPTERA:						
small	Ether	Air drying	Ether in vacuo	Wet		
large	Ether	Mounting—air drying	Ether—soft plastic in vacuo	After draining		
DIPTERA	Ether	Mounting—air drying	Ether in vacuo	Wet		
HEMIPTERA	Ether	Mounting—air drying	Ether—soft plastic in vacuo	Direct		
PISCES	Ether in water	Carl's solution	Alc.—acetone—soft plastic in vacuo	Direct		
AMPHIBIA	Ether	Carl's solution	Water—pure glycerin—draining in abs. alc.—acetone	Direct		
REPTILIA	Ether	Carl's solution	Alc.—acetone—soft plastic in vacuo	After draining		
MAMMALIA, small	Ether	70 % alcohol	Alc. series—acetone—soft plastic in vacuo—draining in acetone	Direct		
ANATOMICAL PREPARATIONS	—	4% formalin	Surficial dehydration in abs. alc.—draining in acetone	Rapid		

Sammanfattning

I föreliggande arbete redovisas en serie nya metoder för ingjutning av biologiska objekt i polyester för studie- och undervisningsändamål. Metoderna ha utarbetats vid institutionen för skogszoologi vid skogshögskolan.

De principiella riktlinjerna för metodiken kunna sammanfattas på följande sätt:

1. De krav som måste ställas på plasttyp för detta ändamål äro i huvudsak följande:
   Den monomera plasten måste kunna polymeriseras utan stark uppvärmning. Den får inte utveckla reaktionsvärme i någon nämnvärd grad. Krympningen vid polymerisationen får inte överstiga 10 %. Den polymeriserade massan måste vara praktiskt taget färglös. Plasten bör kunna polymerisera till kristallint och helst orienterat tillstånd. De krafter, som binda samman de makromolekulära elementen, böra vara så starka, att tryckfastheten och slagfastheten blir tillfredsställande. Hårdhetsgraden måste vara så hög att polymerisatet kan poleras.
   Med hänsyn till dessa krav har en polyester, benämnd Castolite, valts.
2. Den viktigaste principen för behandlingen av biologiska objekt är dehydrering av de delar av objekten, som bringas i direkt kontakt med plastmassan. Detta kan ske genom lufttorkning, behandling med glycerin eller urvattning med alkohol. Speciella metoder härför ha beskrivits.
3. Den vidare behandlingen av objekten före ingjutningen kan ske enligt olika metoder. En av dessa metoder har kallats *etermetoden,* varvid den luft, som befinner sig inuti objektet, ersättes med plastmassa genom en mellanbehandling med eter i exsickator. Metoden beskrives för parasitsteklar, gallsteklar, smärre flugor och myggor.
   En modifikation av etermetoden har utarbetats för ingjutning av fjärilar.
4. Andra objekt förbehandlas bäst med *glycerinmetoden,* varvid stigande glycerinkoncentrationer användas för dehydreringen. Metoden lämpar sig för ingjutning av spindlar, kräftdjur, mångfotingar och tusenfotingar.
5. Många biologiska objekt äro på grund av sin hårdhet svåra att ingjuta i genompolymeriserande plast. För dessa objekt har *mjukplastmetoden* utarbetats. Därvid förbehandlas objektet med monomer plastmassa, som stabiliserats till permanent mjukplast. Det så behandlade objektet ingjutes i polymeriserande plast. Metoden har modifierats efter objektens olika egenskaper. Objekt lämpliga för denna metodik äro många skalbaggar, fiskar, däggdjur och anatomiska preparat.

6. Goda resultat har även vunnits med den s. k. *torrmetoden,* som innebär, att objekten dehydreras med alkohol, behandlas med xylol, lufttorkas och slutligen ingjutas i snabbt polymeriserande plast. Lämpliga objekt för denna metod äro olika slag av insektslarver, även starkt håriga fjärilslarver.
7. I särskilda kapitel redovisas de olika arbetsmomenten vid tillverkningen av gjutformar, ingjutningen, täckningen och slipningen.
8. Slutligen lämnas en översikt över de speciella metoder som utexperimenterats för ingjutning av särskilt viktiga studie- och undervisningsobjekt, grupperade efter objektens art.

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