MASS PREPARATION OF NUCLEI FROM THE LARVAL SALIVARY GLANDS OF *DROSOPHILA HYDEI*

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ABSTRACT

A method has been developed for isolating gram quantities of salivary glands from late third instar larvae of *Drosophila hydei*. The isolated glands have a normal appearance and incorporate RNA and DNA precursors normally. Nuclei can be isolated from these glands in 90% yield with the use of detergents. These nuclei contain morphologically normal giant polytene chromosomes.

INTRODUCTION

The giant chromosomes of the dipteran insects provide a unique system for studying regulation of gene activity, because they are large enough to permit detailed studies of experimentally and naturally induced gene activity at specific chromosomal loci (1). Owing to the very limited amounts of material available, however, it was not possible to study these systems with conventional biochemical techniques until Ristow and Arends (2) recently isolated large quantities of nuclei from *Chironomous* salivary glands.

In order to pursue studies of gene activation, which have been carried out on *Drosophila hydei* in this laboratory,[1] we have developed a technique for isolating mass quantities of nuclei from the salivary glands of this organism. The method draws heavily from the work of Fristrom and Mitchell (3), who first mechanized the preparation of *Drosophila* organs, and from the nuclear isolation technique of Ristow and Arends. One of us has recently described a method for the semi-mass isolation of salivary glands from prepupae of *Drosophila hydei*[2]

[1] H. D. Berendes. Submitted for publication.
[2] J. B. Boyd, Submitted for publication.

MATERIALS AND METHODS

Animal Culture

A wild-type stock of *Drosophila hydei*, which has previously been described by Berendes (4), was cultured in large quantities with the method used by Mitchell and Mitchell (5) for culturing *Drosophila melanogaster*. With *Drosophila hydei*, it was necessary to age the fly culture for 1–2 wk before the eggs were collected. A cake-drying rack was present in the cage for reduction of fly losses. This system produced up to 200 g of synchronized larvae per day. The density of the cultures was reduced when necessary to about 4,000 larvae per 230 sq. cm of medium for insurance of reasonable synchrony. We isolated late third instar larvae weighing about 4.5 mg each by floating the entire culture in saline at a concentration of 80 g of NaCl per liter. All loose food was suspended in the saline by hand, and the suspension was immediately transferred to a 1-liter beaker. The floating larvae were collected in a 1.1-mm mesh vegetable strainer and completely cleaned under a strong stream of water. The average weight of the larvae was determined after they had been dried on filter paper, and the required number of larvae was measured out by weight.

Salivary Gland Isolation

Up to 16 g of damp larvae were placed in the middle of a clean glass plate measuring 33 × 70 cm. We placed two 33-cm-long glass rods 6 mm in diameter on the plate parallel to and about 6 cm from the sides in order to restrict the larvae to the center. 1½ ml of Ringer's solution (5.55 g NaCl, 0.22 g KCl, 0.44 g $CaCl_2$ per liter) were used to wet each 8 g of larvae, and the larvae were distributed in a monolayer over ⅔ of the plate with small brushes. In a dimly lit room, a 150-watt floodlight was directed the length of the plate at a distance of about 90 cm from one end. With the additional aid of small paint brushes, it was possible to orient over 90% of the larvae, with their heads away from the light, within 2–3 min.

The glass rods were removed and a 33-cm-long stainless steel rod was placed on the end of the plate nearest the light. Each end of the rod had a raised lip which kept most of the rod separated from the plate by an interval of 0.12 mm. The diameter of the rod was 2.5 cm, and the lips extended 2.0 cm inwards from the ends. A similar spacing can be obtained by a careful wrapping of the ends of a rod with two windings of cellophane tape. The rod was then firmly, but slowly rolled the length of the plate, squashing the larvae from behind. Since some larvae stuck to the rod, it was wiped clean with a wide paint brush as it rolled.

The glass plate was immediately held vertically, and all tissue was washed into a plastic dish with a strong stream of Ringer's at 0°C. All subsequent operations were carried out at 0–4°C. The suspension was strained through a 1.1-mm mesh screen into a 600-ml beaker. After resuspension in cold Ringer's, the solid material was restrained and washed with a strong stream of Ringer's. Up to this point, the procedure required 8 min. After 2 or 3 min, the fat body was decanted from the glands that had settled to the bottom. The remaining 100 ml were strained through a 0.85-mm mesh screen into a 150-ml beaker, and the residue was washed carefully. Three further cycles of decanting and resuspension yielded a preparation that consisted of about ½ salivary glands.

