OBSERVATIONS ON GROWTH AND MORPHOGENESIS IN CULTURED CELLS OF CARROT (*DAUCUS CAROTA* L.)

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This paper deals with problems of somatic embryogenesis (totipotency) of carrot cells in liquid culture and when dispersed in thin films of nutrient agar. The critical events that intervene between somatic cells and embryo plantlets during morphogenesis are delineated. Factors that affect the course of these events are described with special reference to the stimuli which permit the very smallest units to develop into organized structures. Special attention is directed to the importance of a protracted period of darkness and the use of an 'embryo-conditioned medium' as interacting stimuli that facilitate the early stages of development. Means of arresting and releasing the development of pro-embryonic units are discussed. Aspects of growth and form of the developing cultures are presented photographically at different levels. The behaviour of free protoplasts from pro-embryonic units is described and contrasted with that of intact totipotent cells. The significance of all these observations is examined in the light of practical applications and in relation to problems of development.

1. Introduction: from parenchyma to somatic embryos

(*a*) *Cells* in situ *and in culture*

Mature, living somatic cells of angiosperms may remain quiescent as they fulfil their prescribed rôles in the plant body even though, when removed and appropriately stimulated, they may be able to grow again. In fact, many cells, such as those from the secondary phloem of carrot roots (Steward 1963; Steward, Mapes & Mears 1958*a*; Steward, Mapes & Smith 1958*b*; Steward *et al.* 1961; Steward, Blakely, Kent & Mapes 1963; Steward, Mapes, Kent & Holsten 1964) or from other sites (Steward, Kent & Mapes 1967; and Steward, Mapes & Ammirato 1969) from other umbelliferae (Steward, Ammirato & Mapes 1970) or from other plants (Steward & Mapes 1971*a*, *b*) may, under appropriate conditions, develop into plants. Cultures of carrot (*Daucus carota*) and of water parsnip (*Sium suave*) have multiplied so extensively in suspension cultures and have given rise to embryoids and plantlets in such great numbers that the totipotency of their living cells *in situ* is beyond all doubt (Steward *et al.* 1964; cf. Steward *et al.* 1969, Fig. 13). Thus the original genetic information, variously expressed and repressed during development, must remain intact throughout.

Some 19 years have elapsed since the first laboratory observations of totipotency and somatic embryogenesis of carrot cells, and much work in other laboratories has been stimulated as a result (cf. Steward & Krikorian 1975 and references there cited). The question whether somatic embryos originate from single cells or small groups of cells in cultures has, however, attracted an inordinate amount of attention (for summaries and discussion by other authors see Halperin 1969 and 1970; Vasil & Vasil 1972; Reinert 1973; McWilliam, Smith & Street 1974). But preoccupation with this question has in fact been sterile since neither zygotes, nor free single somatic cells, can ever become embryos without first passing through a small globular multicellular stage. Nevertheless the evident variations in the expression of somatic embryogenesis (Steward *et al.* 1969, 1970) emphasize that there are causal factors that need to be more completely understood and controlled so that 'adventive' or 'somatic' embryos may develop from cells in culture as precisely and infallibly as from zygotes in ovules.

The need for better understanding of somatic embryogenesis in cultures is also emphasized by the many plants which have not yet responded to the existing techniques. Monocotyledons have usually responded with difficulty, although some work on cereals and other grasses (e.g. rice, wheat and *Bromus*) has yielded somatic embryonic forms (cf., for example, Adachi & Katayama 1969; Shimada, Sasakuma & Tsunewaki 1969; Constabel, Miller & Gamborg

1971). In anticipation of practical applications (cf. Nickell & Heinz 1973 and Murashige 1974 and references there cited) many agriculturally important plants have been cultured. Of special significance are extensions of the culture techniques (cf. Street 1973; Gamborg & Wetter 1975) to include the culture of haploid cells from anthers leading to the development of haploid or polyploid plants (cf. Nitsch & Norreel 1973 and Sunderland 1973 and references there cited), the preparation and use of free protoplasts *in lieu* of intact cells (cf. Colloques International C.N.R.S. No. 212, 1973; Cocking 1972, 1973; Potrykus 1973 and references there cited), and the application of cultured cells and protoplasts to genetic studies (cf. Carlson 1973, 1975; Carlson, Dearing & Floyd 1973; Gamborg & Wetter 1975; Melchers & Labib 1974; Morel, Bourgin & Chupeau 1973; Chaleff & Carlson 1974 and Smith 1974 and references there cited). However, before full advantage can be taken of these various developments, embryogenesis in the systems in question needs to be fully and consistently controlled.

This paper, then, surveys results obtained with carrot cultures and appraises the status of these problems. To this end the events involved and the induction of cell growth and morphogenesis are, retrospectively, dissected. The roles of different culture conditions and media are discussed, various factors that affect morphogenesis are described, the characteristics of the resultant forms that emerge are examined, and some present opportunities and limitations in this field of work are faced.

(*b*) *Induction of growth and morphogenesis*

The rigorously programmed development of a zygote normally occurs in an ovule where the environment provides the sequence of conditions that is compatible with embryogeny. Hence the release of the totipotency that persists in mature somatic cells involves a reciprocal chain of events. First, the quiescent somatic tissue must be stimulated to grow and, in so doing, it must be liberated from any limiting restraints *in situ*. The proliferated cells as they multiply must then be submitted to conditions that allow them to stimulate zygotes.

The salient events from the isolation of mature carrot tissue explants (free of cambium) through their growth as tissue cultures to plantlets may be formulated as a sequence of stages (0–(iv)) as in table 1. (Subsequent references to numbered stages 0–(iv) relate to those in table 1.) But, if proliferating and free cell cultures are established directly from developing embryos from immature seeds, their organized growth and somatic embryogenesis, when successful, may derive from tissues which had not fully matured or were not yet quiescent *in situ*; in dicotyledons, it may even be traceable to still active cambial elements. In such situations the need for the inductive events of stages 0–(iv) of table 1 may, in effect, be minimized; this occurred in the first lavish production of carrot embryos on agar from cells from carrot embryos from immature seeds of wild carrot (Steward *et al.* 1964) and in other similar examples.

