

Chapter 11

Methods with Insect Cells in Suspension Culture

I. Aedes albopictus

ALLAN SPRADLING, ROBERT H. SINGER,¹
JUDITH LENGYEL, AND SHELDON PENMAN

*Department of Biology,
Massachusetts Institute of Technology,
Cambridge, Massachusetts*

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I. Introduction

Techniques for growing cells and tissues of insects in culture have improved dramatically in the last 10 years. There now exist established lines derived from numerous species, particularly among the Diptera and Lepidoptera, and the number is rapidly increasing as methods favorable to establishment are refined (reviewed by Brooks and Kurtti, 1971). Eventually, it may be possible to obtain cell lines with known genetic alterations by the choice of an appropriate mutant strain as starting material.

The conditions under which these lines must be grown, however, are not well suited to investigations of insect cell biology at the molecular level. It is not always possible, for example, to grow the cells in conventional suspension culture and thereby obtain the quantities of macromolecules neces-

¹ *Present address:* Department of Anatomy, University of Massachusetts Medical School, Worcester, Massachusetts.

sary for many types of analysis. Even when this can be done, the media commonly used for propagating the cells are expensive and extremely complex, often containing high concentrations of precursor molecules which render radioactive labeling inefficient. In addition, techniques for cellular fractionation analogous to those developed for cultured mammalian cells cannot in general be used for insect lines.

Because it was clear that the new insect cell lines would provide interesting systems for basic studies of cell physiology, we undertook several years ago the adaptation of one such line to growth conditions more suitable to this purpose. Over a period of about a year, cells of the *Aedes albopictus* line established by Singh (1967) were adapted to growth in suspension culture in Eagle's medium supplemented only with nonessential amino acids and serum. Exponentially growing cells of this subline may consequently be labeled with radioactive precursor molecules as readily as cultured mammalian cells. The development of fractionation procedures for the mosquito cells proved to be a considerably more difficult problem, especially for RNA, and in some cases, such as the subfraction of nuclei, the problem is still not satisfactorily resolved.

We describe here the methods utilized for adapting *Aedes* cells and for their fractionation, as well as some of the basic cellular parameters as measured by these techniques. More recently, a similar approach has made possible the adaptation of the Schneider *Drosophila* line no. 2 (Schneider, 1972) to similar conditions of growth, as described in Chapter 12.

II. Adaptation of *A. albopictus* Cells to Desired Growth Conditions

The optimum composition of the medium used to propagate a particular line of insect cells has remained a matter of some controversy on both theoretical and practical grounds (Schneider, 1971; Stanley, 1972). Commonly, it has been suggested that parameters of the medium should closely parallel values found in the hemolymph of the species for which it is used. However, the well-known variability of such hemolymph parameters as ion balance (Florkin and Jeuniaux, 1964), in addition to questions of its physiological significance for the unknown cell types in culture, at present leave this question unresolved. Empirically, many insect lines have been found to grow well in a variety of media, some of which differ considerably from hemolymph (Brooks and Kurtti, 1971; Schneider, 1972). In undertaking this project we attempted to utilize, within limits imposed by a constant karyotype and undiminished growth rate, this apparent plasticity of many insect cells.

The *A. albopictus* line was established using the medium of Mitsuhashi and Maramorosch (1964), originally designed for the culture of leafhopper cells. It contains large quantities of yeast extract and lactalbumin hydrolysate, and is supplemented with fetal bovine serum (FBS) for use with mosquito cells. Since conditions of growth were desired that would permit optimum labeling with nucleotides, a minimum change was the elimination of the requirement for yeast extract. Eagle's minimal essential medium (MEM) (Joklik-modified) was used as a basic medium, since the salts and osmotic pressure are similar to M. + M. media and its additional vitamins might help eliminate the yeast extract requirement. Amino acid concentrations were increased by supplementing with 1% nonessential amino acids and 1% lactalbumin hydrolysate. When *Aedes* cells were seeded into 250-ml Falcon plastic tissue culture flasks and fed with the supplemented Eagle's medium plus 10% FBS, growth continued after a brief delay. The cells were allowed to grow under these conditions for several months. Half the medium was replaced every 3 days. New cultures were seeded by briskly shaking a flask to release loosely attached cells into the medium, which was then added to a new flask.