Up to three such preparations were then submitted to centrifugation in discontinuous Ficoll gradients. Tubes fitting the SW 25, 2 rotor of the Spinco L2 ultracentrifuge were filled with 15 ml each of 32%, 18%, and 0% (w/v) Ficoll solution dissolved in Ringer's. The Ringer's was buffered with 0.01 M Tris at a pH of 7.2 (20°C). The Ficoll had been dialyzed exhaustively against water. The gland suspension was layered into the buffered Ringer's layer in about 5 ml and centrifuged at 0°C to 20,000 rpm and back with the brake on. Material recovered from the top of the 32% Ficoll layer was suspended and decanted five to six times in 100 ml of Ringer's. This preparation, which consisted of over 95% salivary glands, was placed in an ice-cooled petri dish, and the remaining contamination was removed with a Pasteur pipette under a dissecting microscope. Gland recovery was determined by weight after the glands had been packed in a pre-weighed tube at 3,000 rpm for 1 min in a table centrifuge. Most glassware used for containment and handling of the glands had been siliconized.

Nuclear Isolation

METHOD I: This was modified from Prescott et al. (6). 5–10 × 10^3 glands were suspended in 5 ml of the following buffer containing 0.001% spermidine phosphate: 0.11 M NaCl, 0.0019 M KCl, 0.01 M Tris-(hydroxymethyl)aminomethane, 0.001 M $MgCl_2$, pH 7.2 (20°C). After 0.2 ml of 5% Triton X-100 had been added, the suspension was pipetted hard with a Pasteur pipette until more than 95% of the tissue had been completely disrupted. The suspension was then diluted to 25 ml in the above buffer without the spermidine and filtered through a nylon screen having a mesh of 53 μ (Züricher Beuteltuchfabrik AG, Zürich). An additional 5 ml of the buffer was used for washing the screen, and the filtrate was centrifuged at about 500 rpm for 3–5 min. The resulting residue was suspended gently in the same buffer and recentrifuged. All operations were carried out at 0–4°C in siliconized glassware. Unless otherwise stated, all data were obtained with nuclei prepared according to this method.

METHOD II: This was modified from Ristow and Arends (2). Glands were collected by hand in Ringer's buffered at pH 7.3 (1.1 mM $CaCl_2$, 0.11 M NaCl, 1.9 mM KCl, 2.3 mM $NaHCO_3$, 0.08 mM NaH_2PO_4). To 1,000 glands in 1 ml of this Ringer's were added 0.1 ml of 10% Tween 80 and 0.04 ml of 10% sodium desoxycholate. The suspension was shaken gently for 5 min at room temperature before the tissue was disrupted and treated as described above. In this case, the nuclei were centrifuged for 3 min at 1,000 rpm in a clinical centrifuge.

Cytology

SQUASH PREPARATION OF NUCLEI: 1 drop of nuclear suspension was placed on a gelatinized slide. The nuclei were allowed to settle and stick to the glass before the excess fluid was drained off with filter paper. 3% orcein BDH in 70% acetic acid was used for staining the preparation for 1 min. After the stain had been drained, a drop of 45% acetic acid was added, and the preparation was gently squashed under a coverslip.

RADIOAUTOGRAPHY Glands isolated in Ficoll were incubated for 15 min at room temperature in 20 ml of Ringer's containing 1 μc/μl tritiated thymidine or uridine (Thymidine-6-T(n), sp act 16,500 mc/mmole; uridine-5-T, sp act, 16,600 mc/mmole. The Radiochemical Centre, Amersham, England). Sub-

sequent staining, squashing, and radioautography were performed as described by Berendes (7) with an 8-day exposure time.

