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Cell lines derived from late embryonic stages of *Drosophila melanogaster*

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SUMMARY

The development of three cell lines initiated from the late embryonic stages of *Drosophila melanogaster* is described. The primary cultures consisted of trypsinized fragments from embryos 20–24 h old. The length of time between primary culture and subsequent subculture varied from 8 months for the first line to 3 weeks for the third. All three lines have been maintained *in vitro* for more than a year. The characteristics of each line are given and evidence is presented that at least one line is derived from imaginal disc cells. A few comments on insect tissue culture in general are also made.

INTRODUCTION

Within the past year, two timely and commendable reviews have appeared, both of relevance to the present paper. The first served to document the almost unique status of *Drosophila melanogaster* as the organism of choice in studying many aspects of developmental biology (Fristrom, 1970). The second catalogued the rapid advances which have been made in the field of insect tissue culture, especially in the last 5 years (Brooks & Kurtti, 1971). The use of culture systems to augment findings *in vivo* has been extensively employed in developmental studies. With *Drosophila* much more emphasis has been placed on organ cultures, particularly those of the imaginal discs, than on true cell culture. Three factors appear largely responsible. Until recently, the successful establishment of insect cell lines has entailed extensive periods of adaptation, often 3 or more months in duration. This and the second factor, the almost inevitable emergence of highly polyploid lines, have served as effective deterrents. Finally, because earlier reports of long-term cultivation of *Drosophila* cells tended to be more speculative than substantive, later reports of success met with considerable skepticism.

Bona fide cell lines of *D. melanogaster* were eventually established by Kakpakov, Gvosdev, Platova & Polukarova (1969) and Echalier & Ohanessian (1970), but as noted above, the length of time from primary culture to subsequent subculture was rather prolonged. Since recent work with cell lines from other

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dipteran species indicated that such a lag need not exist (Schneider, 1971*b*) it was of interest to determine whether the same held true for *Drosophila*.

This paper reports the development of three cell lines from *D. melanogaster*, each of which successively required less time to adapt to conditions *in vitro*. Hopefully, the evidence presented will convince investigators that the culture of *Drosophila* cells is now a feasible and reliable technique and hence can serve as one more tool in probing the developmental biology of this organism.

MATERIALS AND METHODS

Drosophila melanogaster (Oregon-R) eggs, collected over a 4 h interval, were dechorionated in 2·5 % aqueous sodium hypochlorite and surface-sterilized by immersion in 70 % ethanol for 20 min followed by an additional 20 min in 0·05 % $HgCl_2$ in 70 % ethanol. After being rinsed thoroughly in sterile distilled water the eggs were transferred to Petri dishes containing sterile Metricel black filters backed with Millipore prefilters, both previously wetted with culture medium. The eggs were placed overnight in a 22 °C incubator and removed for culturing when 20–24 h old.

The embryos were each cut into halves or thirds, then placed in 0·2 % trypsin (1:250, Difco) in Rinaldini's salt solution (Rinaldini, 1954) for 20–45 min at room temperature. From 100–300 embryos were used to initiate each culture. After the addition of fetal bovine serum (FBS), the fragments were centrifuged at 100*g* for 2–3 min, resuspended in 1·25 ml culture medium and seeded into glass T-9 flasks. The cultures were maintained at one of two temperatures, $22 \pm 0{\cdot}5$ °C or $27 \pm 0{\cdot}5$ °C, with a gaseous phase of ambient air.

Schneider's culture medium (Schneider, 1964, 1966) containing an additional 500 mg bacteriological peptone per 100 ml medium and supplemented with 15 % inactivated FBS was used for the first two lines. The medium for the third line also contained a 1 % solution of NCTC-109 vitamins (× 10) (Evans, Bryant, Kerr & Schilling, 1964). The pH of 6·7–6·8 was monitored with 0·01 % phenol red.

The cell lines have been maintained by subculturing every 3–7 days. The cells readily attach to the glass but not so firmly as to require trypsin treatment; simple pipetting is adequate to flush most of the cells from the bottom of the flasks.

Estimation of the growth rate was made by haemocytometer count every second day. A 0·01 % crystal violet solution in 0·1 M citric acid served as the diluent.

For cytological study, the cells were fixed in methanol:acetic acid (3:1) and stained with Delafield's hematoxylin or Giemsa. Chromosome counts were made on cells with colchicine-blocked metaphases.

Cells from the first two lines have been frozen at −68 °C, in culture medium containing 10 % glycerol, and successfully regenerated. Cells from the third line have not, as yet, been subjected to this treatment.