The summary in table 1 of the definitive events along the route from tissue explants to plantlets via cultured cells anticipates some of the new evidence but it serves as a useful guide to the interpretation of the data which is to be presented.

The starting material for the sequence described in table 1 was first the standard explants (about 2.5 mg) of secondary phloem cut at 1–2 mm from the cambium between the lateral roots of mature carrots. As so cut, the explants may contain about 2.5×10^3 cells which have acquired their mature characteristics and, normally and *in situ*, they would not grow again. They are, in fact, quiescent, even dormant, mature cells. Nevertheless, when the carrot

phloem explants are appropriately stimulated their cells may multiply and yield up to 2–3 million cells per explant in about 21 days.

This use of carrot root phloem explants as a standard source of cells to be cultured is largely a matter of convenience. Often cells from other sources in the plant body or from embryos or germinated seeds may be intercepted in their normal course of development and caused to

TABLE 1. STAGES AND INTERVENING EVENTS IN THE INDUCTION OF GROWTH AND MORPHOGENESIS IN SOMATIC CELLS AND TISSUE

(Based on carrot, *Daucus carota*, and water parsnip, *Sium suave*.)

intervening events	stage no.	description	status of cells and/or cultured units
	0	mature tissue *in situ*	quiescent totipotent cells limited by location and prior development
aseptic isolation from tissue of origin	(i)	isolated small explants	mature somatic cells receptive to exogenous growth promoters†
rapid growth induced	(ii)	cultured explants	rapidly metabolizing, randomly proliferating, attached cells
liberation of cells, individually and in small clusters	(iii)	suspension cultures	populations of growing unorganized units propagated by sub-cultures
cell multiplication and morphogenetic induction‡	(iv)	propagated, pro-embryonic stocks (to be graded by filtration)	population of units with varying capability for independent organized growth
embryogenesis promoted by media‖ and environment		(iva) smaller units	isolated units in which development is arrested§
		(ivb) larger units	pro-embryonic globules and larger§ cell aggregates (nodules) in which development is induced
	(v)	embryo cultures	organized units in various stages of somatic embryonic development
plant development	(vi)	propagated crops of embryos and plantlets	plantlets in profusion to cultures committed *en masse* to organized growth

† Synergistic combinations of substances (natural or synthetic) here interact with nutrients (including trace elements) and environment (e.g. light and temperature) to regulate growth, metabolism and composition of the cultured explants.

‡ Sequential treatments, involving different synergistic combinations of growth factors, changes in the nutrient medium (e.g. its concentration and composition), etc., variously induce a morphogenetic capacity in preference to mere proliferation: this capacity may be revealed best in liquid cultures in bulk, which are heterogeneous in unit size and in which larger organized growths foster development from repetitively liberated cells.

§ When isolated into liquid, or on to semi-solid media, the smallest units (free cells and clusters up to 30–50 small dense cells) are apt to be arrested in their multiplication and/or morphogenesis; the more the larger units have advanced in the bulk culture the less demanding are their subsequent requirements in terms of the nature of the medium (see ‖ below) and of the physical environment (e.g. with respect to light and darkness).

‖ Factors conducive to organized growth, especially of small units, may be furnished to fresh culture medium by the addition of medium pre-conditioned by the prior growth of embryos and plantlets in ungraded cultures. Factors in the environment which promote morphogenetic induction involve long exposure to prior darkness at temperatures above a minimum. The effects of darkness and conditioned medium interact. Early stages, as at (iva) and (ivb), the progress of development may be arrested at will and the units maintained reversibly 'poised'. The cultures may exist in this 'poised' state for long periods, e.g. in a medium which is concentrated by sorbitol and with its sucrose reduced, but they may be re-activated when the conditions are reversed.

grow in culture with less drastic inductive stimuli. By contrast, cells from some other flowering plants may remain viable in culture but are unable to respond to conditions that have sufficed for somatic embryogenesis in carrot.

Differences in responses at stages 0–(iv) (table 1) of explants from different carrot roots are attributable to conditions that obtained during the development of a given storage root in this biennial plant. The known variability in the quantitative responses of explants from different carrot roots of the same harvested stock (overcome in the carrot assay system for growth factors by using populations of explants cut from a single root; for references see Steward & Krikorian 1971) may be attributable to microclimatic and storage factors which endow each plant root with somewhat different propensities; these possibilities were, in fact, investigated by Craven (1972, cf. p. 244 and following) together with one of us (F.C.S.). Nevertheless, the conditions for the growth of the excised explants can be controlled.

But in the outcome, the morphogenetic events as they occur in liquid cultures *en masse*, dramatic as they may be (Steward 1970*b*; Steward *et al.* 1970), really conceal factors which emerge only when the responses of the smallest units (individual cells and small clusters) of the composite population are traced. It is then apparent that the entire population allows smaller units to develop with greater ease than when they are isolated in a freshly prepared medium. In other words the population, in effect, 'conditions' the medium in ways that promote the growth and morphogenesis from the smallest units. Thus, a definitive event in what is conveniently referred to as 'somatic embryogenesis' is the conversion of free cells and small units in suspension cultures to the obviously more highly organized units from which embryos and plantlets develop. Cultures of such units which may multiply in liquid media, are 'pro-embryonic' (i.e. they are at stage (iv)) in the sense that they have the capacity to give rise, profusely, to embryos and thence to plantlets.

Therefore the aim was to follow, separately, the fate of the smaller (i.e. free cells and small aggregates) and the larger units in such cultures for, when they are isolated, the development of the former is liable to be arrested, and to require special morphogenetic stimuli (stages (iva)–(v)), whereas the ability for organized development of the latter (stages (ivb)–(v)) is more apt to be self-induced.

Work with suspended cells and small units graded from stock suspension cultures has, in fact, shown:

(*a*) the rôle of the medium in so far as it may be 'conditioned' by the free growth in it of larger units which spontaneously organize into plantlets;

(*b*) the unexpected rôle of protracted darkness which induces isolated small units, which would otherwise remain arrested, to develop;

(*c*) the fact that, during their development, the potentially embryonic cultures may be arrested (i.e. 'poised'), or again restored to full activity, by suitable but reversible controls over the concentration and composition of the medium. (To arrest growth and development of pro-embryonic units the total concentration of the medium is increased, as by sorbitol, while that of sucrose is reduced; later, and to reverse this effect, the sucrose is restored and the high sorbitol is removed.)