After it was clear that a subline capable of growth in the new media had been obtained, adaptation to suspension culture was undertaken. Because conditions in a suspension culture are physically rigorous, it did not seem advisable to begin this step until a well-adapted line had been obtained in monolayer culture. Indeed, several additional months of passaging between flask and suspension cultures followed before a subline was obtained that grew readily in suspension.

At this point it was determined that supplementation with lactalbumin hydrolysate was no longer necessary. It thus became possible to label cell protein with radioactive amino acids (e.g., leucine), either directly or by preparing the corresponding amino acid-deficient medium. Elimination of the nonessential amino acid supplement caused the cells eventually to stop growing, which suggests that at least one of the seven amino acids not required for growth by mammalian cells is a required amino acid for mosquito cells. Elimination of the serum also resulted in a slow decline in the growth rate of the culture. The nutritional requirements of the cells were not examined further.

III. Characteristics of the Cell Line

The *A. albopictus* subline obtained as a result of these procedures seems to have undergone few changes in its measurable properties. Morphologically, the cells from suspension culture are of two basic types. Spherical cells

about $10\ \mu\text{m}$ in diameter predominate over a similar class of cells which contain one or more spinelike projections, commonly referred to as spindle-shaped cells. Each contains a single large nucleus, usually with one prominent nucleolus. The cells display a strong tendency to attach to glass or plastic surfaces and to each other. Thus, in suspension cultures, clumps form, some of which contain many hundreds of cells.

If suspension-grown cells are seeded at a relatively low density into a plastic tissue culture flask, virtually all the cells rapidly attach to the surface. As the culture grows, dense, often multilayered arrays of cells are produced. In addition, a much wider variety of cell morphology is present under these conditions. Cytoplasmic bridges connecting widely spaced cells, multinucleate syncytia, and other complex forms are often common in such a culture.

The growth rate of the cells at 25°C is exponential, with a doubling time of approximately 21 hours. However, as might be expected for cells from a poikilothermic organism, exponential growth is observed over a considerable range of temperature. *Aedes* cells maintained at 32°C , the highest temperature investigated, multiply more rapidly than at the lower temperature, doubling about every 15 hours. Suspension cultures have also been maintained at 20°C with good growth.

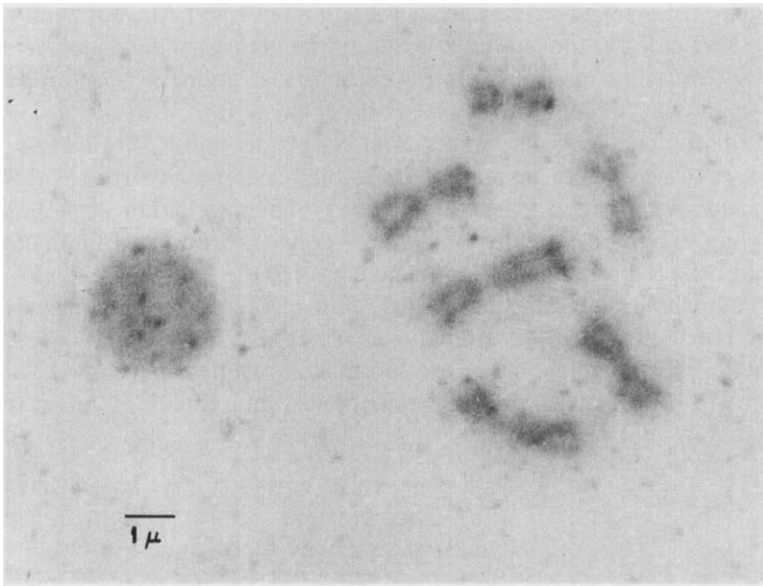


FIG. 1. Metaphase from adapted cells. *Aedes* cells (10^7) from an exponentially growing suspension culture were arrested overnight with $1\ \mu\text{g/ml}$ vinblastine sulfate. The cells were hypotonically treated and then fixed in 3:1 ethanol-acetic acid. Slides were prepared by the air-drying technique and stained with Giemsa. $\times 6400$.

