

LARGE-SCALE ISOLATION AND FRACTIONATION OF ORGANS OF *DROSOPHILA MELANOGASTER* LARVAE

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ABSTRACT

Methods for the mass isolation of diverse organs from small animals are described. They involve novel devices: a mechanical dissecting system, a centrifugal agitator for the separation of fibrillar from globular particles, and a settling chamber for the fractionation at unit gravity of particles with sedimentation velocities above the useful range for centrifugation. The application of these methods to the isolation of polytene and nonpolytene nuclei from *Drosophila melanogaster* larvae is described.

INTRODUCTION

Biochemical studies of small organisms such as insects have been largely restricted to whole animal preparations, because the amount of pure organs obtainable by manual dissection is small. In order to study proteins of polytene and nonpolytene nuclei of *Drosophila melanogaster*, we have been developing methodology for the isolation of gram quantities of pure organs. A method described by Fristrom and Mitchell (1) allows the isolation of imaginal discs but is not suitable for other organs, while a recently published method by Boyd et al. (2) for the isolation of larval salivary glands is applicable to *Drosophila hydei* but not to other species.

In this laboratory we have previously devised a method for the rapid isolation of salivary glands from moderate quantities of *D. melanogaster* larvae (3). The isolation of imaginal discs could be carried out simultaneously, but the yield and degree of purity of the discs were quite low.

We now report a method for the isolation of a variety of organs from large quantities of larvae with good yield and high degree of purity. The method consists of four principal steps, (a) automated continuous dissection, (b) fractionation by centrifugal agitation, (c) fractionation by settling

at natural gravity, and (d) isopycnic centrifugation in discontinuous density gradients. These procedures provide an approach to the general problem of doing biochemical studies with specific tissues of small organisms. We also describe modifications to a previously published method for the isolation of nuclei from *Drosophila* salivary glands and imaginal discs (3).

MATERIALS

Third instar larvae of *Drosophila melanogaster*, strain Oregon R, were thoroughly washed with distilled water, then drained, and placed into ice-cold organ medium (25 mM disodium glycerophosphate, 10 mM KH_2PO_4 , 30 mM KCl, 10 mM MgCl_2 , 3 mM CaCl_2 , 162 mM sucrose pH 6.8 [3]). The weight of the larvae is approximately half the settled volume. Coarse nylon net was obtained as curtain material. Glass-filled Delrin, a product of E. I. du Pont de Nemours & Co., Inc., Wilmington, Del., was used because of its low coefficient of thermal expansion. Stainless steel #1 fine intestinal needles were purchased from Richard Allen Mfg. Co. (Stratford, Conn.) and were hand sharpened. Ficoll was obtained from Pharmacia Fine Chemicals, Inc. (Uppsala, Sweden), disodium glycerophosphate from the Dajac Laboratories, Philadelphia, Pa., and crystalline bovine albumin from Pentex Biochemical,

Kankakee, Ill. All of these reagents were pretreated to remove trace metals as previously described (3).

METHODS

During all operations the fractions were kept at a temperature of 0–4°C. Larvae, as well as isolated organs, can be stored in ice-cold organ medium (3) for several hours without noticeable influence on microscopic appearance.

The Automated Dissecting System

A successful method to liberate the organs should combine high yield with minimal damage to the tissues. After trying many different types of homogenizers, we found the most satisfactory system to be an automated dissecting device that works on the same principle as the manual dissecting method; first, opening the animals with a needle, and then, gently squeezing out the organs.

The system is illustrated in Fig. 1. A buffer reservoir containing organ medium pressurized with nitrogen at 5 psi is connected air-tight to a two-neck round-bottom flask (A) containing the larvae in organ medium. Mounted air-tight in the main neck of the flask with a rubber stopper are an air vent and the continuous dissecting device (B) (see Figs. 2 and 3). The flow of the pressurized medium and a rotating stainless steel cylinder transport the animals up the grooves of the stationary plexiglas sleeve,

over needle points that protrude into these grooves, where they are torn open. The collapsed carcasses then tend to roll over the ridges in the upper part of the grooves, starting a gentle squeezing process that is continued by stainless steel discs above the transporting cylinder, thus liberating the individual organs with little mechanical stress. The mixture of emptied carcasses and organs is swept through the overflow into the upward filtration system (C), where the empty carcasses are retained, while the organs flow through the net into a trap (D). Because of the reduced flow rate in the wide upper part of the trap, the organs remain near the bottom, while soluble material, small fragments, and fat bodies flow over to be collected if desired.

