Quantitative tissue isolation from *Drosophila* freeze-dried in acetone

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Freeze-drying procedures were developed to enable collection of tissues from *Drosophila* flies. The flies were frozen in acetone at -86 or -94 °C, and dehydrated therein. After drying, many tissues could be easily taken in entirety and free of neighbouring tissues without action of degradative enzymes. Seven polypeptide species specific to retina, and nine specific to cornea, were identified on two-dimensional electrophoretograms. Phospholipids of the dried tissues could be studied by t.l.c., and phosphatidic acid of the fly head was found to occur predominantly in the retina. Activity of three enzymes in the dried tissues could be assayed. The results of protein, phospholipid and enzyme analyses were corroborated by analyses by 'genetic dissection' using an eyeless mutant line.

INTRODUCTION

Drosophila melanogaster is a unique experimental animal in which a number of classical genetic manipulations can be combined with methods of biochemical analysis. This advantage has become even more significant in recent years, as recombinant-DNA techniques have come to be applied with utmost sophistication in this insect (Spradling & Rubin, 1982).

However, the small size of the fly, weighing about 1 mg, continues to pose formidable obstacles to biochemical analyses of specific tissues. It is virtually impossible to collect particular tissues fresh in sufficient quantity for preparative or most analytical purposes. Homogenization of the whole animal is bound to release a host of powerful digestive enzymes of the alimentary tract. Neither is it possible to obtain by surgical dissection a particular tissue in its entirety and free of neighbouring tissues, such as would be required to establish specific occurrence of a substance or an activity in that tissue.

In the course of our attempt at molecular characterization of *Drosophila* mutations in visual and contractile functions (Hotta, 1979), we developed a method to circumvent the above difficulties. Here we describe the procedure, in which fruit flies are rapidly frozen in acetone/solid CO₂ mixture and dehydrated at -20 °C. From flies dried in this way, it was found that many tissues can be cleanly detached without action of endogenous degradative enzymes. Applicability of the method is demonstrated by analyses of protein and phospholipid composition, and of enzyme activities in the tissue samples thus collected.

Preliminary accounts of portions of the work have appeared in abstracts (Fujita & Hotta, 1978*a,b*).

EXPERIMENTAL

Flies

Flies of *Drosophila melanogaster* Canton-Special strain, wild-type or mutant, were raised at 25 °C on cornmeal-agar-yeast medium. In mutant flies of *sine oculis* (*so*) strain, ocelli are absent and compound eyes are either absent or reduced to small groups of ommatidia (Lindsley & Grell, 1968). Individuals lacking compound eyes were selected for analyses described below. For phospholipid analysis, flies were fed for 1 day with [³²P]P_i as described by Yoshioka *et al.* (1984).

Chemicals

Reagents used in electrophoresis were from the same sources as those in O'Farrell (1975). Solid CO₂ and liquid N₂ were from local commercial sources. The radio-labelled enzyme substrate phosphatidyl[¹⁴C]inositol (270 mCi/mmol) was purchased from Amersham, and [γ -³²P]ATP (2900 Ci/mmol) and cyclic [³H]AMP (38.1 Ci/mmol) were from New England Nuclear Corp. Other chemicals used were of reagent grade.

Electron microscopy

Freeze-dried flies and tissues were coated with platinum to 20 nm thickness, and viewed with a Hitachi S-700 scanning electron microscope at 15 kV.

Two-dimensional gel electrophoresis

This was performed as described by O'Farrell (1975). Tissues collected from dried flies were homogenized in 60 μ l of lysis buffer (9 M-urea, 2% Nonidet P-40, 2% Ampholine pH 3.5–10, and 5% mercaptoethanol) with glass micro-tissue grinders (Kontes). After centrifugation at 1300 g for 5 min, 25 μ l portions of supernatant were

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loaded into electrofocusing tubes. Ampholine pH 3.5–10 was used at 2% for the first dimension. Second-dimension electrophoresis in the presence of SDS was in 12% polyacrylamide gels. The spots were developed by staining with Coomassie Brilliant Blue R-250.

T.I.c.