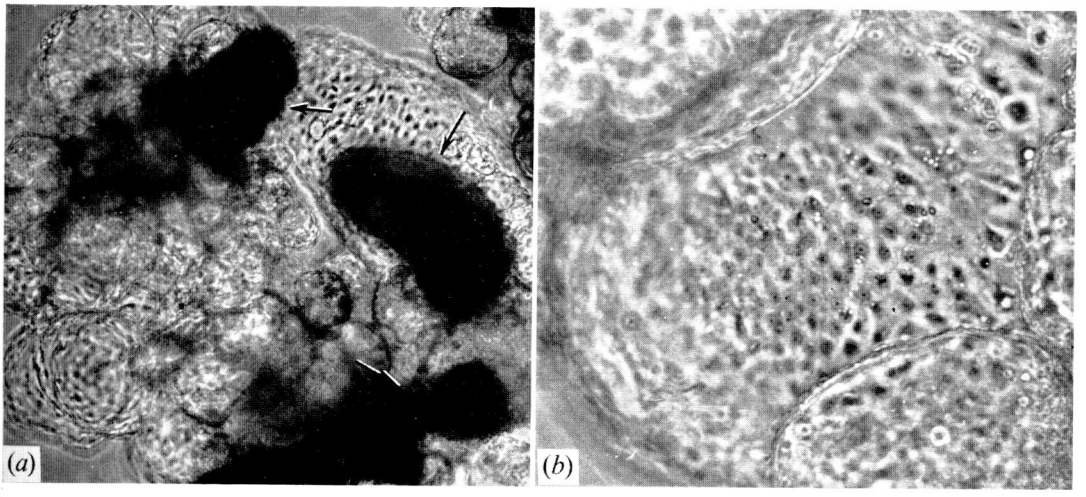
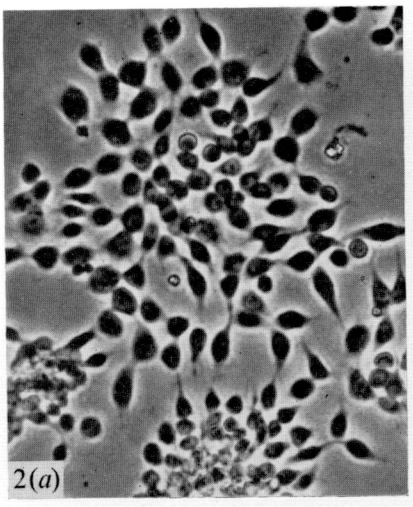


Fig. 1. Cellular spheres developing from embryonic fragments of *D. melanogaster*. Phase contrast. (*a*) Three fragments, indicated by arrows, in a 25-day-old culture. Note size of spheres relative to that of the fragments, particularly the one in the upper right-hand corner. × 150. (*b*) Spheres at a higher magnification showing the individual cells. × 300.

RESULTS

Primary cultures for the three cell lines were initiated in August 1969, December 1969 and February 1970, respectively. All three cultures followed the same pattern of development previously described for cell lines of *Aedes aegypti* (Singh, 1967), *Anopheles stephensi* (Schneider, 1969), *Culex tritaeniorhynchus* and *C. salinarius* (Schneider, 1971*a*). The most prominent characteristic of the primary cultures was the growth of cell spheres issuing from the cut ends of the embryonic fragments. Usually 2–3 weeks elapsed before the spheres were apparent. The size of the spheres varied considerably as did the cells from one sphere to the next. Fig. 1*a* shows three fragments with the accompanying spheres. At a higher magnification the individual cells within the spheres can readily be seen (Fig. 1*b*). It was not uncommon to find fragments almost completely engulfed by the hollow, cellular spheres with the diameters of the latter often greatly exceeding those of the former. (Some bleb formation was also encountered but there was no difficulty in distinguishing these from the cellular spheres.)