Chemical Analysis

After centrifugation in Ficoll solution 100 whole glands were counted out and homogenized in 0.5 ml of 10% trichloroacetic acid. Samples of this suspension were taken for the independent determination of protein, RNA, and DNA. Protein was determined by the procedure of Lowry et al. (8) after the suspension was pelleted and the residue was dissolved in N NaOH. Vacuum-dried bovine serum albumin was used as the standard. RNA was determined by the modification of the Schmidt and Thannhauser procedure used by Huberman and Attardi (9). The concentration of the standard RNA solution was determined spectrophotometrically. For DNA measurements, the suspension was pelleted, and the residue was washed with ethanol-ether (3:1). This residue was well suspended in 0.5 M $HClO_4$ and heated at 96°C for 45 min according to the procedure of Wannemacher et al. (10). Incubation at 70°C for 15 min in perchloric acid hydrolyzes only 75% of the DNA that is hydrolyzed by incubation at 90°C for 15 min in 5% trichloroacetic acid or by the incubation conditions recommended by Wannemacher et al. DNA was determined by the diphenylamine procedure of Burton (11) with D-deoxyribose as a standard.

Nuclei recovered from about 150 mg of glands were uniformly suspended in 20% trichloroacetic acid and analyzed for protein, RNA, and DNA. The sample taken for protein analysis was centrifuged and washed with ether before being analyzed by the procedure of Huberman and Attardi (9). DNA and RNA were determined as described for the gland analyses.

RESULTS AND DISCUSSION

Glands—Purity and Composition

The general appearance of a gland preparation is indicated in Fig. 1. Although a number of the glands are fragmented by the rolling procedure, the bulk of the tissue retains the normal morphological appearance. A small percentage of glands, which cannot be distinguished in this photograph, have an opaque appearance which probably arises during the rolling step. The amount of fat body adhering to the glands after centrifugation is always much less than 5% of the total tissue. A small amount of intestinal contamination, which is recovered with the glands from the Ficoll gradient, has been removed with a capillary pipette.

Salivary glands also have been purified successfully on gradients containing 75, 65, and 20% sucrose buffered at pH 7.0 with 0.02 M Tris. These glands were satisfactory for some biochemical purposes, and morphologically normal

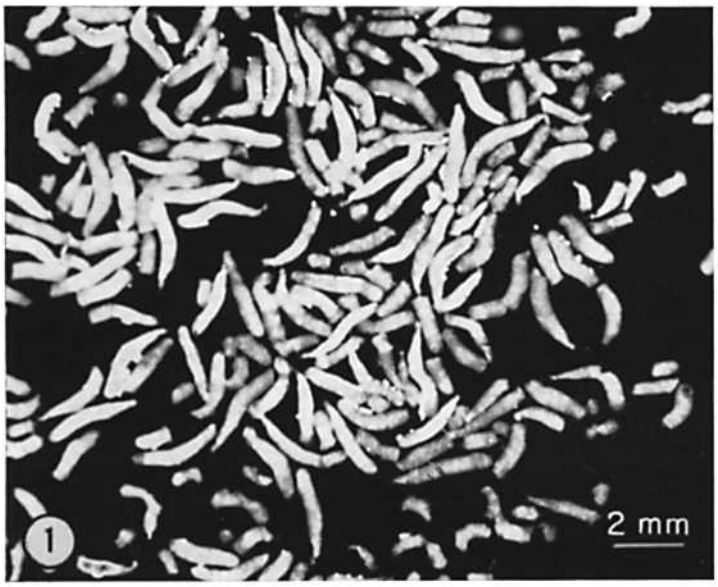

FIGURE 1 Mass-isolated salivary glands. Polaroid photograph. Glands were isolated from late third instar larvae weighing an average of 4.6 mg each. The smaller pieces are fragments of salivary glands. The white material seen attached to some glands is fat body. × 5.

nuclei could be obtained from them. The morphology of the glands themselves, however, was far inferior to that of glands obtained with Ficoll.

The chemical composition of the isolated glands is given in Table I. The fact that the glands vary considerably in size is reflected in the variation in the DNA composition. The more consistent composition ratios are characteristic of a normal differentiated tissue.

Gland—Recovery

Two methods which have been used for monitoring the recovery of glands from the larvae are compared in Table II. Although protein measurement is undoubtedly the most accurate, wet weight measurements are the most convenient and have been used routinely. These and other data have shown that with this method it is possible to obtain 30–45% of the glands from 3,000 larvae in less than an hour. For every centrifugation cycle about 10,000 larvae can be handled in less than 2 hr, and 2.4 g of glands can be recovered per day.