After all the larvae are dissected, any free organs remaining in the filtration system are decanted and washed out with organ medium through a double layer of the nylon net into a siliconized glass beaker (3). After settling for 20 min, the supernatants of the organ trap and the filter washes are removed carefully. The sedimented organs are then washed twice with at least 20 times their volume of fresh medium in 1500 ml siliconized glass beakers, waiting 15 min each time before removing the supernatant.

In later steps of purification the organs tend to stick to the glassware. This can be reduced by rinsing the glassware with the original supernatant and then with medium.

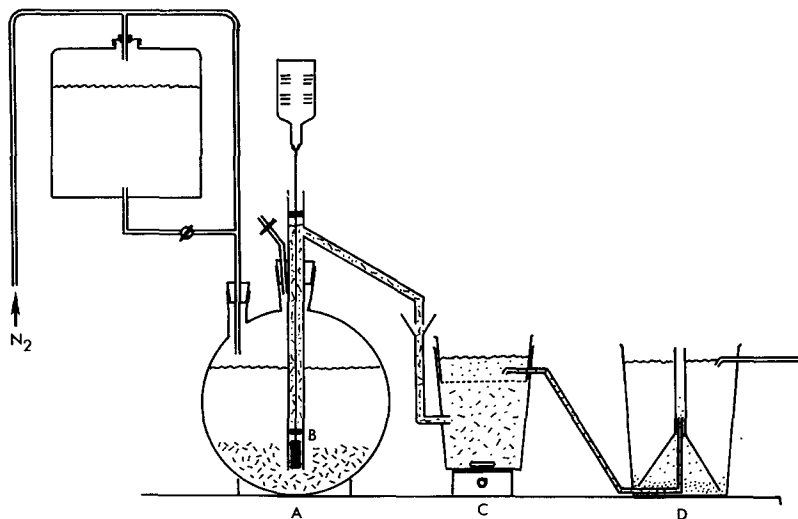


FIGURE 1 Schematic diagram of the automated dissecting system. A, larvae container; B, continuous dissecting device (see also Figs. 2 and 3); C, upward filtration system on magnetic stirrer, consisting of a 2-liter conical polypropylene beaker with a coarse nylon net (1 mm pores) held in place by the sawed-off rim of another beaker; D, an organ trap consisting of a 4-liter conical polypropylene beaker containing an inverted 6" plastic funnel with extended stem and scalloped rim.



FIGURE 2 Lower end of continuous dissector: (left) stationary part, showing the grooved sleeve with three or four rows of needles protruding into the grooves; (right) rotating part with transporting cylinder, four squeezing discs and one of the plastic spacers that center the rotating part within the stationary part during operation.

Centrifugal Agitation

Based on the earlier experience that gut could be separated from salivary glands and imaginal discs in a rotated beaker (3), a large plexiglas beaker was constructed with a bottom that rises in three steps to the wall (Fig. 4). A plexiglas tube is placed on the first step, the chamber outside the tube is filled with organ medium, and the inside of the tube is filled with a suspension of organs of about 100 g of larvae. After 15 min of settling time the tube is removed carefully. The beaker is placed on a level surface of crushed ice and first rotated slowly about 1 inch back and forth to facilitate the separation of different layers within the sediment. Then the beaker is rotated with short but smooth hand strokes at about 10–15 rpm. The gut rises over the steps and up the side of the beaker, from where it is periodically transferred to a suction flask by means of a Pasteur pipette, without stopping the rotation. After the

most easily disturbed gut is removed the rotation is stopped and the pile of organs at the very center of the beaker is gently dispersed with glass rod. The fractionation is resumed, first with a back and forth motion and then by rotation as before. Under proper conditions practically all of the gut can be removed within 10 min, leaving salivary glands, imaginal discs, and most of the Malpighian tubules at the bottom of the beaker.