Karyotypes as determined from metaphase-arrested cells passaged about 1 year in suspension culture indicate that the population consists of approximately 70% diploid (six chromosomes), 30% tetraploid, and a small number of cells of higher ploidy. This is similar to the karyotypic frequencies reported by other workers (Stevens, 1970; Bianchi *et al.*, 1971). Even though the number of chromosomes appears to be normal, considerable alteration of the genome can occur as a result of extensive chromosomal translocation and rearrangement. Indeed, some studies of the original Singh line indicate that changes of this nature are detected with high frequency (Bianchi *et al.*, 1971). Although we have not made an extensive analysis, translocations in the adapted cells are easily detected. A typical metaphase is shown in Fig. 1.

Knowledge of the karyotype makes feasible a measurement of the genome size for this species. Diphenylamine assays yield a value, corrected for the fraction of polyploid cells, of 5.7×10^{11} daltons DNA per haploid genome. Since the relative lengths of the stages in the cell cycle have not been determined, however, some error would be introduced should they differ significantly from CHO cells used for comparison. The value obtained is in approximate agreement with a kinetic complexity of 4.1×10^{11} daltons determined by renaturation kinetics of DNA from the cell line (Spradling *et al.*, 1974).

IV. Cell Fractionation and Other Procedures

In many respects the extraction of macromolecules from the *Aedes* cell line proved to be routine. Total nuclear DNA, for example, can be prepared in good yield from isolated nuclei by standard sodium dodecyl sulfate (SDS)–high salt procedures (Marmur, 1961). However, it became clear very early that virtually any procedure that ruptured the cell membrane simultaneously released a RNase activity that rendered recovery of intact molecules of some types of RNA very difficult.

Two tests were employed to guide our efforts at overcoming this problem. First, direct lysis in SDS of whole cells labeled briefly with uridine indicated that *Aedes* nuclei contain RNA molecules of very large size (greater than 50 S), hence any procedure that did not result in a similar recovery of large molecules was clearly inadequate. Second, direct tests of RNase activity were made by coextracting ^3H -labeled *Aedes* cells and ^{14}C -labeled HeLa cells. A decrease in the size of the appropriate ^{14}C HeLa fraction compared to a similar preparation of HeLa RNA extracted alone was taken as evidence of degradative activity.

By utilizing this approach, several procedures have been developed for the fractionation of *Aedes* cells based on standard methods used for mammalian cells (Penman, 1966). One of two methods is employed to lyse the cell membrane. Treatment of cells in an isotonic buffer [100 mM NaCl, 10 mM MgSO₄, 30 mM tris (pH 8.3)] with 0.5% of the detergent NP40 yields 100% cell lysis, leaving nuclei with virtually no cytoplasmic contamination visible by phase microscopy. Alternatively, when detergents must be avoided, as in the preparation of a mitochondrial fraction, a hypotonic medium is used [20 mM NaCl, 5 mM MgSO₄, 10 mM tris (pH 8.3)]. *Aedes* cells are very unstable at this tonicity, and a few strokes with a Dounce homogenizer results in nearly complete lysis. Nuclei are pelleted by centrifugation at 800 g for 5 minutes. If the resulting supernatant is centrifuged an additional 10 minutes at 8000 g, a crude mitochondrial fraction is obtained.

Early experiments in which poly(A)-containing mRNA was prepared by passing RNA purified from the cytoplasmic fraction over oligo-dT cellulose consistently yielded preparations with a mean sedimentation coefficient of about 15 S compared to 18 S for similar preparations from mammalian cells. Coextraction of *Aedes* and HeLa cells revealed that the RNA was not, however, being extracted intact, despite this uniformity of results. Extensive experimentation suggests that such degradation is totally eliminated by the