(*c*) *Culture conditions and media*

In this laboratory the conditions that have best fostered the first rapid proliferative growth (i.e. stages (i) and (ii)) of carrot explants occur in liquid nutrient media which are appropriately supplemented by growth factors and which are contained in slowly rotated tubes or flasks

(Steward & Shantz 1955; Steward 1963). These flasks accommodate up to 100 of the carrot explants. The composition of the modified basal medium of White (B_W) (see table 1 of Mott & Steward 1973) when supplemented with 10 % coconut milk (or water, i.e. the liquid endosperm of the nut) induces these explants to proliferate and the slow rotation (1 rev/min) releases some superficial cells into the ambient medium (stage (iii)). The nutrient conditions that promote the rapid proliferation of carrot explants have also permitted liberated cells and small aggregates to grow in suspension and to furnish cultures that may be serially propagated (stage (iv)). But the conditions of culture that procure rapid organized proliferation of carrot or other cultures are not necessarily the best for their organized growth. Therefore, from time to time, advantages have accrued from the following:

(*a*) The use of different basal nutrient media, especially the one ($B_{M.S.}$) described by Murashige & Skoog (1962). This medium has greater total concentration, greater potassium concentration relative to calcium and relies more on reduced nitrogen (NH_4^+) than on nitrate and supplies iron in a different form.

(*b*) The use of synergistic combinations of whole coconut milk (CM) with other growth factors, especially of the auxin type (notably naphthaleacetic acid, NAA) or with casein hydrolysate (CH) as a general source of reduced nitrogen.

(*c*) The knowledge, summarized by Steward & Krikorian (1971 and references there cited), that the complex of growth factors that resides in coconut milk, or other effective fluids such as that from *Aesculus* fruits, can be partially resolved into separately acting, but complementary, synergistic systems. These have been shown to consist, as in system I, of growth factors (AF_1), that require the so-called neutral fraction (NF) which is replaceable by myo-inositol and others (AF_2) that are replaceable by zeatin (as in system II) and auxin (IAA).

The results have shown that the size and form of carrot embryos, developed from free cells and small units, are affected by the osmotic concentration of the medium (Ammirato & Steward 1971), that cultures of free cells of some plants may best be obtained by the use of synergistic combinations of growth factors (CM and NAA; CM and 2,4-D) and that successful and profuse embryo development in otherwise recalcitrant cells may be promoted by sequential treatments involving basal media with different synergistic combinations of such growth factors, or with different levels of growth promoting stimuli, such as $B_{M.S.} + CM + NAA \rightarrow B_{M.S.} + CM \rightarrow B_{M.S.}$, etc. (cf. Steward *et al.* 1967; Steward & Mapes 1971*a*, *b*; Steward & Krikorian 1971 and references there cited).

Whereas most of the evidence on the growth and morphogenesis of carrot cells emerged from liquid suspension cultures the techniques have here made more use of semi-solid agar media in small (50 mm diameter) disposable petri dishes; this permits many combinations of experimental variables to be tested simultaneously. From stock suspension cultures, graded as to unit size by filtration, aliquots are accurately measured and uniformly distributed within the appropriate medium. This technique allows individual treatments to be replicated many times so that representative examples may be arranged for examination and photography. The results are here recorded photographically, with explanatory legends. The visible consequences of the applied treatments to which attention is directed over-ride any minor variations as between replicate dishes or within the crop as grown in any given dish. Thus the aim has not been to compile rigorously quantitative data for, in fact, the criteria of somatic embryogenesis do not yet readily lend themselves to such measurement. Instead an overall visual view of the responses of the system is presented.

2. Factors that control behaviour of the cultures

(*a*) *Clonal variations*

At the first stages (0 through (i) and (ii)) of their responses to exogenous growth factors, explants from different carrot roots show evident clonal variations. The responses of a given clone (figure 1)† of carrot explants on agar media under ten combinations of various factors, are shown after 8 weeks of their growth; the responses of eight distinct clones (figure 2a–h) of carrot explants to those treatments ($B_{M.S.}$ + NAA, $B_{M.S.}$ + CM 1 % + NAA) which, as seen in figure 1, were the most conducive to their organized growth on agar are also shown. It is apparent that marked differences obtained in the ease with which such cultures proliferated or acquired organization, even on the same medium. Nevertheless, cultures derived from each of the eight clones of figure 2 eventually gave rise, albeit at different rates and to different degrees, to cultured units which grew in an organized way (figures 3–7) and from which pro-embryonic stock cultures (figures 8–11) were derived. Thus, stock carrot cultures, grown in liquid, and that are 'potentially embryonic' or 'pro-embryonic', may be established in a variety of ways. These cultures are pro-embryonic in the sense that they contain units, which range from cells to aggregates of varying size, out of which profuse growth in the form of embryos and plantlets (figures 12 and 13) may be obtained.

However, for the purposes of the work here described it was sufficient that stock cultures of a given strain of carrot were grown and activated to the approximate level of stage (iv) of table 1 irrespective of the empirical means adopted for the purpose.

The cultures actually used (as at stage (iv)) were maintained in flasks in 250 ml of medium ($B_{M.S.}$ + NAA), into which they could be so sparsely inoculated that, at the outset, their liquid appeared clear; nevertheless, their rate of growth (at 21 °C in constant light) was such that they developed dense crops which were heterogeneous as to their unit sizes. The units in these crops ranged from free cells to cell clusters, or aggregates, which varied in their need for further inductive treatments to permit their continued organized development. The data are recorded in plate 2 (figures 14–26) which, with explanatory legends, reveal the salient factors that trigger the definitive events formulated in table 1, especially those involved in stages (iv)–(v).

(*b*) *Sizes of units and inocula*

The total amount of growth which pro-embryonic cultures produce and their degree of organization are greatly affected by time and treatment as well as by the size of the units in the inoculum.