Settling at Natural Gravity

The difference in size between imaginal discs and salivary glands allows their separation by sedimentation. Since even the lowest controllable speed on available centrifuges is too high, we used sedimentation at unit gravity through a stabilizing gradient, a principle which has been employed previously for cell separation (4). The high sedimentation rate of whole organs necessitated the design of a special settling chamber to permit rapid layering of the sample on the gradient and rapid collection of the separated organs. The settling chamber consists of a plexiglas block (Fig. 5) with a cylindrical cavity and slots for metal sheets that can be pushed across the cylindrical bore to separate the different zones of the chamber. With the compartments almost closed, the chamber is filled with organ medium containing a stabilizing linear gradient of Ficoll (2–5%). All of the compartments are then opened to release bubbles. The top partition is pushed in to separate the sample (top) compartment from the gradient. The organ suspension is poured in and then hand-stirred slowly while the partition is opened. This creates a sharp boundary between the sample and the stabilizing gradient. After about 5 min the lowest compartment is closed to separate any small larvae, food particles, and white Malpighian tubules. After about 15 min of settling the next two compartments are closed, the lower one containing the salivary glands, the upper one containing small fragments of salivary glands and some of the largest imaginal discs. After about 25 min of settling, the fourth compartment containing the main imaginal disc fraction is closed. The Malpighian tubules being of variable size and density are distributed throughout the gradient and are subsequently separated in an isopycnic centrifugation step. After the supernatant containing small and light particles has been poured off, the lower compartments are opened one after the other and their contents are rinsed into silicized glass beakers with organ medium. Each fraction is then washed once with organ medium to remove the Ficoll.

The imaginal disc fraction and salivary gland fractions are separately subjected to isopycnic cen-

trifugation in density gradients suited to the particular fraction, as described below.

Isopycnic Centrifugation

The discontinuous Ficoll gradients used by Frisstrom and Mitchell (1) and Boyd et al. (2) were slightly modified. Purified Ficoll was dissolved in organ medium and the density of the stock solution was adjusted with organ medium to 1.1175 g/ml at room temperature (25°C).¹ The salivary gland fraction is layered onto a discontinuous gradient (Gradient I) consisting of 15 ml of undiluted Ficoll stock solution (Ficoll A) and 20 ml of Ficoll B (Ficoll A diluted with ½ volume of organ medium) in 50-ml polycarbonate centrifuge tubes. For imaginal discs (Gradient II) Ficoll B is used as the lower layer and Ficoll C (Ficoll A diluted with 1.5 volumes of organ medium) for the upper layer of the discontinuous gradient. All boundaries are broadened slightly by gentle stirring to prevent aggregation. After standing vertically for 2 min, the tubes are centrifuged at 40 *g* for 1 min, rotated 180° to prevent packing on one side during acceleration, and centrifuged another 3 min at 2500 *g*.

The gradient for salivary glands separates glands associated with parts of fat body together with some imaginal discs and gut pieces (upper band) from the main salivary gland fraction (lower band) and Malpighian tubules as well as mouthparts (pellet). The gradient for imaginal discs separates testes associated with fat bodies (upper band) from the imaginal discs (lower band) and Malpighian tubules together with fragments of salivary glands (pellet). The bands are recovered with a wide-mouth, polypropylene pipette and washed twice with gland medium to remove the Ficoll.

The success of isopycnic centrifugation depends on the precise adjustment of the density of the different layers. The densities of the organs may vary with the size, age, and growth conditions of the larvae. In addition, previous exposure of organs to solutions containing Ficoll or sucrose tends to increase these densities. We therefore recommend a pilot experiment to determine whether adjustment of the densities is required.

RESULTS OF FRACTIONATION OF *DROSOPHILA* LARVAE

In Fig. 6 is shown a flow-sheet of the fractionation of organs of late third instar larvae of *D. melanogaster*. In this diagram, the major fraction for each organ type is indicated by a box.

¹The densities of Ficoll solutions were measured by weighing 15 ml samples dispensed by a calibrated syringe with Chaney adapter.

Salivary Glands (Fig. 7)

The method as described yields, per 500 g of larvae, approximately 2 g of salivary glands, of which about half are broken, mainly near the neck. As judged by microscope observation, this fraction contains about 2-5% contaminating tissue mass, mainly attached fragments of fat body, mouthparts, and fragments of gut. Practically all free contaminants can be removed by a rerun on the isopycnic gradient or by hand under a dissecting microscope. If desired, whole glands can be separated from gland fragments by appropriate fractionation in the settling chamber.