The number of glands actually freed from the mouth parts of the larvae depends, in part, on the spacing between the glass plate and the roller. A spacing of 0.065 mm releases up to 75% of the glands, as tested by counting the glands freed from 50 perfectly oriented larvae. Such glands, however, are badly damaged and contaminated with other tissues. By the same criterion, a spacing of 0.12 mm frees 40–55% of the glands in good condition. Another factor which strongly determines the number of glands released during rolling is the orientation of the larvae with respect to the roller. Any larvae not facing the direction of rolling do not yield any glands. Although it is more difficult to control this variable, 85–95% of the larvae can generally be induced by the light to orient in the proper direction.

TABLE I

Chemical Composition of Isolated Salivary Glands from Late Third Instar Larvae

Sample No.	DNA/salivary gland (mμg)	mg RNA / mg DNA	mg Protein / mg DNA
1	48	16	100
2	48	16	108
3	43	17	109

Analyses were performed as described under Materials and Methods. Three batches of glands obtained from larvae weighing 5.3 mg each were purified in Ficoll and analyzed independently. The DNA values are the averages of duplicate determinations. All other values are the averages of quadruplicate determination.

TABLE II

Recovery of Glands from Late Third Instar Larvae

Sample No.	Wet-weight of recovered glands	Recovery (by weight)	Soluble protein recovered	Recovery (protein measurements)
	g	%	*mg*	%
1	0.111	35	2.94	41
2	0.105	33	3.06	43

Salivary glands were prepared from two groups of 1760 larvae weighing an average of 4.5 mg each. The average wet weight of a single gland was 0.09 ± 0.01 mg as determined by weighing groups of 100 glands. Similarly, the soluble protein per single gland was found to be 2.0 μg by analyses of groups of 100 glands. The averages of duplicate determinations are presented for the protein measurements.

The conditions of centrifugation must also be carefully controlled for the achievement of a maximum yield of glands with minimum contamination. The fractionation is so sensitive to the Ficoll concentration and the temperature of centrifugation that the proper conditions must be redetermined for each centrifuge. Centrifugation conditions have also been found which yield virtually pure glands at the expense of losing one-half of the glands applied to the gradient. Under these conditions, the glands which are completely free of fat are separated from those with some contamination.

Glands—Functional State

The functional state of the isolated glands has been investigated by determining the patterns of incorporation of RNA and DNA precursors. The radioautographs presented in Fig. 2 demonstrate that the capacity of the glands to incorporate these precursors has remained intact during the isolation procedure. In these experiments, the Ficoll-isolated glands were incubated 2 hr after the larvae had been sacrificed, and yet the incorporation patterns are identical with those obtained from glands that were incubated immediately after hand dissection. In the case of uridine incorporation, the normal pattern is one of high specific incorporation into the nucleolus