Phospholipids were extracted and separated by the method of Jolles *et al.* (1979), with modification described by Yoshioka *et al.* (1983). The silica-gel plate (Merck, Art. 5721) was developed in the first dimension with chloroform/methanol/28% NH_4OH (13:7:1, by vol.), and in the second dimension with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.). The phospholipids were detected by autoradiography.

Enzyme assays

Heads or tissues were taken from flies either freeze-dried as described below or frozen at -80°C after light diethyl ether anaesthesia. Assay reactions were at 25°C .

For determination of phosphatidylinositol phospholipase C (EC 3.1.4.10) and diacylglycerol kinase (EC 2.7.1.107) activities, 40 heads or corresponding amount of tissue were homogenized in $230\ \mu\text{l}$ of the media detailed in Yoshioka *et al.* (1984, 1985), and $100\ \mu\text{l}$ of the homogenates were used as enzyme samples. Phospholipase C was assayed by formation of [^{14}C]inositol phosphate from phosphatidylinositol (2 mM), as described by Eichberg *et al.* (1981) with a slight modification as described by Yoshioka *et al.* (1985). Diacylglycerol kinase was measured by incorporation of ^{32}P into phosphatidic acid with [$\gamma\text{-}^{32}\text{P}$]ATP and 1,2-diolein as substrates, by the method of Lapetina & Hawthorne (1971) with modifications described by Yoshioka *et al.* (1984).

Cyclic AMP phosphodiesterase (EC 3.1.4.17) was determined essentially as described by Byers *et al.* (1981); 20 fly heads or equivalent were homogenized in $250\ \mu\text{l}$ of 80 mM-Tris/HCl buffer, pH 8.0, containing 20 mM- MgCl_2 , 4 mM-dithiothreitol and 2 mM-5'-AMP. Then $100\ \mu\text{l}$ of the homogenate was mixed with $100\ \mu\text{l}$ of 0.2 mM-cyclic [^3H]AMP in water to start the reaction. The reaction was terminated by addition of ZnSO_4 and $\text{Ba}(\text{OH})_2$ to 0.05 M (Pösch, 1971), and radiolabelled 5'-AMP formed was measured.

Freeze-drying procedures

Procedure A was initially developed and used for the experiments described below. Procedure B is a more recent improvement, and is simpler and safer.

Procedure A.

(1) About 70 ml of acetone is poured into a 125 ml Pyrex conical flask holding chips of solid CO_2 .

(2) Up to a few hundred anaesthetized flies are put into the flask in small portions through a funnel.

(3) The flask is placed in a chest-type explosion-proof freezer at -25 to -20°C . After the solid CO_2 is gone and the flask contents have reached the freezer temperature, the flask is stoppered.

(4) During the following 7–10 days, the acetone in the flask is twice replaced with fresh and chilled acetone. This is done in the freezer to avoid thawing of the sample.

(5) Let the flask warm up to room temperature before

removing acetone. The sample is placed on filter paper to let acetone evaporate. For storage the sample should be completely dried in a conventional freeze-dryer.

Procedure B.

(1) A 125 ml Pyrex conical flask with about 70 ml of acetone and 6 g of anhydrous Na_2SO_4 is placed in liquid N_2 held about 2 cm deep in a foamed-polystyrene box.

(2) The flask is swirled occasionally until about half the acetone has solidified at the bottom. The melting point of acetone is -94°C . The flask is removed from the liquid N_2 bath, and a few hundred flies are put into the flask in portions.

(3) The flask is placed in an explosion-proof freezer at -25 to -20°C . After the flask has warmed up to the freezer temperature, it is stoppered.

(4) After 7–10 days (without change of acetone), the flask is taken to room temperature, and the sample dried and stored as in Procedure A.

Comments

(1) Anhydrous Na_2SO_4 must not be used in Procedure A, as it causes spattering of acetone.

(2) Immediately after step (2) of either procedure, frozen flies may be fragmented by mechanical agitation for efficient collection of head and appendage structures.

(3) The minimum time required in step (4) will depend on the sample. A longer time is allowed for flies of larger species.

(4) The dried material can be stored over silica gel at -20°C for at least 1 year without changes in two-dimensional-electrophoretic polypeptide pattern.