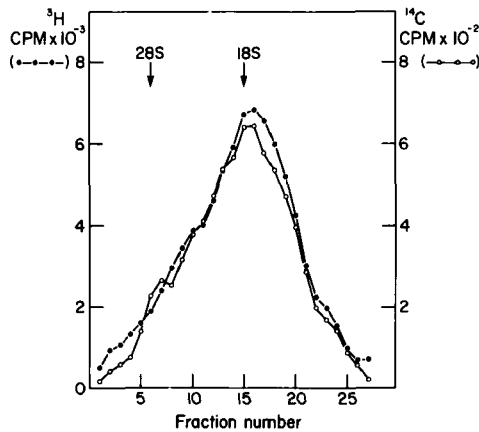


FIG. 2. Coextraction of *Aedes* and HeLa mRNA. *Aedes* cells (2.5×10^7) concentrated $5\times$ to a density of 5×10^6 cells/ml were labeled 2 hours with $10 \mu\text{Ci/ml}$ adenosine-³H (New England Nuclear, 26 Ci/mmmole). HeLa cells (1.2×10^7) were concentrated to 2×10^6 cells/ml and labeled for 2 hours with $0.5 \mu\text{Ci/ml}$ adenosine-¹⁴C (New England Nuclear, 50 mCi/mmmole). The two cultures were then mixed, and a cytoplasmic extract prepared as described for *Aedes* cells. mRNA was purified by phenol extraction and oligo-dT cellulose chromatography. Closed circles, mRNA-³H eluted from oligo-dT cellulose; open circles, mRNA-¹⁴C eluted from oligo-dT cellulose.

addition of polyvinyl sulfate (PVS) (25 $\mu\text{g}/\text{ml}$) and diethylpyrocarbonate (0.5%) to the lysis medium just prior to use. Spermidine (35 $\mu\text{g}/\text{ml}$) is added as well, since this polycation counteracts the destabilizing effect of PVS on cell nuclei.

By using these procedures, mRNA from *Aedes* cells labeled for 1 hour with uridine- ^3H is found to sediment with a mean at about 18 S, very much like mRNA from mammalian cells. This result is probably not due to aggregation of RNA, since the sedimentation distribution is unchanged relative to rRNA when sedimentation is carried out under denaturing conditions in 99% dimethyl sulfoxide (DMSO). The steady-state size distribution of *Aedes* mRNA is somewhat smaller than that of newly labeled material, however (Spradling and Penman, 1974). Figure 2 shows by coextraction that *Aedes* mRNA prepared by these techniques is probably totally intact. mRNA extracted from polysomes (Fig. 3) has a sedimentation distribution identical to that prepared from whole cytoplasm. Treatment of the cytoplasmic extract with EDTA prior to centrifugation causes more than 90% of the polyosomal O.D. and the associated poly(A)-containing RNA to sediment near the top of the gradient.

Two properties of *Aedes* rRNA are worthy of note. Figure 4A shows a typical preparation of cytoplasmic RNA coelectrophoresed with HeLa rRNA. Both the *Aedes* species are seen to differ in apparent size from HeLa rRNA, a conclusion also supported by cosedimentation in sucrose gradients. Furthermore, as indicated in Fig. 4A, the relative amounts of large and small rRNA are generally not found in the expected 2:1 ratio. The origin of

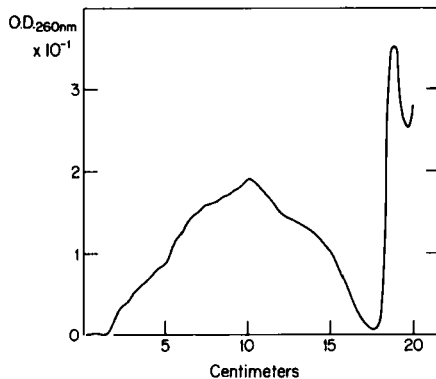


FIG. 3. *Aedes* polysomes. A cytoplasmic extract was prepared from 2×10^7 *Aedes* cells in 1 ml as described in the text. It was layered over a 16.5-ml, 15–30% (w/w) sucrose gradient in lysis medium and centrifuged 2 hours at 25,000 rpm in a Spinco SW27. Absorbance was monitored by pumping the gradient from the bottom through a continuous-flow spectrophotometer.