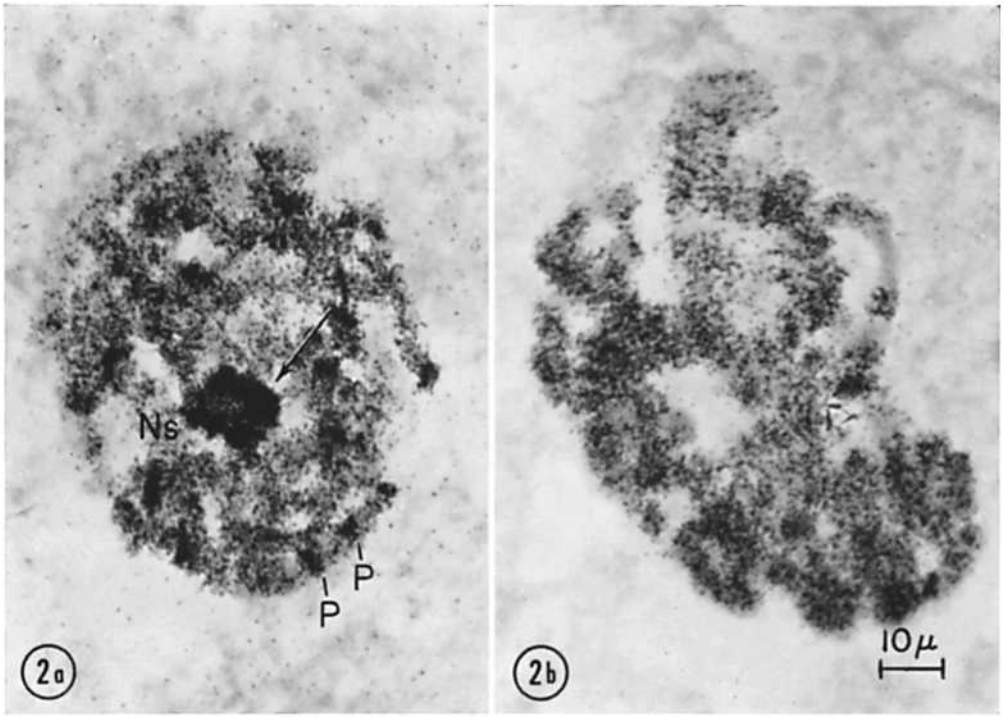

FIGURE 2 Labeling patterns of mass-isolated salivary glands. *a*, Tritiated-uridine incorporation into the nucleus. Specific labeling is found in the chromosome puffs (*P*) and in the nucleolus (*Ns*). *b*, Tritiated-thymidine incorporation into the nucleus. Discontinuous labeling can be seen in the spread chromosome on the right. × 800.

and the puffed regions of the chromosomes. Over 90% of the nuclei in the isolated glands showed the normal intensity and distribution of label. The failure of about 10% of the nuclei to incorporate uridine is also a normal phenomenon.

Glands from late third instar larvae were used for the thymidine incorporation studies. As Plaut and Fanning (12) have shown for *Drosophila melanogaster*, glands taken from this stage of development normally display a pattern of discontinuous labeling of the chromosomes. The percentage of labeled nuclei in the isolated glands was similar to that of labeled nuclei in glands of larvae injected with the precursor 15 min before preparation for isolation, i.e. about 20–30% (7).

As another test of gland integrity, the deoxyribonuclease activities of hand-dissected and mass-prepared glands are compared in Fig. 3. The single enzyme activity is retained in the mass-prepared glands at roughly the same specific activity that is found in glands immediately after hand dissection. Thus, at least one of the enzymes of the glands is retained during the isolation procedure.

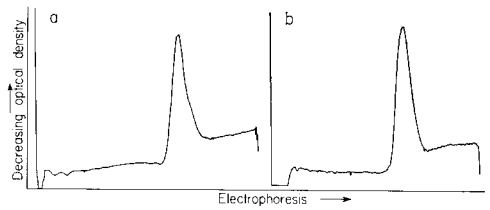

FIG. 3 Deoxyribonuclease activity in hand- and mass-prepared salivary glands. Enzyme activity was detected in acrylamide gel after disc electrophoresis according to Boyd and Mitchell (13). Gels containing native DNA were incubated for 2 hr at ph 4 in the presence of EDTA. *a*, Hand-Prepared glands; *b*, Ficoll-prepared glands.

Nuclei—Morphology and Composition

The chromosomes of nuclei obtained from glands isolated with both detergent methods appear normal when observed under phase con-