Figures 14–16 show the amount of growth obtained with time (figure 15) when the small inoculum of the stock culture (figure 14) was introduced into the culture medium. Figure 16 shows a representative sample (1 %) of the final crop; figures 17–20 show that the units which composed these crops had not only multiplied in the course of 21 days but that they had also formed structures reminiscent of the torpedo stage of normal embryonic development. Figure 21, however, emphasizes that subcultures grew more readily if they included a random sample of the larger units in the stock (> 180 μm, figure 21*a*, *b*) and that less growth was obtained when the units inoculated were smaller (figure 21*c*–*f*); in fact, such growth was not visible, even after 21 days (figure 21*f*) if the units inoculated (fraction 8) were smaller

† Figures 1–69 appear on plates 1–8.

than those (order of 75 μm) retained by the smallest sieves used. (The description of figure 21 relates the sieves by numbers to the unit size of the particles they transmitted or retained.)

(*c*) *Conditioned media and darkness*

The growth of inocula from the stock pro-embryonic culture into fresh culture medium ($B_{M.S.}$) is not solely a function of the unit size of the inocula as in figure 21; this is shown in figures 22 and 23. The smaller units (fractions 6–8, with units in the size range of 150 μm to less than 75 μm as in figure 21*d–f*), which did not respond when they were inoculated into fresh liquid media and cultured in the light (figure 22*b–d*) did grow well in the dark (figure 23*b–d*), in a medium of the same composition ($B_{M.S.}$) but which was 'pre-conditioned' by the prior growth in it of a crop of carrot embryos.

Three prominent factors (size of initial units, darkness, and conditioning of the medium) affect the passage of units from stage (iv) through (v) to (vi). The ambient medium of cultures at stage (iv) becomes 'conditioned' by the progressive development of the potentially embryonic stock. The larger units (figure 24) that this medium contained passed spontaneously (especially on prolonged culture and in the absence of NAA) to the status of pro-embryonic globules and developing embryos (i.e. from stages (iv) to (v)). The smaller units in the mass culture benefited by the 'conditioning' of the medium. The smaller the units that were isolated from the stock culture (cf. figures 24–26), the more they required some further developmental stimuli which were communicated to them by darkness and by the use of a pre-conditioned medium.

The rôle and use of various complex media is a very familiar feature of the culture of mammalian cells (Harris 1964). Although completely definable media have long been the laudable objective for the culture of plant cells and organs, the effects of suitably conditioned media are nevertheless real for they may promote the growth of randomly proliferating plant

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Description of plate 1

Figure 1. The growth and development from small explants (2.5 mg) all of the same clone: representative cultures showing the effect of different conditions after 8 weeks on agar. Calibration: 10 mm.

Aa, B_W + CH (200 p.p.m.)	Ba, $B_{M.S.}$
Ab, B_W + CH + NAA (2 p.p.m.)	Bb, $B_{M.S.}$ + NAA (2 p.p.m.)
Ac, B_W + CH + CM (1 %) in the dark	Bc, $B_{M.S.}$ + CM (1 %) in the dark
Ad, B_W + CH + CM (1 %)	Bd, $B_{M.S.}$ + CM (1 %)
Ae, B_W + CH + CM (1 %) + NAA (2 p.p.m.)	Be, $B_{M.S.}$ + CM (1 %) + NAA (2 p.p.m.)

Figure 2. The range of variation in the response of 8 clones (a–h) of carrot explants on agar after 8 weeks of treatments (Bb and Be of figure 1) which were conducive to their growth and development. Row A, representative cultures showing responses of the 8 clones (a–h) to the treatment Be of figure 1. Row B, representative cultures showing responses of the 8 clones (a–h) to the treatment Bb of figure 1. Calibration: 10 mm.

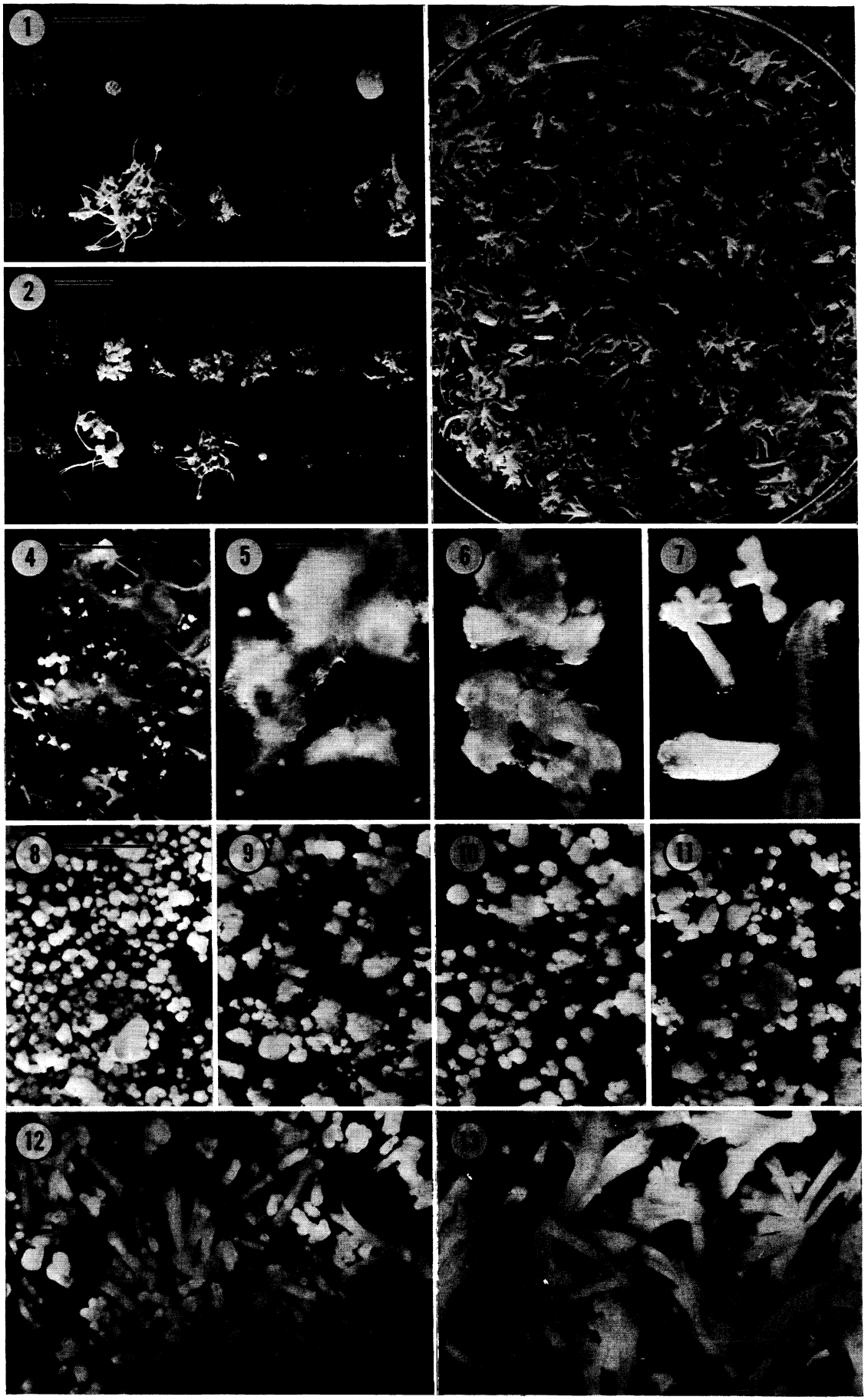
Figure 3. The crop, grown in 250 ml of liquid medium, from a sparse inoculum of organized units from clone f treated as in row A at figure 2.