If the fragments remained in a healthy state, the spheres were excised about the third or fourth week of culture and returned along with the fragments to the original flask. Under such circumstances new spheres arose from the fragments, in number and size equal to or surpassing those of the initial growth. This procedure was repeated two or more times at weekly or bi-weekly intervals until some 100 or more spheres were present in each primary culture. Fragments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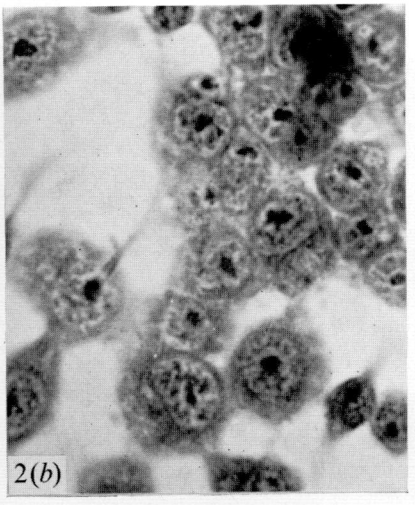
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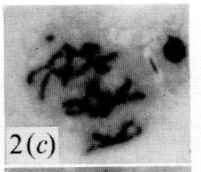
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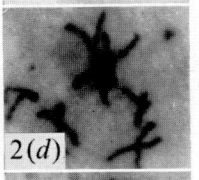
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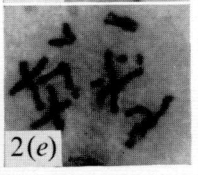
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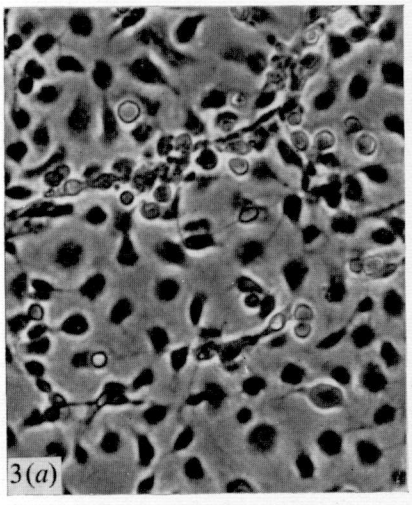
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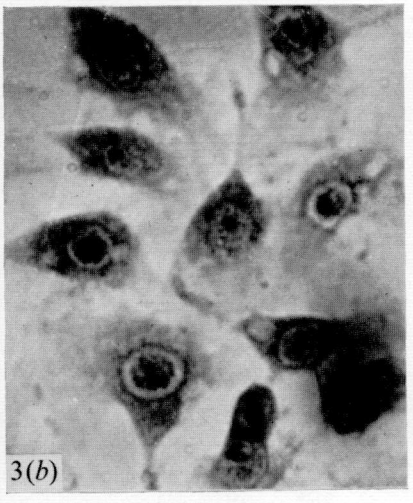
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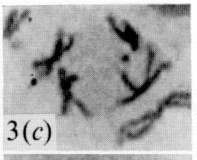
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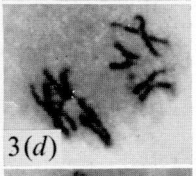
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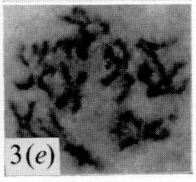
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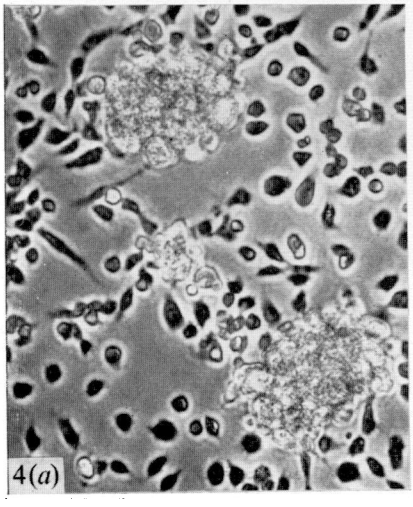
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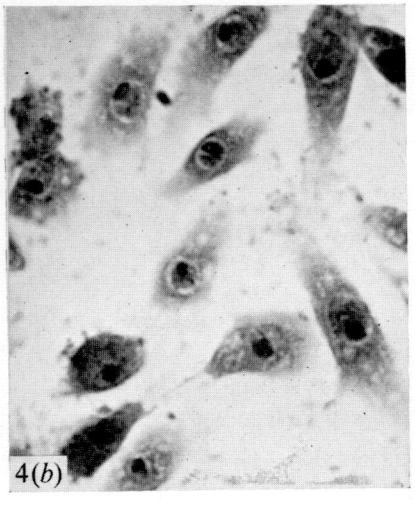
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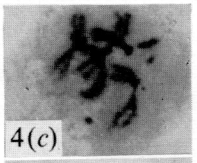
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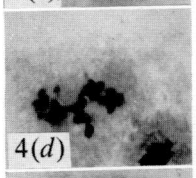
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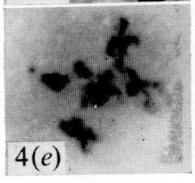
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were considered healthy if they retained a transparent appearance and if melanization did not occur at the cut ends. The excised spheres may be trypsinized to release the cells or, better, simply teased apart. With either method such cells usually attached to the bottom of the flask and began multiplying. During this time the medium was partially renewed at intervals varying from 1–2 weeks.