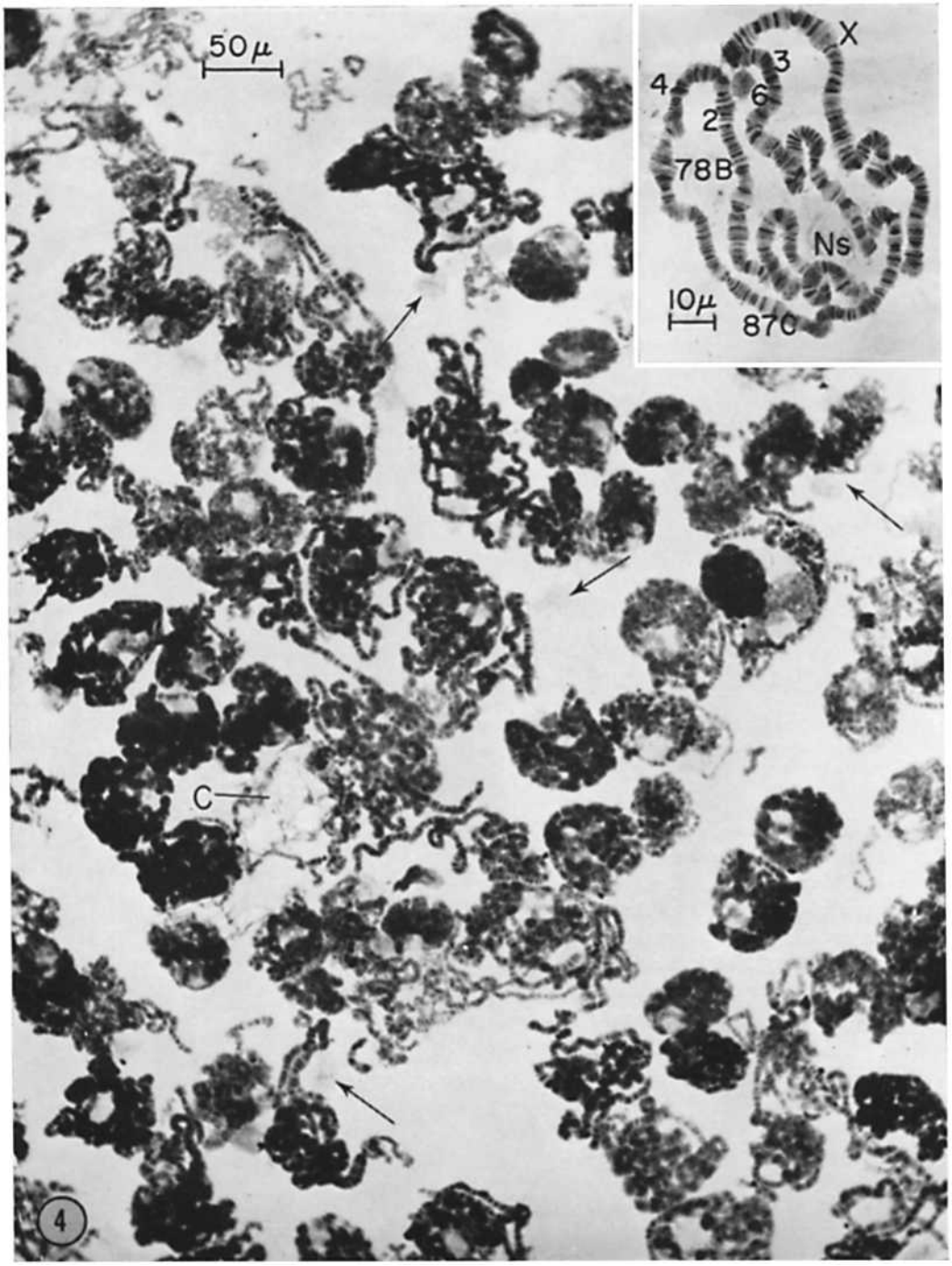

FIGURE 4 Isolated nuclei, gently squashed after orcein staining. Cytoplasmic contamination is indicated by *C*. Nucleoli, which are easily separated from the chromosomes by squashing, are designated by arrows. Insert, salivary gland chromosomes of an isolated nucleus obtained from late third instar larvae. The locations of the nucleolus (*Ns*) and some of the prepupal chromosome puffs are indicated. Chromosome numbers are given according to Berendes (14). × 220. Insert, × 600.

trast. The micrograph in Fig. 4 further demonstrates that the chromosomes of stained squash preparations of isolated nuclei also look perfectly normal. In some preparations, the chromosomes appear to be stiffer and more brittle than those in normal squash preparations of whole salivary glands. Squash preparations of isolated nuclei, which were subsequently stained by the Feulgen reaction and fast green (FCF, 0.1%, pH 2.4), revealed that the isolated nuclei also contain the acidic protein that is normally located on the chromosome puffs and on nucleoli.[1]

By visual estimate, the nuclear preparations never contain more than 10% cytoplasmic contamination. Such contamination appears to be derived almost entirely from the outer membrane of the gland. If the nuclei are prepared by more gentle pipetting, contamination is considerably reduced at the expense of nuclear yield. It is highly probable that all cytoplasmic contamination could be removed by centrifugation of the nuclei through a dense medium.