RESULTS

Examination of the freeze-dried flies under a binocular microscope revealed that the external morphology of the sample was well preserved, except for occasional cracks seen in the fly abdomen or larval body (Figs. 1a and 1b). When nicks were made into the cuticle with a pointed razor blade, the dried tissues tended to crack along tissue boundaries under mechanical stress delivered through a tungsten needle or fine forceps. Compound eyes, for example, could be removed from the head in this manner. Usually cracks developed along the interface between the retina and the first optic ganglion, so that the brain as well, complete with the ganglion, could be lifted out of the head (Fig. 1c). The corneal layer of the eye could be easily dismantled to yield the neural retina (Fig. 1d). Muscles broke off the cuticle at their attachment sites, and could be collected singly and *in toto*. Fig. 1(e) shows some flight muscles in the left thorax. Many other tissues, such as thoracic ganglia, alimentary tract and ovaries, could be similarly taken. Tissue samples thus collected could be stored over silica gel, allowing accumulation of the material up to desired quantities. Head tissues were studied below for protein and phospholipid composition and enzyme activities.

Electrophoretic analysis of tissue proteins

Heads taken from the freeze-dried flies were analysed for protein composition by two-dimensional electrophoretic analysis (O'Farrell, 1975). The electrophoretogram of the analysis of 16 whole dried heads is shown in Fig. 2(a). About 400 spots could be counted. The

Fig. 1. Scanning electron micrographs of freeze-dried *Drosophila* fly and tissues taken therefrom

(a) Whole fly. (b) Head. (c) The isolated brain, with the first optic ganglia at both ends. (d) The isolated reticular cell layer after removal of corneal layer. (e) The six dorsoventral indirect flight muscles (asterisks), and the jump muscle or tergosternal depressor of trochanter (star), *in situ* in the leftmost quarter of the thorax, viewed from inside. The white bar in panel (a) denotes 300 μm , and that in all others 100 μm .

pattern of the spots was reproducible as long as the strain and the age of the flies were controlled. Fresh and dried whole heads analysed in parallel gave virtually identical electrophoretograms (not shown), showing that our procedure does not entail covalent alteration or selective loss of polypeptides detected in Fig. 2(a).

With analysis of fresh samples, we had occasionally experienced anomalies in the spot pattern, with many high- M_r spots diminishing in intensity, accompanied by appearance of a number of low- M_r spots. In a control experiment such an alteration was suppressed to a large extent, but not eliminated, by inclusion of 2 mM-phenylmethanesulphonyl fluoride and 5 mM-EDTA in the sample homogenization buffer, indicating involve-

ment of endogenous proteinases in the anomaly. With freeze-dried samples, however, such an anomaly was never encountered.

In the head of dried flies, a nick in the cuticle at the perimeter of the compound eye facilitated removal of the entire compound eyes. Electrophoretograms (Figs. 2a–2c) show results of analysis in parallel of 16 whole heads, 16 heads from which compound eyes had been removed, and 70 compound eyes respectively. All the spots in Fig. 2(a) can be found in Figs. 2(b) and 2(c), or both. Spot-by-spot comparison revealed 29 spots that were present in Figs. 2(a) and 2(c), but absent from Fig. 2(b). These polypeptides are thus called ‘eye-specific’. The more prominent of these are marked by arrowheads

Fig. 2. Two-dimensional gel-electrophoretic identification of eye-specific proteins of *Drosophila* flies

Heads, compound eyes, retinæ and corneas were dissected from freeze-dried flies as described in the text. Protein staining was with Coomassie Brilliant Blue. (a) 16 whole heads; (b) 16 heads with compound eyes removed; (c) 35 pairs of compound eyes; (d) 70 pairs of retinæ; (e) 60 pairs of corneal layers. Arrowheads point to spots detected in (a) and (c), but not in (b), and thus called eye-specific. In (d) and (e), spots are numbered with R or L, indicating specificity to the retina and lens respectively. Approximate values of pI ('IEF') and M_r are indicated beside panel (a).

in Figs. 2(a) and 2(c). These spots were absent from the electrophoretogram for an eyeless mutant line, so (Hotta, 1979).