Choosing the appropriate time to attempt subculturing was necessarily subjective but was based on the number of cells attached to the bottom of the flask, their rate of multiplication, the rapidity with which the cell spheres grew as well as the overall appearance of the primary culture. Not surprisingly, the successful reading of such criteria was considerably enhanced by experience in handling the cultures. This was well documented in the present study: the first line was not subcultured until 8 months had elapsed whereas the second was successfully subcultured after 3 months and the third after 3 weeks. Since the primary cultures were essentially identical with respect to size of original inoculum, trypsin pretreatment and medium utilized (see Discussion section regarding the NCTC supplement), the decrease in the interval prior to subculturing for the successive lines can be attributed almost entirely to increasing familiarity with the culture system.

Line 1. Three morphologically distinct cell types occur in this line: (1) round to ovoid cells, varying from 5–15 μm in diameter, (2) spindle-shaped cells, ranging from 5–9 μm in diameter and 10–40 μm in length and (3) 'macrophage-like' cells which assume a multiplicity of shapes and sizes but invariably are much larger than the first two types, averaging 25 μm in diameter and 60 μm in length.

Cells of this line never form a monolayer but instead are usually grouped in colonies of widely disparate sizes in which the round cells predominate (Fig. 2*a*). The spindle-shaped cells are more common at the periphery of the colonies or in regions where the cell density is low. Both cell types have a very large nucleus often occupying the greater portion of the cell. A prominent nucleolus is also present (Fig. 2*b*). Another characteristic of the line, confined to the spindle-shaped cells, is their tendency to grow in a unidirectional manner. It is not uncommon to see such cells lined up like 'beads on a string', 4–8 cells long.

The 'macrophage-like' cell is similar in appearance to one of the five cell types described for primary cultures of *D. melanogaster* embryos by Shields & Sang (1970). The outstanding characteristic of this cell type is the very extensive,

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FIGURES 2–4

Figs. 2–4. Cells and chromosome complements of the three cell lines of *D. melanogaster*. (2*a*, 3*a*, 4*a*) Cells of lines 1, 2 and 3 in 36th, 33rd and 21st passages, respectively. Phase-contrast. × 600. (2*b*, 3*b*, 4*b*) Hematoxylin-stained cells of the three respective lines at a magnification of × 1650. (2*c–e*, 3*c–e*, 4*c–e*) Typical karyotypes of the three lines. Colchicine-treated and Giemsa stained. × 1132–1800.

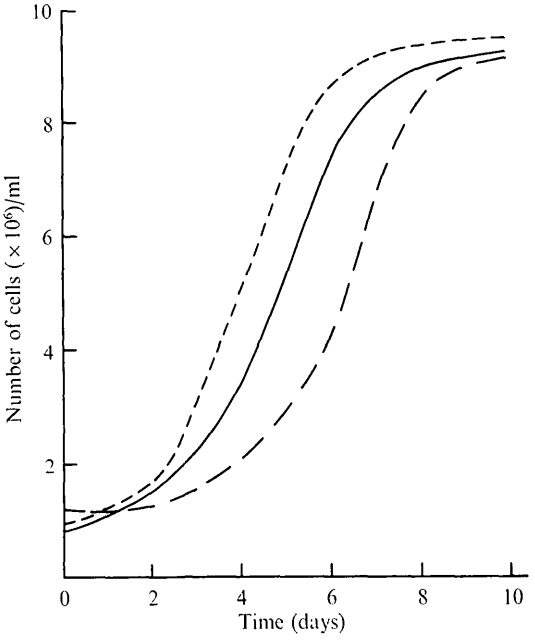


Fig. 5. Growth curves for the three *D. melanogaster* cell lines. Cell counts were made in subcultures 44, 36 and 23 for lines 1, 2 and 3, respectively. ——, Line 1 (27 °C); - - -, line 2 (27 °C); – –, line 3 (22 °C).

flattened cytoplasm surrounding a prominent nucleus. Although very common (30–40 % of the total number of cells) in the primary culture and early subcultures, this cell type decreased in frequency as the passage number increased: at the 40th subculture such cells comprised only 5 % of the total population.

A typical growth curve for line 1 is given in Fig. 5. The chromosome number has remained fairly constant at 8 (Fig. 2*c*, *d*) or 8 plus one or more unidentifiable fragments (Fig. 2*e*).