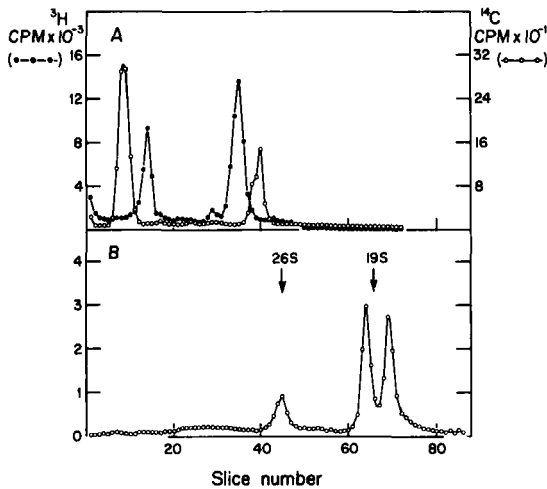


FIG. 4. *Aedes* rRNA. (A) HeLa uridine- ^3H -labeled whole cytoplasmic RNA (approximately 10^3 cpm) was combined with about 5×10^3 cpm of a similar preparation of *Aedes* RNA labeled with uridine- ^{14}C . The sample was electrophoresed as described in Hirsch *et al.* (1974) on a 3.5% polyacrylamide gel for 7 hours. (B) Approximately 2×10^4 cpm of uridine- ^3H -labeled 26 S *Aedes* rRNA prepared by pooling the appropriate fractions from a sucrose gradient was combined with 10^3 cpm of uridine- ^{14}C -labeled *Aedes* cytoplasmic RNA as a marker. The sample was electrophoresed as above, except that the acrylamide concentration was 2.8%.

this anomaly is illustrated in Fig. 4B. 26 S rRNA isolated from a sucrose gradient was heated at 70°C for 5 minutes and then electrophoresed on a 2.8% acrylamide gel. The bulk of the 26 S species dissociated into two component molecules, one of which migrates slightly faster and one somewhat slower than 19 S rRNA. Some breakdown occurs during cell fractionation, and under most conditions the product species comigrate with the small rRNA thus giving rise to the anomalous ratio. The instability of the large rRNA of many invertebrate species has been described previously (Applebaum *et al.*, 1966; Shine and Dalgarno, 1973).

Appropriately labeled whole *Aedes* cells or isolated nuclei, when lysed directly with an SDS-containing buffer, are found to contain large heterogeneous nuclear RNA as well as rRNA precursors. So far, however, it has not been possible to subfractionate the nuclei into nucleolar and nucleoplasmic fractions consistently while maintaining the full size of the HnRNA.

When working with an unfamiliar cell line, often the most elementary procedures can cause problems. For example, early measurements of the growth characteristics of the *Aedes* cells yielded erratic results. Eventually, it became clear that this was due to the tendency of the cells to clump to-

gether while growing. Big clumps tend to be lost from a sample, since they settle rapidly. In addition, unless the cells are stirred at rapid rate, a portion of them will attach to the culture bottle.

These problems were overcome by a combination of care in maintaining constant conditions of culture agitation and by lysing aliquots of cells with NP40 and counting cell nuclei rather than cells in the hemocytometer. The detergent plus a brief vortexing disperses the clumps and yields accurate counts.

Stocks of *Aedes* cells may be stored frozen at -80°C (or preferably -170°C) if 10% glycerol or DMSO is added as a cryoprotectant. Even when cells are simply allowed to stand in a flask at room temperature without feeding, they remain viable for very long periods of time (greater than 6 months).

V. Conclusion

The development of an adapted line of *A. albopictus* that can be manipulated in much the same manner as cultured mammalian cells should significantly increase the utility of this line in many areas of cell biology. The RNA metabolism of togaviruses during infection of insect cells, for example, has not been well characterized, at least in part because of a lack of the methods described here. Yet such studies might lead to a better understanding of the mechanisms that result in lytic infection of vertebrate hosts by togaviruses which establish a persistent infection in the cells of their insect vectors (Stollar *et al.*, 1972). Another use of the line is in making comparisons between cells from organisms that are widely divergent on an evolutionary scale. Several aspects of RNA metabolism have already been examined in this regard (Hirsch *et al.*, 1974; Spradling *et al.*, 1974). The fact that we have been able to adapt two lines (see Chapter 12) to favorable growth conditions suggests that many insect lines could be similarly propagated. The availability of a variety of such cell lines would undoubtedly be a stimulus for increasing the contributions of work on cultured insect cells to our understanding of eukaryotic molecular biology.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (NIH 5 R01 CA08416; NIH CA12174) and from the National Science Foundation (NSF GB37709X). J.A.L. is the recipient of an NIH postdoctoral research fellowship (GM54218-02).

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