Figure 4. Early, globular, embryonic forms and young plantlets, as they occurred in a crop such as that shown at figure 3. Calibration: 5 mm.

Figure 5–7. The range of forms, at higher magnification, which can occur in such cultures as in figure 4. Calibration: 0.5 mm.

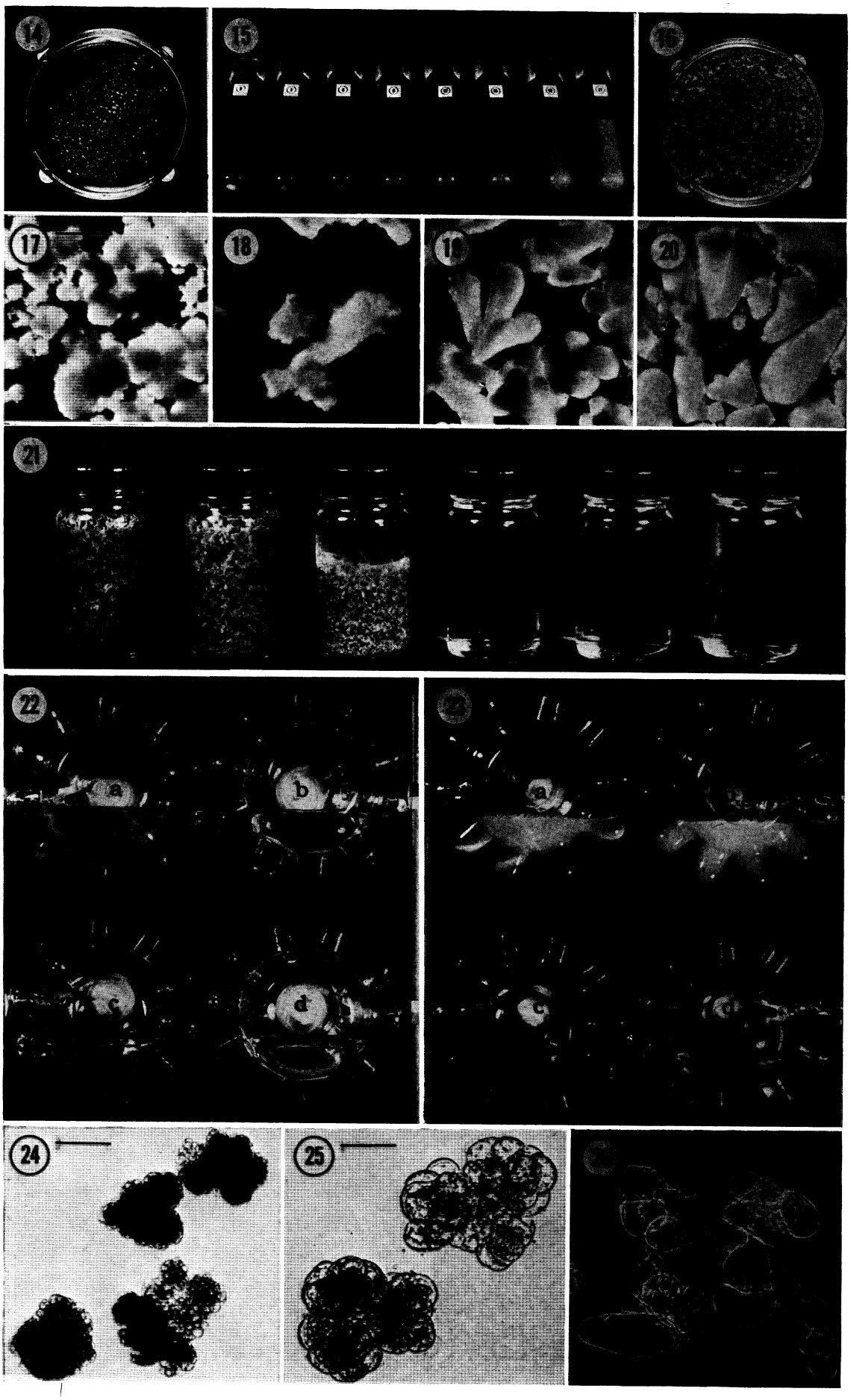
Figures 8–11. Different 'pro-embryonic' stocks, comparable to the smallest units of figure 4, which were derived from different clones of figure 2. Calibrations: 5 mm.

Figures 12–13. The embryos and young plantlets which were produced from the 'pro-embryonic stock cultures' illustrated at figures 9 and 11 respectively (cf. figure 3).



FIGURES 1–13. For description see opposite

(*Facing p.* 40)



FIGURES 14–26. For description see opposite

cultures even on otherwise balanced and complete media (cf. Muir, Hildbrandt & Riker 1958; Blakely & Steward 1964; Stuart & Street 1969; Barker 1970).

Based on the implications of figures 14–26 various other experiments were performed although these will not be described in detail.