Imaginal Discs (Fig. 8)

From 500 g of larvae, about 0.4 g of imaginal discs are obtained. Contamination, as estimated by microscope observation, is about 20% by mass. The yield of the disc fraction can be increased by slower and longer rotation of the centrifugal agitator. The purity can be improved by allowing the salivary glands to settle to the bottom of the settling chamber, thereby leaving more of the chamber available for the separation of discs from other particles. This also permits separation of discs of different sizes. These modifications are recommended when the purity of the salivary gland fraction is not of primary importance.

Testes (Fig. 9)

A small but quite pure fraction of testes is obtained at the top of the isopycnic gradient for imaginal discs, due to their association with small fat bodies. The procedural modifications recommended for the imaginal discs apply to the testis fraction as well.

Other Organs

The described procedure emphasizes the isolation of salivary glands and imaginal discs. Other organs are obtained at lower degrees of yield and purity. Although we did not pursue the purification of these organs in detail, we can recommend the following purification procedures:

MALPIGHIAN TUBULES (FIG. 10): The high density of the Malpighian tubules can be used to purify relatively crude preparations by isopycnic centrifugation. Thus the Malpighian tubule-rich fractions at the top of the settling chamber and in the pellet of isopycnic gradient for both salivary glands and imaginal discs can be washed free of Ficoll and then banded in a discontinuous Ficoll gradient between layers with densities of 1.1175 and about 1.122 g/ml.