The corneal layer of the dried compound eye could be easily separated from the reticular cell layer. Separate analysis of 140 retinæ and 120 corneas yielded results shown in Figs. 2(d) and 2(e) respectively. The pattern of spots in Fig. 2(e) is grossly different from that of others, showing a highly specialized nature of the corneal tissue. The eye-specific spots appeared in Fig. 2(d) or Fig. 2(e), but not in both, so that they could be called retina-specific (Fig. 2d) or cornea-specific (Fig. 2e). The spots were accordingly named as indicated. Clear separation of

retina- and cornea-specific spots demonstrates clean surgical resolution of the two tissues made possible by our freeze-drying procedure.

When a library of monoclonal antibodies (MAbs) was constructed against *Drosophila* head by using immuno-histochemical screening, one of the photoreceptor-specific MAbs, MAb 24B10, was found to bind specifically in immunoblot experiments to the retina-specific spot R1 (Zipursky *et al.*, 1984). This 160 kDa polypeptide has been affinity-purified and partially sequenced (Zipursky *et al.*, 1985). Another, MAb 3F11, histochemically specific to cornea, bound to a family of diffuse spots, L1-L3 (Fujita & Zipursky, 1982), shown

Fig. 3. T.l.c. of phospholipids from *Drosophila* heads and eyes

The samples, in amounts that represented phospholipids in 20 heads, 40 eyes or 20 brains, were spotted to the lower left of each panel. The chromatogram was first developed toward the top and then to the right. Genetic subtractive dissection (*a*, wild-type heads; *b*, heads of eyeless mutant *so*) and surgical dissection of freeze-dried wild-type flies (*c*, compound eyes; *d*, brains) concur to demonstrate that phosphatidic acid (PA) occurs for the most part in the eyes. Other abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

here to be cornea-specific. Thus our drying method provides opportunities for independent tests of histological specificity of antibodies, and for quantitative analyses of the antigens defined by immunohistochemistry.

Drosophila opsin has been shown to form a spot at pI 7.8 and 39 kDa (Nichols & Pak, 1985). As our two-dimensional gels here resolve spots up to pI of approx. 7.5, none of the spots R1–R7 are likely to represent opsin.

Chromatographic analysis of tissue phospholipids

Flies were fed with [^{32}P]phosphate for 1 day. Our experiments showed that under our conditions radioactivity of the major phospholipid species paralleled the phosphorus content, indicating that the radioactivity may be taken as a measure of the amount of the phospholipid. Fresh whole heads of wild-type and eyeless mutant (*so*) flies yielded the thin-layer chromatograms shown in Figs. 3(*a*) and 3(*b*) respectively, after homogenization and chloroform/methanol extraction. The extract of the heads of the wild-type freeze-dried flies gave a pattern (not shown) virtually identical with that in

Fig. 3(*a*), indicating that major phospholipid species are not extracted from the fly tissues by acetone under the conditions of our freeze-drying procedure. Chloroform/methanol-extractable radioactivity recovered from acetone was less than 2% of that found in the dried flies. For the five major phospholipids labelled in Fig. 3, radioactivity recovered from the spots on the chromatogram accounted for similar fractions of the amount loaded in samples taken from both frozen and freeze-dried heads. Previously Ostrowski *et al.* (1962) found no loss of phosphorus and nitrogen from rat liver tissues in acetone at -72°C , in contrast with methanol.

When compound eyes and brains were separately collected from the dried flies and similarly analysed for phospholipid, Figs. 3(*c*) (eyes) and 3(*d*) (brains) were obtained. The spot patterns were similar to each other, and both also resembled that of fresh heads (Fig. 3*a*). The content of phosphatidic acid was, however, different; most of the acid in the head occurred in the eyes.

This finding was corroborated by an analysis of the fresh heads from *so* flies carrying a mutation that nearly eliminates compound eyes in the adult fly (Lindsley & Grell, 1968). Only a trace amount of phosphatidic acid

Table 1. Enzyme activities in head and tissues of the dried *Drosophila* flies

Samples of homogenates of whole heads, eyes or brains were assayed for enzyme activities as described in the Experimental section. Values represent amounts of enzyme in a single head, a pair of compound eyes, or a single brain, and are means $\pm$ S.D.