The experiments in question utilized similar pro-embryonic stock cultures graded to control the size of their units and, consequentially, the ease or difficulty with which they grew when plated in agar media. The observations showed how the subsequent growth on agar in the light could be related to the composition of the medium, the duration of a prior dark period and the temperature conditions during that dark period. The conclusions were as follows.

A stock culture grown on an appropriate medium ($B_{M.S.}$) could furnish otherwise viable small units (fraction 7), which would not grow in continuous light even in a period as long as 6 weeks. However, the recalcitrance of such small units, toward immediate growth in the light, could be overcome if the agar medium ($B_{M.S.}$) also contained filtered 'conditioned medium' on which an abundant crop of embryos had previously grown in liquid. The 'embryo-conditioned medium' so obtained therefore substituted for the otherwise inductive dark period,

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Description of plate 2

The behaviour of a pro-embryonic stock culture in a medium ($B_{M.S.}$) which supports morphogenesis.

Figure 14. A representative sample, comprising 4 ml of the stock culture in a 5 cm diameter disposable petri dish.

Figure 15. The crops which show the time course of growth in darkness when the stock culture was inoculated (2 ml) into 250 ml of the medium. Each tube, a–h, of diameter 25 mm, contained the total yield from two flasks (i.e. 500 ml of medium initially inoculated with 4 ml of stock culture, as at figure 14) after 0, 3, 6, 9, 12, 15, 18 and 21 days respectively.

Figure 16. A representative fraction (1 %) of the total crop after 21 days (figure 15 h) grown in 500 ml from the total inoculum shown in figure 14.

Figures 17–20. Typical units from the crops shown at figure 15*a*, *d*, *e*, *h*, respectively. Calibration: 0.5 mm. *Note*: there is an obvious transition from small globular aggregates in the original inoculum to more highly organized clusters of embryonic units as the cultures progressed.

Figure 21. The effects on growth when the units of the inoculum were graded as to size by the use of eight sieves. Material was collected by successive use of sieves numbered 1, 4, 5, 6, 7 and 8 which gave fractions with particles of the following sizes > 840 μm, 250–180 μm; 180–150 μm; 150–90 μm; 90–75 μm; < 75 μm. The material as collected on the sieves was aseptically re-suspended in 30 ml of medium; and 2 ml of this was used to inoculate a series of 250 ml liquid cultures which yielded the crops a–f after 4 weeks at constant light and temperature. *Note*: under these conditions negligible growth occurred from units less than 150 μm in size (i.e. 21 d–f); subsequent figures will show how these very small initial units may be induced to grow. In this later work the various fractions of specified unit size are identified by the number of the sieve used in their isolation, i.e. fractions no. 4–8 respectively.

Figures 22–23. The conditions necessary to cause liquid cultures of the smaller units (cf. figure 21 d–f) to grow.

Figure 22 a–d shows the responses of fractions 5–8 respectively in the basal medium after 4 weeks in the light.

Figure 23 a–d shows the responses of the same fractions 5–8 after 4 weeks in the dark in the basal medium supplemented by a medium pre-conditioned by the prior growth of embryos.

Note: whereas virtually no growth occurred in the light from the smallest units (fractions 6–8, figure 22 b–d) a substantial growth did occur from these units in the preconditioned medium in the dark (figure 23 b–d). The somewhat larger units (fraction 5) could grow substantially in the light both with (figure 23 a) and without (figure 22 a) the use of pre-conditioned medium.

Figures 24–26 show representative units of the fractions nos. 4, 7 and 8, used as inocula as in figure 21 b, e and f. Figure 24, calibration: 200 μm; figures 25 and 26, calibration: 50 μm. *Note*. The larger units shown at Fig. 24 grew readily (cf. figure 21 b) whereas the development of the smaller less organized ones, as at figures 25 and 26, was arrested and they only grew under the more special conditions of figure 23 c and d.

however, media conditioned by randomly proliferating carrot explants were not as efficient, in this respect, as those in which a copious crop of embryos had grown.

The small units (fraction 7) were less demanding of prior darkness on some media ($B_W + CH$) than on others ($B_{M.S.}$) and, therefore, they were then less responsive to the conditioned media although their growth was not so well organized. The inductive effect of darkness upon the subsequent growth of small units (fractions 7 and 8) in the light did not occur at a lowered temperature (4 °C). Presumptively some metabolism was necessary in the induction during the dark period. The minimum duration of the effective dark period was, in turn, a property of the stock in question but for the one most investigated, its effect was completed between 6 and 14 days after a prior period of 6 weeks in continuous light.

Small units (fractions 7 and 8) retained their ability, without growth, during a long period in the light to respond to a period of dark induction and this could be administered at different times along the quiescent period in the light.

In retrospect, therefore, one can now appreciate why, in an earlier study of the behaviour of isolated cells on agar media, some cells unaccountably remained quiescent for very long periods even though eventually they could spring into growth unpredictably (Blakely 1964).

The conclusions referred to above are both confirmed and consolidated by the evidence in plate 3 (figure 27) for this presents, in a Latin-square diagram of 30 treatments, results which show how (*a*) the degree of conditioning of the medium (due to the prior growth upon it of embryos) and (*b*) the exposure to prior darkness, break the inability of small graded carrot units to grow in the light on the appropriate basal medium ($B_{M.S.}$). Thus the effects attributable to darkness and to the use of the conditioned medium are supplementary when they interact.

(*d*) *'Poised' cultures: development arrested and released*

The course of development (stage (iv)–(v)) may be reversibly arrested and restored, whether it passes through the larger units (that develop spontaneously) or the smaller ones (that need to be induced). When the course of the development is arrested, the cultures are said to be 'poised', for they will resume their growth and development when the appropriate conditions are restored, as indicated below.

Description of plate 3

Interactions of environmental factors and media upon the behaviour of a graded pro-embryonic stock culture.

Figure 27. This symmetrical design shows the interaction between the degree to which the medium was 'preconditioned' and the duration of an initial dark period upon the subsequent growth (total period 56 days) of units of fraction 7 in the light. The different degrees of conditioning were obtained by adding the medium taken from cultures of embryos grown from 0, 3, 6, 9 and 12 days to the plates shown at (a) to (e) respectively. The effects of the initial dark periods (0, 1, 2, 4, 8 and 16 days) are shown at rows A–F respectively.