TABLE III

Chemical Composition of Isolated Nuclei

Sample No.	mg RNA / mg DNA	mg Acid-soluble protein / mg DNA	mg Nonacid–soluble protein / mg DNA
1	0.37	1.0	1.7
2	0.31	1.3	1.4

The DNA values are the averages of duplicate determinations. All other measurements were made in quadruplicate. The sample No. refers to independently prepared samples of nuclei.

The data in Table III show that nuclear isolation results in a roughly 40–50-fold decrease in the ratio of protein to DNA. The nuclei themselves contain four to five times as much protein as RNA. Earlier experiments indicated that chemical differences observed among samples of nuclei are attributable primarily to inaccuracy in the micro methods of analysis. The protein values are lower than those obtained for HeLa cell nuclei by the same analytical methods (9). This difference is probably real, however, because the method used for obtaining nuclei in the present study (6) has been shown to permit the retention, in nuclei isolated from Amoeba, of greater than 80% of the original nuclear protein (15). The relatively low amount of nonacid–soluble protein in the giant nuclei is partially attributable to the fact that detergent removes the outer nuclear membrane (16), which was not removed in the case of the HeLa cell nuclei. In addition, the larger nuclei require less membrane area to contain a given amount of DNA.

Nuclei—Recovery

Because the nuclei tend to be sticky when isolated in the absence of sucrose solutions, several complementary methods were used to establish the efficiency with which nuclei are recovered from glands. The results obtained with three methods (Table IV) all indicate a recovery of 90%. Additional experiments have further shown that very forceful pipetting does not reduce this yield. Thus, in spite of their large size, the nuclei are not sensitive to the forces encountered during pipetting. As mentioned before, nuclear contamination can be reduced by more gentle pipetting which reduces the yield to as low as 60%.

TABLE IV

Recovery of Nuclei from Glands

Nuclei count

	Calculated maximum	Capillary count	Hemocytometer count
Nuclei/ml	9.5×10^4	8.5×10^4	8.2×10^4
Yield		90%	86%

DNA measurement

DNA in 1 mg of glands	DNA in nuclei derived from 1 mg of glands	Yield
μg	μg	%
0.30	0.27	90

For the counting experiments, nuclei were prepared from a known weight of glands in a known volume and counted directly in the detergent. Nuclei were counted in 1-μl capillaries (Drummond, Broomall, Pa.) under a dissecting microscope. The maximum yield was calculated from the known number of glands/mg (Table II) and from the number of cells/gland (4). DNA determinations were performed as described in Materials and Methods.

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REFERENCES

1. BEERMANN, W. 1966. *In* Cell Differentiation and Morphogenesis. North-Holland Publishing Co., Amsterdam. 24.
2. RISTOW, H., and S. ARENDS. 1968. *Biochim. Biophys. Acta.* **157**:178.
3. FRISTROM, J. W., and H. K. MITCHELL. 1965. *J. Cell Biol.* **27**:445.
4. BERENDES, H. D. 1965. *Chromosoma.* **17**:35.
5. MITCHELL, H. K., and A. MITCHELL. 1964. *Drosophila Inform. Serv.* **39**:125.
6. PRESCOTT, D. M., M. V. N. RAO, D. P. EVENSON, G. E. STONE, and J. D. THRASHER. 1966. *In* Methods in Cell Physiology. Academic Press Inc., New York. **2**:131.
7. BERENDES, H. D. 1966. *Chromosoma.* **20**:32.
8. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
9. HUBERMAN, J. A., and G. ATTARDI. 1966. *J. Cell Biol.* **31**:95.
10. WANNEMACHER, R. W., JR., W. L. BANKS, JR., and W. H. WUNNER. 1965. *Anal. Biochem.* **11**: 320.
11. BURTON, K. 1956. *Biochem. J.* **62**:315.
12. PLAUT, W., and F. FANNING. 1966. *J. Mol. Biol.* **16**:85.
13. BOYD, J. B., and H. K. MITCHELL. 1965. *Anal. Biochem.* **13**:28.
14. BERENDES, H. D. 1963. *Chromosoma.* **14**:195.
15. GOLDSTEIN, L., and D. M. PRESCOTT. 1967. *J. Cell Biol.* **33**:637.
16. HOLTZMAN, E., I. SMITH, and S. PENMAN. 1966. *J. Mol. Biol.* **17**:131.