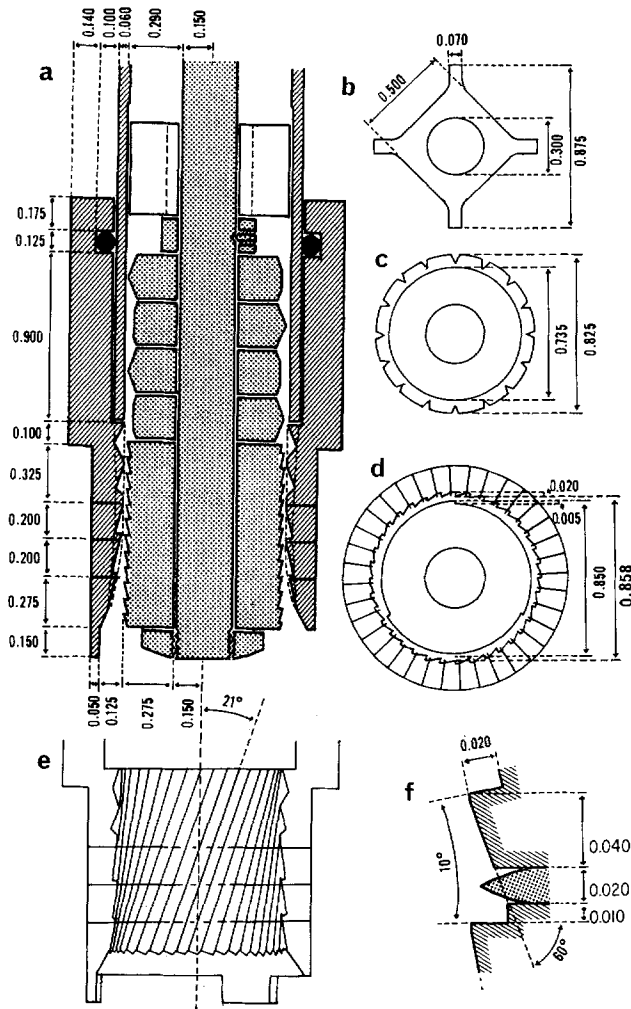


FIGURE 3 Details of the continuous dissecting device, with dimensions suitable for late third instar larvae of *D. melanogaster*. (a) Longitudinal section showing the plexiglas stationary part (diagonal shading), consisting of a grooved sleeve attached with an O-ring (black) to the machined end of the main tube, as well as the stainless steel rotating part (stippled), consisting of a transporting cylinder with a triple buttress thread directed upwards (three leads, 0.25 inches pitch, 0.008 inches deep, with back surface at a 5.5° angle to the axis of the cylinder), and four squeezing discs (0.25 inches thick) mounted on a shaft. The main tube is 16 inches long and is made from 1 inch outer diameter plexiglas, machined 2 inches at each end to 0.875 inches inner diameter, in such a way that the internal bores of the two ends are in precise alignment. The lower 1.50 inches of the main tube is machined on the outside as well, just enough to be perfectly round and aligned with the bore, in order to secure a proper fit and alignment of the grooved sleeve. The outer diameters of the four steel discs are (counting from the top) 0.840, 0.830, 0.825, and 0.820 inches. The edge of these discs is broken by grooves (eight in the upper two, 16 in the lower two discs) to prevent clogging (Figs. 2, 3 c). In operation the shaft is centered within the stationary part by two Delrin spacers (one of which is seen in the figure, unhatched), and attached through a flexible joint to a variable-speed, high-torque motor turning clockwise at about 60 rpm; (b) cross-section through a spacer; (c) top view of a stainless steel squeezing disc; (d) cross-section through the grooved sleeve and the transporting cylinder at the level of the middle needle row; (e) view of the inside of a grooved sleeve, the front half of which is cut away. In order to cut the 36 grooves, the sleeve is mounted, narrow part up, on a rotary table of a drill-press and tilted 21 degrees, at right angles to the crossfeed. A sharp cutting tool, shaped to fit the cross-section of a groove as illustrated in Fig. 3 f, is then used to cut with vertical motions a straight groove into the inner wall of the sleeve, to a depth of 0.020 inches at the shallowest point (middle of the narrow part of the sleeve). The sleeve is then turned 10 degrees and the next groove cut in the same way. This results in an interesting and desirable change in the cross-sectional shape and depth of the grooves as well as in an increase in the inner diameter of the sleeve, particularly towards the lower end (as indicated in the longitudinal section of the sleeve, which cuts several grooves obliquely). In addition, the lower end is beveled on the inside and provided with three feet to facilitate entry of the larvae. Three or four rows of needle tips are inserted through the sleeve so that they protrude into and are protected by the grooves (see Fig. 2, horizontal lines in Figs. 3 a and 3 e, radial lines in Fig. 3 d, and crosshatched in Fig. 3 f).

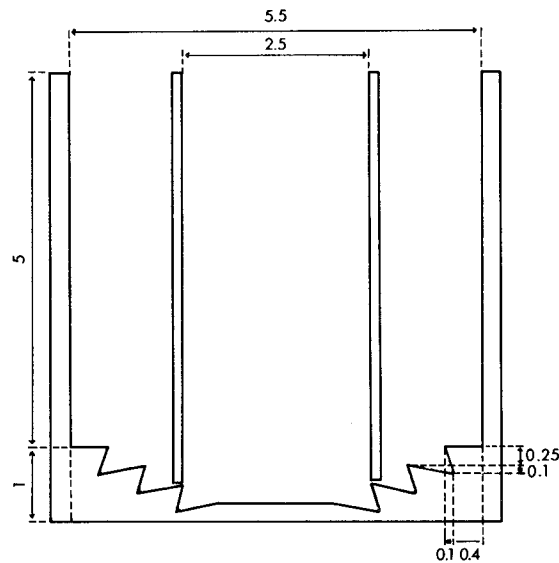


FIGURE 4 Centrifugal agitator, consisting of a plexiglas beaker, the bottom of which rises to the wall in three steps, and a plexiglas tube used for loading the organs into the center compartment. All measurements are given in inches.

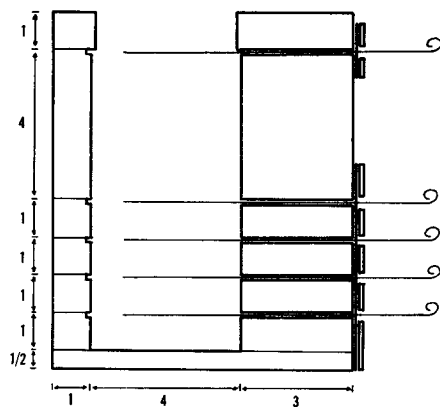


FIGURE 5 Diagram of the settling chamber, consisting of a plexiglas block with a cylindrical cavity and metal sheets for the separation of different zones of the density gradient in the chamber. Note that the sample (top) compartment is smaller in diameter than the others, to prevent organs from touching the wall during settling. The metal sheets are lubricated with silicone oil, and the slots are sealed with a rubber gasket held in place by an aluminum plate. Measurements are in inches.

GUT: The bulk of the gut is recovered from the centrifugal agitator by collection in a trap. It can be purified as well as fractionated in the settling chamber.

FAT BODIES: These can be concentrated from the overflow of the organ trap, either by continuous

flow centrifugation or by flotation. The fat bodies are mostly broken into relatively small fragments.