Note: on normal, i.e. unconditioned medium, column a, a long period (8 to 16 days) of initial darkness was necessary to induce embryonic growth (Fa). Under continuous light (row A) a well-conditioned medium (Ae) was necessary to induce embryonic growth without the stimulus of darkness. Conversely, a longer period of darkness enabled these cultures to develop in the unconditioned medium (Fa). In combination, shorter periods of dark induction interacted with less intensively conditioned media to promote the development of the cultured units (e.g. Eb, Dc, Cd). Thus the effects due to preconditioning the medium, and to the period of initial darkness, interacted positively when in combination, to release the growth potential of the small units.

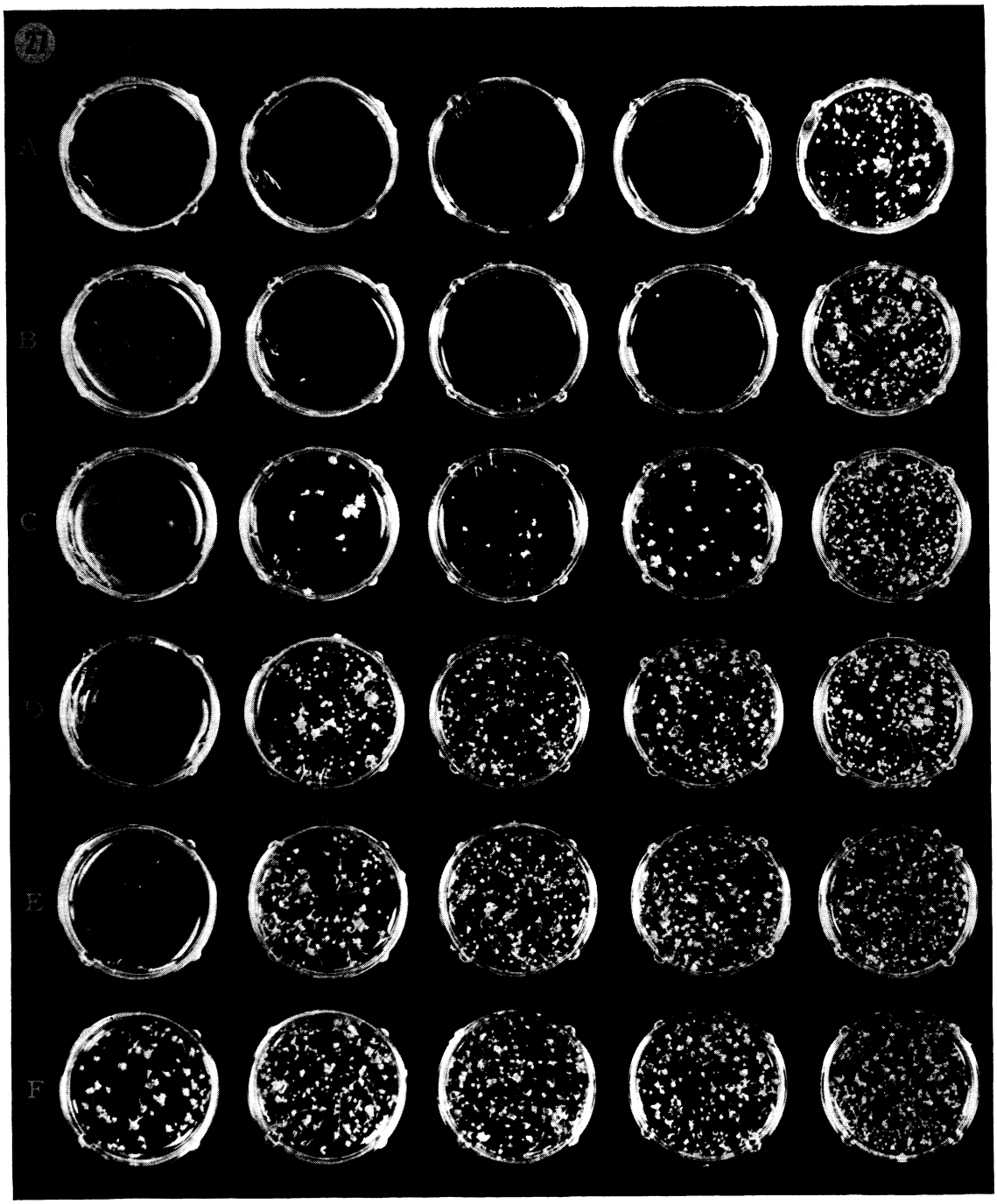
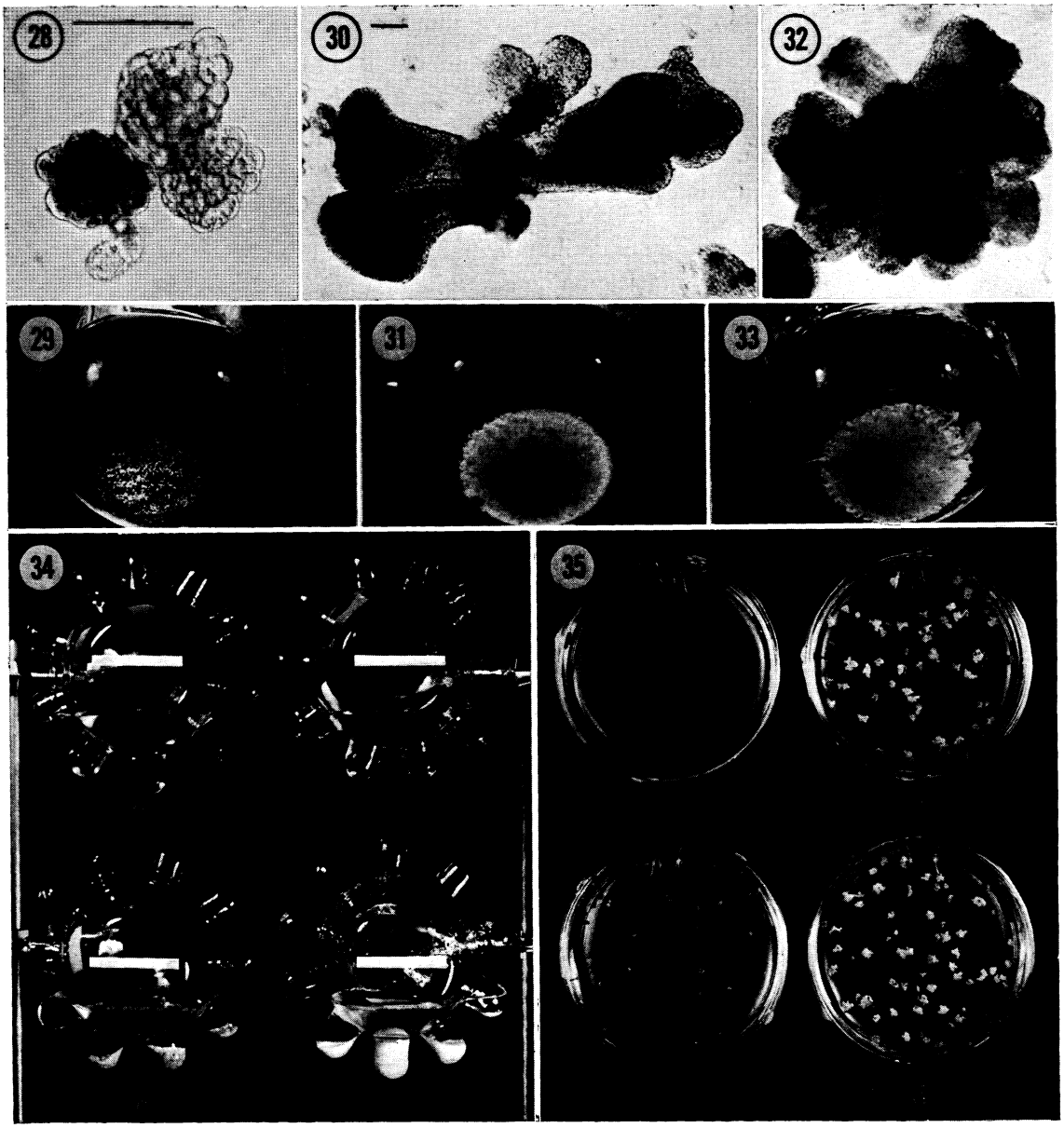