	Phospholipase C (nmol/5 min per head)	Diacylglycerol kinase (nmol/5 min per head)	Cyclic AMP phosphodiesterase (nmol/20 min per head)
Enzyme activity in			
(a) Frozen head	1.59 $\pm$ 0.21 (n = 8)	0.303 $\pm$ 0.077 (n = 8)	0.551 $\pm$ 0.069 (n = 6)
(b) Dried head	0.49 $\pm$ 0.12 (n = 4)	0.087 $\pm$ 0.024 (n = 4)	0.263 $\pm$ 0.047 (n = 6)
(c) Dried eye	0.35 $\pm$ 0.11 (n = 4)	0.043 $\pm$ 0.007 (n = 4)	0.060 $\pm$ 0.014 (n = 4)
(d) Dried brain	0.04 $\pm$ 0.01 (n = 4)	0.003 $\pm$ 0.001 (n = 3)	0.127 $\pm$ 0.015 (n = 4)
Recovery in dried head			
(e) $\left(\frac{b}{a} \times 100\right)$	31%	29%	47%

was detected in the heads of these flies, which had an otherwise similar composition (compare Fig. 3*b* with 3*a*).

These parallel results obtained between surgical and genetic dissections show that the phospholipid difference detected between eye and brain (Figs. 3*c* and 3*d*) is not an artefact of the freeze-drying procedure. Conversely they demonstrate that a great decrease in phosphatidic acid in *so* heads is a consequence of the absence of the eye tissue and is not due to some alteration in phospholipid metabolism in the remaining tissues caused by the mutation.

Enzyme activities in the dried tissues

Enzyme activities in the whole head were studied for phospholipase C, diacylglycerol kinase, and cyclic AMP phosphodiesterase, and compared between frozen flies and those dried by our procedure. Fly heads or collected dry tissues were homogenized, and samples were assayed for enzyme activities as described in the Experimental section to yield the values in Table 1. Recoveries of enzyme activity in the dried head (row *b*) relative to that in the frozen head (row *a*) was 31% for phospholipase C, 29% for the kinase, and 47% for phosphodiesterase (row *e*). Thus an appreciable fraction of the activities survived our drying procedure. This point is considered in the Discussion section.

Enzyme activities could be assayed also in eye and brain tissues collected from dried flies (Table 1, rows *c* and *d*). The activities found in the eyes represented 71% of that of the whole dried head for phospholipase C, and 49% for diacylglycerol kinase, whereas only very small fractions were detected in the brain for the two enzymes. In contrast, 23% of phosphodiesterase activity of the whole head was accounted for by that in the eyes, and 48% by that in the brain.

The fraction of activities not accounted for by those in the two major neural tissues of the head may be ascribed to other lesser tissues, such as chemosensory organs and fat-bodies. Only low amounts of the three enzyme activities were detected in the dried proboscis, which contains most of the head musculature. Loss of small fragments of dried tissues that had crumbled off during

dissection and collection may have contributed to the discrepancy.

The fraction of enzymes contained in the eyes was independently assessed by analysing frozen heads of flies of the *so* strain, in which eyes had been genetically eliminated, and comparing them with the frozen wild-type heads. The eyeless mutant head had 6% phospholipase C, 1% diacylglycerol kinase and 63% phosphodiesterase activities of the wild-type head, in basic agreement with the results of Table 1 in that the brain contained little phospholipase C and kinase, whereas up to twice as much phosphodiesterase was contained in the brain (and other non-retinal tissues) than in the eyes.

Thus our findings demonstrate that our drying procedure can provide a basis for a direct measure, albeit coarse, of tissue distribution of certain enzymes in the small insect.