Salivary Gland and Imaginal Disc Nuclei

Nuclei were isolated by essentially the same method described previously (3), except that, for the first homogenization, bovine albumin (1 mg/ml) was added to the medium (0.1 M Na₂ glycerophosphate, 50 mM sucrose, 4 mM MgCl₂, and 0.4 mM CaCl₂, pH 6.8), in order to minimize proteolysis. Moreover, no detergent was used until the nuclei had been thoroughly washed to remove the albumin and cytoplasmic organelles. The nuclei were then suspended in 0.5% Triton X-100 in the same medium, stirred for 1 min, and centrifuged (164 g for 1 min). The Triton was removed by washing twice. Polytene and nonpolytene nuclei obtained by this procedure are shown in Fig. 11.

DISCUSSION

The problem of obtaining sufficient tissues for biochemical experiments is frequently encountered in studies with small animals. The procedures described herein help to solve this problem, and moreover provide the means for the isolation of a number of different organs in the same operation. In adapting these procedures to different organisms, the principles involved in their design must be considered.

An important advantage of the continuous dis-

secting device is its gentle action, which results in minimal damage to the organs and minimal loss of soluble cellular components. The dimensions are such that the maximal space between the stationary part and the rotating part is about equal to the diameter of the organism and the minimal space is one quarter the diameter of the organism, to avoid cutting the organs. The length of needle protruding into the grooves is one quarter the

diameter of the organism. Under these conditions over half of the *Drosophila* larvae are emptied. Although it is possible to obtain complete emptying of all of the larvae by increasing the sizes of the squeezing discs, this is not recommended (except perhaps for the isolation of cuticles) because the increased yield represents mainly crushed tissues. If these considerations are fulfilled, the dissecting device should be adaptable to other small animals, provided the organs are easily liberated when the outer covering is torn. To this end, the dissecting device has been constructed in such a way that all critical parts can be exchanged easily. To adjust it to relatively small differences in animal size, it is sufficient to use a transporting cylinder and squeezing discs of different diameter, while for much larger or smaller animals an additional modification of the depth and number of grooves in the sleeve is necessary.

The three methods described for the separation of organ types rely on different parameters, namely shape, size, and density, and their combined resolving power is therefore much greater than that of any one of them. Furthermore, the removal of gut by centrifugal agitation eliminates clumping in the subsequent steps, while the preliminary separation of salivary glands and imaginal discs by means of the settling chamber permits the use of different density gradients for these two tissues in the isopycnic sedimentation step.

The efficacy of these procedures depends on the species. For example, in the case of *Drosophila virilis*, which has long, narrow salivary glands, we have found that neither the centrifugal agitator nor the settling chamber is very effective in separating salivary glands from gut, although the settling chamber was effective in separating these two tissues from imaginal discs. In this species, therefore, the resolution of salivary glands and gut relies mainly on isopycnic sedimentation.

These methods have been used for studying nuclear proteins² and nucleic acids³ of diverse organs of *Drosophila* larvae, and have also been modified for the isolation of mature eggs from adult flies of the same genus⁴. Although some initial

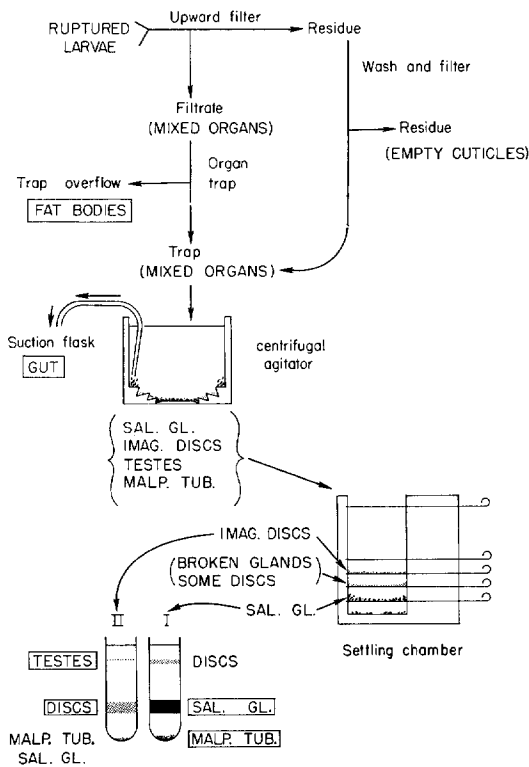


FIGURE 6 Flow-sheet of the isolation of organs and tissues of *Drosophila melanogaster* larvae. The principal fraction for each tissue is indicated by a box. The times required for the steps in the isolation of nuclei from salivary glands (*sal. gl.*) and imaginal discs (*imag. discs*) of 500 g of larvae are:

1.5–2 hr for dissecting the larvae; 1 hr to wash the crude organ mixture; 1–1.5 hr to remove the gut using two centrifugal agitators alternately; 1 hr to separate discs and glands and to wash the fractions free of Ficoll; 0.5 hr to separate the salivary glands and imaginal discs from Malpighian tubules (*Malp. tub.*) and testes by isopycnic sedimentation; 0.5–1 hr for final purification by resedimentation or under the dissecting microscope; and 1 hr for isolating nuclei.

² Cohen, L. H., B. V. Gotchel, and A. Zweidler. Unpublished data.

³ Travaglini, E., and J. Schultz. Private communication.

⁴ Mahowald, A. Private communication.

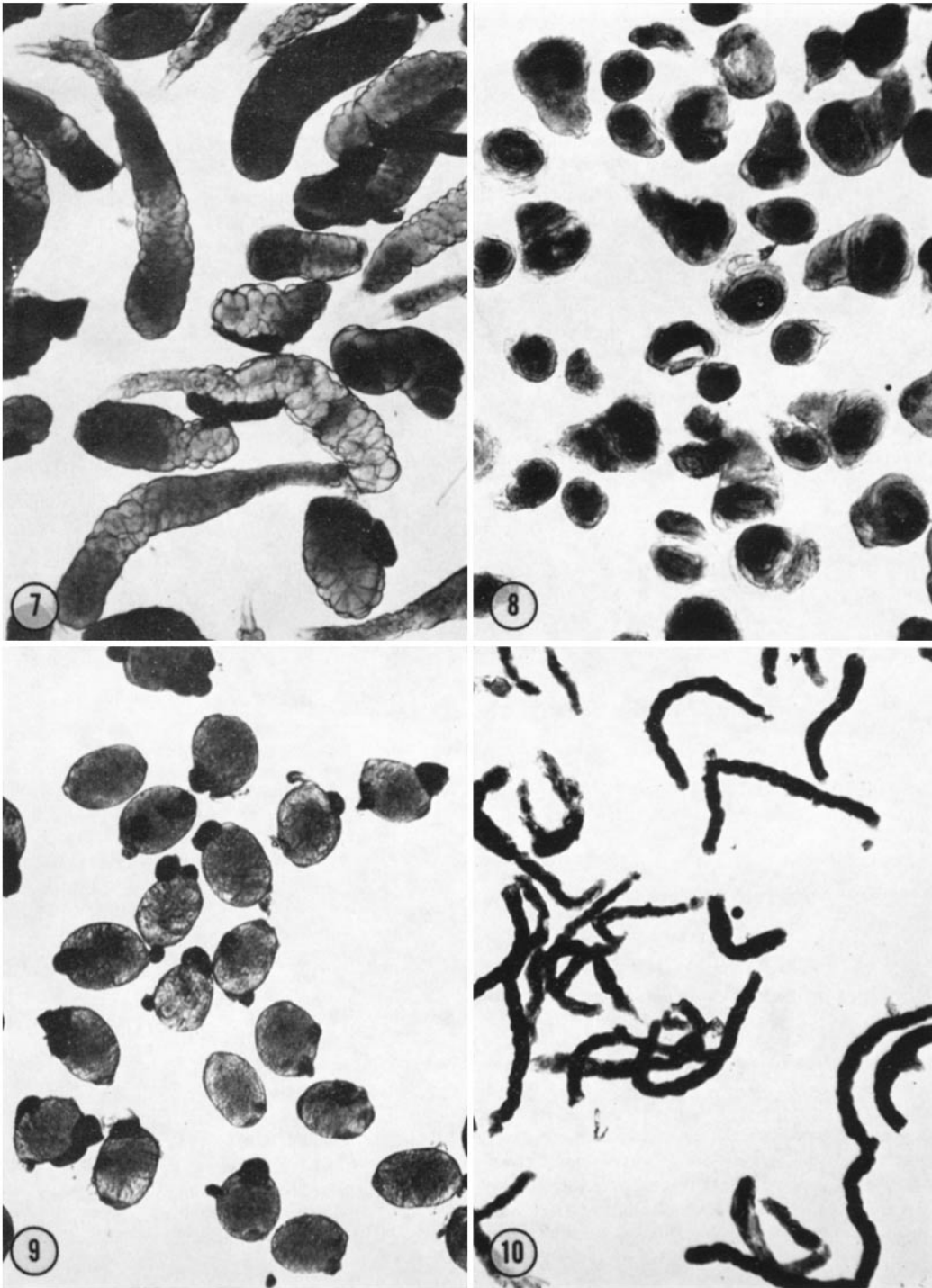


FIGURE 7 Light micrograph of isolated salivary glands. $\times 35$.

FIGURE 8 Light micrograph of isolated imago discs. $\times 35$.

FIGURE 9 Light micrograph of testes with associated fat body (black). $\times 35$.

FIGURE 10 Light micrograph of Malpighian tubules. $\times 35$.

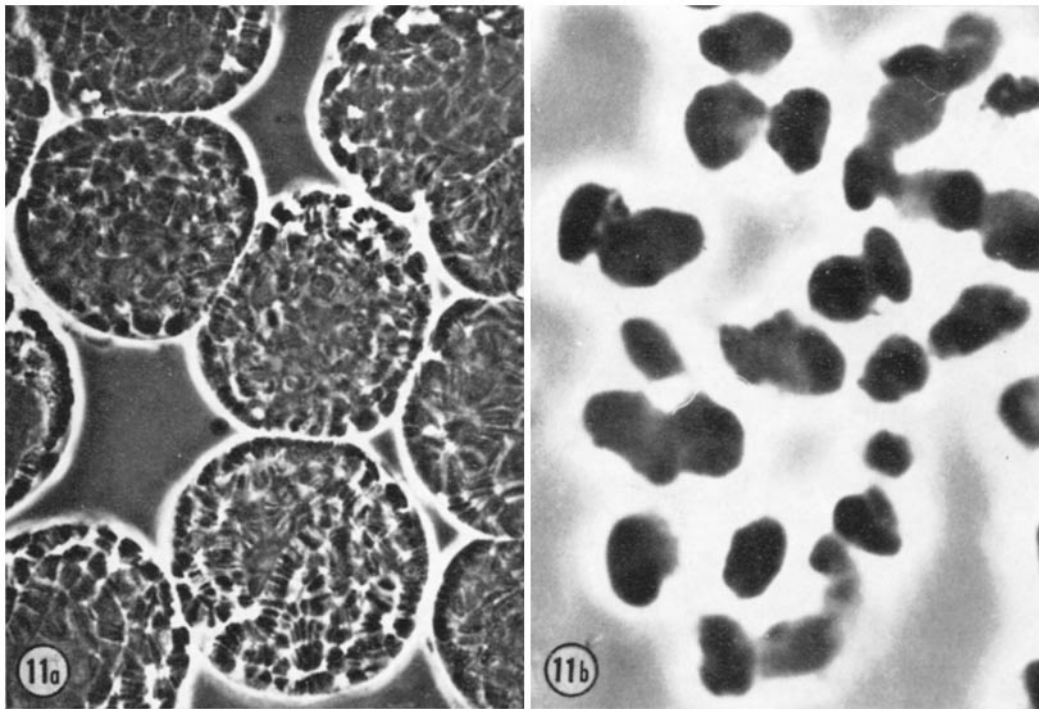


FIGURE 11 Phase contrast micrograph of unfixed nuclei: a. Salivary gland nuclei, $\times 900$; b. Imaginal disc nuclei, $\times 3000$.

effort will be required to adapt them to other organisms, we believe that they should be useful for many applications.

The success of this work depended on the personal effort and technical expertise of William Hafner in constructing the dissecting device and Robert Ellis in making many prototypes and the final versions of the centrifugal agitator and the settling chamber. Inquiries about these items should be directed to them. The invaluable assistance of Judy Spokas in raising and collecting the huge numbers of larvae is acknowledged. We would also like to thank Barbara Gotchel for excellent advice and assistance and Elizabeth Travaglini and Dana Tartof for advice about raising larvae. We are indebted to Dr. Jack Schultz for his continued interest and encouragement, and for making available facilities for growing larvae.

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