Line 2. The cells of this line are less diverse in their morphology than those of the first. The cells grow in a loose monolayer with little tendency to pile up at central foci. They are predominantly epithelial-like in appearance and range from 5–11 μm in diameter and 11–35 μm in length (Fig. 3*a*). Small pockets containing 10–20 round cells, 5–10 μm in diameter, are dispersed at random among the other cells. The nuclei are perhaps a little smaller than those of the first line but the nucleoli are equally prominent (Fig. 3*b*). The cells multiply at a rate somewhat higher than those of line 1 (Fig. 5).

Initially both *XX* and *XY* cells were present. However, after approximately 15 subcultures the *XY* cells were apparently eliminated: all cells scored since the 16th passage have been *XX* with either a remaining normal chromosome set or one that is haplo-4 (Fig. 3*c*, *d*). However, the cells have a tendency to become tetraploid if seeded too lightly on transfer and often, but not always, if grown in plastic flasks (Fig. 3*e*).

Line 3. The third line is characterized by a very loose network of cells with a

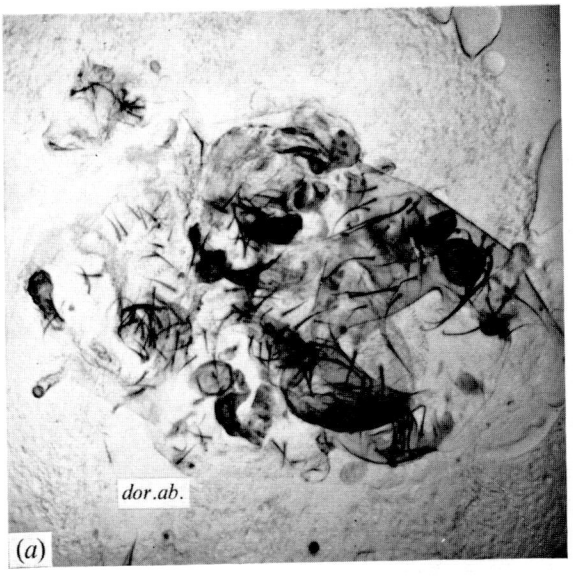
pronounced tendency to aggregate into central foci (Fig. 4*a*); a true monolayer is never formed. The size of the cells varies from 5–9 μm in diameter and 10–30 μm in length. The cells are very irregular in shape, most often having an angular appearance. Nuclei and nucleoli are large and distinct; the cytoplasm immediately surrounding the nucleus stains fairly darkly but becomes progressively fainter towards the periphery of the cell. In most instances, the boundaries of the cells appear to taper off into rhizoidal-looking processes, often with filamentous connexions between adjacent cells (Fig. 4*b*). The rate of growth is shown in Fig. 5. (This line grows somewhat erratically at 27 °C; hence it is maintained at 22 °C to ensure its continuence as a line.) The chromosome set is diploid with a great preponderance of *XY* cells (Fig. 4*c–e*).

No attempt was made to determine the origin of the cells in the first two lines. The relatively slow growth of the cell spheres in the primary cultures precluded using any of the former for transplantation purposes. No such limitation was present in the primary culture of the third line. During the 2nd, 8th and 10th month of culture, 15 cell spheres were excised from fragments chosen at random and transplanted whole or in part into wild type or *yw* third-stage larvae. All of the implants recovered (12, 8 and 11 for the 1st, 2nd and 3rd transplant series, respectively) had metamorphosed into adult structures indicative of imaginal-disc origin: head cuticle, antennal segments, anal plates, lateral plates and ventral and dorsal abdominal segments (Fig. 6*a–e*). Moreover, spheres arising from the same fragment always metamorphosed into the same type of adult structure. This was true even when the spheres were injected as much as 8 months apart. (The primary cultures, if contamination is avoided, can be maintained in perfectly healthy condition for a year or longer with no apparent diminution in the ability of the fragments to put forth more spheres.)

One cell mass, of 15 from the first subculture, when transplanted into a third-stage larva also underwent metamorphosis (Fig. 6*f–h*). However, individual cells from the same culture, scraped from the bottom of the flask and centrifuged into a compact pellet, did not form any recognizable structures after implantation (0 out of 15 implanted pellets). This loss of differentiation capacity extended to all cells from the second and subsequent transfers.