DISCUSSION

Freeze-drying is in fact an old art in biological research. Biochemists have long employed the procedure to concentrate and preserve the preparations (Everse & Stolzenbach, 1971). Some have taken advantage of the technique to dissect minute neural tissues for microchemical analyses (Lowry, 1953). Histochemists have used it to avoid covalent fixatives in fixing tissues for enzyme histochemistry, and developed a variation called freeze-substitution, whereby the frozen tissue is dehydrated not *in vacuo* but in organic solvents at low temperatures (Pearse, 1968; Burstone, 1962). We have now shown that freeze-substitution in acetone causes weakening of tissue boundaries in small insects, enabling clean and quantitative collection of tissue samples for biochemical analyses. Our freeze-drying method has three strong advantages. (i) Various tissues of the fly can be separated cleanly, and recovered in entirety and free of neighbouring tissues. (ii) Proteins and phospholipids, and presumably other acetone-insoluble molecules, can be recovered quantitatively for analysis without covalent alterations caused by degradative enzymes such as

proteinases. (iii) The collected specimen can be stored desiccated, and accumulated to a quantity required for analysis.

It has been further found that a measurable fraction of enzyme activity survived the drying procedure for the three enzymes tested. This fact is not surprising, in view of the fact that the earliest methods of enzyme purification involved extraction from acetone-dried powder and precipitation by acetone (Morton, 1955; Green & Hughes, 1955). In general, one should expect that recovery of activity will vary greatly, depending on the stability of the particular enzyme considered. In addition, certain parameters of the drying process, such as the rate of freezing and reagent purity, can influence the results. It must be further noted that, as the above values were determined in tissue homogenates, they may reflect an altered state of endogenous inhibitors, activators or proteinases that affect the intrinsic activity of the enzymes rather than recovery of the enzyme molecules themselves. In any event it should be possible, where required, to optimize relevant variables to achieve increased or even quantitative recovery of the enzymes in the dried tissues.

During the course of the development of our drying procedure, freezing in liquid N₂ (−196 °C) was tested among others in an attempt to achieve rapid freezing. Such experiments invariably yielded deformed flies, presumably owing to an insulating layer of gas formed when the object came in contact with liquid N₂. Acetone in our procedure thus serves not only as a dehydrating agent but as an efficient heat conductor.

We described two procedures, A and B. The data shown above were obtained with procedure A. Procedure B is an improvement, and gives the same results; it is simpler and safer in operation and more reliable in results. It is free from possible contaminants in the preparation using solid CO₂. The former requires solid CO₂, and the latter liquid N₂. The two procedures have a drawback of possible fire hazard associated with inappropriate handling of acetone in a freezer. It should be possible to overcome this by constructing a thermally insulated box with materials resistant to acetone, equipped with a heat sink such as solid CO₂, such that a chamber can be maintained below −20 °C for several days. We have not studied the minimum time in the freezer required for dehydration. It should depend on the actual temperature and the nature of the tissue. Our standard condition of 1 week at −25 °C to −20 °C has been found sufficient for most adult tissues, except for mature eggs in female abdomen. Longer periods of time were allowed for larvae and pupae. Prior dechoriation may be necessary for early fly embryos.

The usefulness of our freeze-drying method was illustrated above by identification of retina-specific proteins, localization of most of the phosphatidic acid in eyes, and demonstration of certain enzyme activities in the dried tissues of the *Drosophila* head. The method has also been used in our demonstration of 12 polypeptides that are specific to fibrillar-type muscles and absent from tubular-type muscles in the fly (Mogami *et al.*, 1982). Matsumoto *et al.* (1982) took advantage of the method in showing light-induced modification of retinal polypeptides. Brains and retinae taken from the dried flies were used as immunogens to develop a library of monoclonal antibodies against *Drosophila* nervous system (Fujita *et al.*, 1982). Since it is likely that the polynucleotides

remain intact in the dried tissues, the method should also prove useful in obtaining mRNAs specific to particular tissues of *Drosophila*.

Applicability of the method was tested in animals of several other species. Larger-sized flies (*Sarcophaga peregrina*), weighing 300–400 mg, were successfully freeze-dried with a dehydration period of 1 month. Small fish (about 1 cm) were dried, and their retinae could be taken with ease. But the muscles could not be removed cleanly. With chick embryos, shrinkage occurred upon evaporation of acetone, and tissues could not be easily detached. Thus certain modifications of the method need to be devised for general application to the vertebrates.

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