DISCUSSION

With few exceptions, the successful establishment of dipteran cell lines has resulted from the use of immature stages, especially embryonic stages or neonate larvae, as primary explants. In *D. melanogaster* embryos, mitotic activity is very prominent for the first 6–8 h, whereas in the later stages emphasis switches to differentiation of the various organ systems. Assuming that a high mitotic rate *in vivo* favors continued proliferation *in vitro*, the very young embryos should prove to be the most suitable donors. From the results of an admittedly small number of experiments however, this may not necessarily be true.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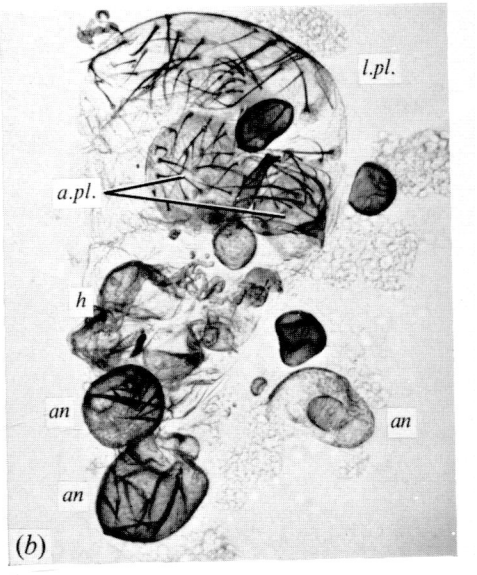
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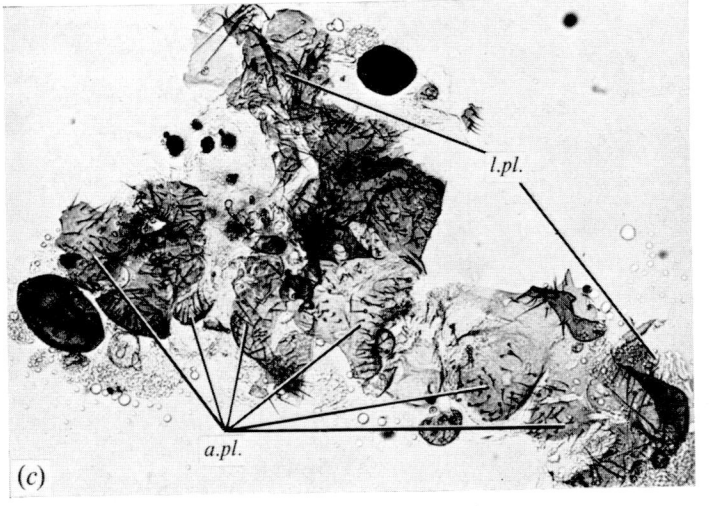
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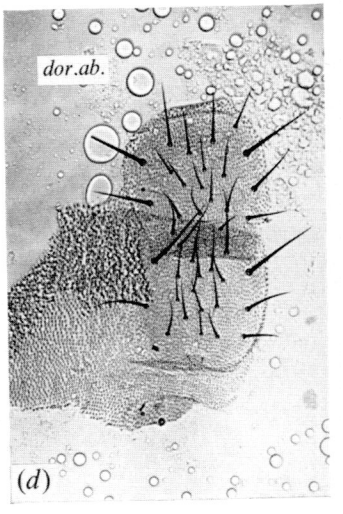
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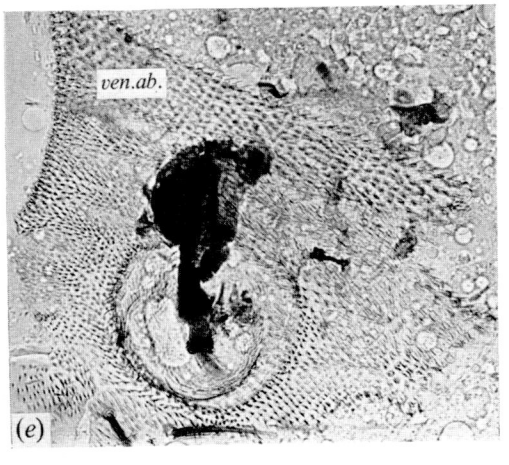
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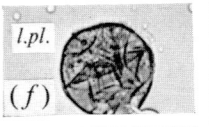
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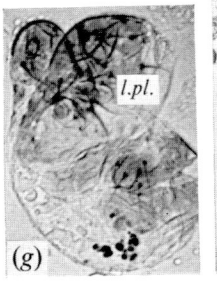
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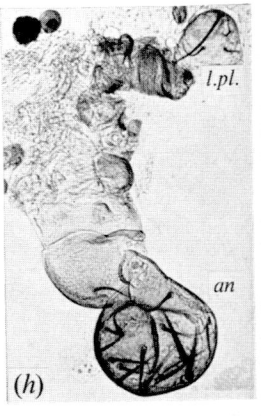
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Using embryos at the stage of blastoderm formation (Kuroda, 1963, 1969) or those 8 h post fertilization (Horikawa & Fox, 1964; Horikawa, Ling & Fox, 1965), primary cultures of short-term duration were readily obtained. However, the evidence supporting their claims for long-term cultivation was not entirely convincing. More recently, Shields & Sang (1970) made a thorough study of cultures initiated from 6–8 h embryos and described in detail the behavior, morphology and survival time of five distinct cell types. None of these cell types persisted longer than 10 weeks *in vitro* and often the interval was considerably less.

The first *bona fide* cell lines of *D. melanogaster* were initiated from lightly homogenized 6–12 h embryos by Kakpakov *et al.* (1969) and Echalier & Ohanessian (1970). The transition period from the primary cultures to the subsequent subcultures was quite prolonged, invariably requiring some 5 or 6 months in the latter study. Although an even longer period, 8 months, was required for the first line reported here, the succeeding lines took 3 months and 3 weeks, respectively. Obviously a decision as to the relative merits of any one embryonic stage to serve as the primary explant must await a systematic study. But the results so far obtained suggest that late embryos may prove as good or better a source than the very young stages.

Among the few cell types which retain the ability to undergo normal cell division rather than polyploidization in the late embryonic stages are the imaginal-disc cells. Hence it is not particularly surprising that the third line was found to be derived from such cells. Whether the first two lines were so derived is open to question, but the probability that they were appears rather high. Spheres chosen at random from a number of primary cultures not associated with the present cell lines also metamorphosed upon implantation into structures indicating imaginal-disc origin. Although some discs were not represented in the metmorphosed implants, e.g. wing, leg and haltere discs, the number of spheres transplanted represented a very small percentage of those present in the primary culture. A more extensive transplantation series might have revealed metamorphosed structures encompassing the entire range of discs.

The design of media for invertebrate cell cultures with attendant pros and cons for patterning the media after the appropriate hemolymph has been adequately reviewed by Jones (1966), Schneider (1967, 1971*a*) and Brooks & Kurtti (1971). All three media currently being used to support the growth of

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FIGURE 6

Fig. 6. Range of metamorphosed structures found in adult flies after implantation of spheres, or portions of spheres, into third-stage larvae. × 110. (*a*) From 2-month culture, (*b*) from 8-month culture, (*c–e*) from 10-month culture, (*f-h*) from cell mass or collapsed spheres in first subculture. Abbreviations: *an*, antenna; *a.pl.*, anal plate; *dor.ab.*, dorsal portion of abdomen; *h*, head cuticle; *l.pl.*, lateral plate; *ven.ab.*, ventral portion of abdomen.

Drosophila cell lines *in vitro* were based to some extent on analyses of larval hemolymph since comparable information for the embryonic stages is lacking. Readings on a Fiske osmometer gave values of 438, 373 and 360 m-osmoles, corresponding to freezing-point depressions of −0·81, −0·70 and −0·67 °C respectively, for the media of Gvosdev & Kakpakov (1968), Echalier & Ohanessian (1970) and Schneider (this paper). Although the values for this parameter are in fairly close agreement with one another, especially for the last two media, the inorganic and organic components chosen to attain these values varied considerably among the three, both qualitatively and quantitatively. It was therefore of interest to determine whether a cell line adapted to grow in the one medium could be maintained in the other two. Cells of line 2 survived three passages in the first medium and apparently indefinitely in the second. These latter cultures were terminated after eight consecutive passages at which time the growth rate was still equal to that of the controls. (A tendency towards polyploidy and/or aneuploidy was fairly pronounced but this also occurs to some extent in the original medium.) These results suggest that more latitude can be used in the design of media than has generally been assumed, particularly with regard to the relative contributions of the ions, sugars and amino acids in attaining the appropriate osmolality.

Since the NCTC supplement proved of value in maintaining cell lines of *A. stephensi* (Schneider, 1969) it was incorporated into the medium for the third *Drosophila* line in an effort to stimulate more rapid growth. It is questionable whether it did so. The much shorter transition period from primary culture to subculture for the third line has already been attributed to increasing competence in handling the cultures. This assumption is reinforced by recent work with primary cultures in which the emergence and growth of the spheres was comparable in media with and without the supplement. As such, it should be considered non-essential and efforts are being made to adapt the third line to grow without it.

The size of the inoculum had a considerable effect on the development of the primary cultures and on the ease with which the cells could be subcultured. The larger the number of fragments seeded per flask, within limits, the sooner the spheres became apparent and the more numerous they became per fragment. For example, a T-9 flask seeded with fragments from 100 embryos usually took 3–4 weeks before the spheres were either numerous or large and the numbers per fragment tended to be 5 or less. In contrast, flasks seeded with fragments from approximately 300 embryos produced spheres within 10–14 days and the numbers per fragment often exceeded 15. Larger numbers, however, did not lead to further improvement and frequently had a detrimental effect. From the foregoing, one might easily conclude that conditioned media would prove beneficial in stimulating growth in the primary cultures. But in the few trials made with such media the results were not too encouraging.

Pretreatment with trypsin proved to be indispensable with the older embryos,

serving to reinforce the assumption that once organization into specialized tissues and organs is complete, some dissociation is necessary before cell migration and division take place *in vitro*. Fourteen cultures were initiated without subjecting the fragments to trypsin and in no instance did the spheres become large enough to provide the necessary cell density for maintaining the primary culture. Usually there was no proliferation at all. The trypsin treatment served only to loosen the matrix between the cells as dissociation was by no means complete. Visually the fragments were identical in appearance before and after treatment.

Assuming that explants from 6–12 and 20–24 h embryos are equally amenable to culturing, the relative advantages of homogenizing the former or trypsinizing the latter appear fairly well balanced. The one great advantage of the homogenization technique is that it is much less time-consuming. With the trypsinization technique the possibility of determining the origin of the cells is much greater, at least in those species where transplantation is feasible, and secondly, it is possible to avoid the tedious process of plating individual cells if clones are desired since the cells issuing from any one fragment will be genotypically identical and these can readily be accumulated for subculturing.

With one exception – the *Aedes aegypti* cell line developed by Grace (1966) – all of the dipteran lines so far established have retained a predominantly diploid chromosome set whereas polyploidy has been the rule for the great majority, if not all, of the cell lines established from other insect orders. (Two other dipteran cell lines, purportedly originating from *Aedes vexans* and *Culiseta inornata* (Sweet & Dupree, 1968; Sweet & McHale, 1970), bear a disconcerting similarity in morphology, growth characteristics, chromosome numbers, immunology and medium utilized to the cells of Grace's line. Hence some reservations must be expressed concerning their identity.) Previously it was noted that the polyploid lines showed an initial spurt of activity followed by a lag or cessation in mitosis before growth was resumed. In contrast, the diploid cell lines maintained a relatively steady rate of growth after the first few days *in vitro* (Schneider, 1969). This suggested that prolonged adaptation of a cell line was contingent, at least in part, upon the appearance of polyploid cells. Since diploid cell lines have now been obtained despite discontinuous growth over a period of 5–6 months (Echalier & Ohanessian, 1970) this supposition is no longer wholly tenable.

The present culture system seemed to be a stable one in that the fragments in the primary cultures were capable of issuing successive cellular spheres for more than a year after the cultures had been initiated and, secondly, that the spheres retained the ability to differentiate *in vivo* irrespective of whether they appeared in the primary culture after 1 month or 10 months *in vitro*. Interestingly there was no evidence of transdetermination in the cells (Hadorn, 1965; Gehring, 1968): successive spheres from the same fragment invariably metamorphosed into similar adult structures. On occasion more than one sphere from different

fragments was inadvertently implanted into the same host; not surprisingly this often resulted in metamorphosed structures from more than one type of disc being present in a single implant (Fig. 6*b*).

With one possible exception, only spheres from the primary culture were capable of metamorphosing *in vivo*. One 'cell mass' out of 15 tested, from the first subculture, did undergo metamorphosis. Due to its size the mass was divided into three parts prior to transplantation and adult structures were recovered from all three (Fig. 6*f–h*). But the possibility exists that in reality the mass was composed of a number of collapsed spheres since a few of the primary fragments were still present in this particular culture. By the second subculture, all fragments were absent and all injected cell masses from this and subsequent cultures failed to metamorphose.

It is possible the spheres could be induced to metamorphose *in vitro* by the introduction of ecdysone into the culture medium since earlier studies have clearly demonstrated the influence of endogenous hormone (Gottschewski, 1960; Schneider, 1964) and exogenous hormone (Mandaron, 1970) on the development of intact imaginal discs *in vitro*. If the spheres are found responsive, it should be readily possible to attack the problem of hormonal control of differentiation at the cellular and subcellular levels.

The fact that the transition from primary culture to subculturing now requires as little as three weeks hopefully means that the adaptation process is neither as extensive nor drastic as in the case of cells requiring 5–6 months (Echalier & Ohanessian, 1970) or the 8 months required for the first line reported in this study. If so, nutritional studies might profitably be undertaken and a more adequate medium reflecting the true requirements of the cells designed.

Although all of the work reported here was limited to wild-type *Drosophila* there are no obvious reasons why the technique could not be extended to mutant strains as well. Assuming this is feasible, then the possibilities for further studies in genetics and developmental biology are extensive indeed.

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