FIGURE 27. For description see opposite

(*Facing p.* 42)



FIGURES 28–35. For description see opposite

By increasing the total concentration, by using a metabolically relatively inert solute (e.g. sorbitol), and simultaneously reducing the concentration of sucrose, the potentially embryonic cultures are arrested in their development (cf. plate 4, figure 34*a* and *c*; figures 30 and 31 with 28 and 29 and with 32 and 33). Pro-embryonic clusters (as at figure 28), which were intrinsically capable of growth and development (as at figures 30 and 31 or 32 and 33), were precluded from doing so by the concentration of sorbitol and lack of sugar in the medium. The cultures in the state of arrested development (stage (iva), as at figure 28) were 'poised' in that they merely awaited the appropriate signal (by removing sorbitol and replenishing sugar) to develop. Even when sparsely inoculated cultures are poised (as described), they may also be stimulated by darkness during this period so that, when subsequently released (i.e. by removing sorbitol and replacing sugar), their growth in the light (stage (iva)–(v)) may far outstrip that of cultures which received no dark induction (cf. figure 34*c*, *d*).

Description of plate 4

'Poised' cultures: treatments which reversibly arrest and release development from pro-embryonic cultures.

Figures 28–33. The combined effects, after 6 weeks of a depleted carbon source (sucrose) and an increased osmotic pressure (via sorbitol), which may 'poise' the cultures, i.e. arrest the development even of the units of an ungraded stock culture.

Figures 28 and 29. Typical units 'poised' and which remained unchanged for 6 weeks in the light on a basal medium ($B_{M.S.}$) with sorbitol (3 %) *in lieu* of sucrose. Figure 29 shows the total crop on 10 ml of medium in a culture tube. Figure 28, calibration: 100 μm.

Figures 30–31. Units (cf. figure 28) which were previously poised for 2 weeks and subsequently developed in the medium ($B_{M.S.}$) with sucrose in the light for 4 weeks.

Figure 31 (cf. figure 29) shows the total crop grown, on 10 ml of medium, in a culture tube.

Figures 32–33. The units which developed during 6 weeks in the light on a medium ($B_{M.S.}$ with sucrose) which did not arrest their development, i.e. they were not poised.

Figure 33 shows the total crop grown (cf. figures 29 and 31) on 10 ml of medium in a culture tube.

Note: all the units, irrespective of size, may be reversibly arrested (i.e. 'poised') by the use of sorbitol *in lieu* of sucrose and they may be released for growth by replacing the sorbitol with sucrose. The requirements of the smallest units for darkness and preconditioned media still obtained after they were subjected to poisoning by sorbitol.

Figure 34. This figure shows, by the growth in flasks after 5 months, the interaction of darkness with conditions that poise cultures inoculated with units of the ungraded stock.

Figures 34*a*, *c*. These cultures were poised in the light for one month; sucrose released the development of (*c*) and the growth is shown after a further 4 months in the light, while (*a*) remained in sorbitol as the control.

Figures 34*b*, *d*. These cultures were poised in the dark for one month; sucrose released the development of *d* and the growth is shown after a further 4 months in the light, while (*b*) remained in sorbitol as the control.

Note: although development was arrested during 'poising' (figures 34*a*, *b*), nevertheless darkness during this period enhanced subsequent growth when units were released by sucrose in the light (cf. figure 34*d*, *c*).

Figure 35. Shows the growth after 5 weeks on a fully conditioned agar medium inoculated with units of fraction 4 from the stock culture. Dark induction during the first 3 weeks at 1 °C was not effective in promoting the subsequent growth during 2 weeks at 25 °C in the light (*a*), although, when the dark induction was at 25 °C it was effective (*b*). Cultures that had received the cold treatment in the dark at 1 °C and subsequently did not grow in the light, as at (*a*), could nevertheless respond to dark induction and grow if the 2 week period at 25 °C was in the dark as at (*c*), although dark induction and growth at 25 °C throughout the full 5-week period, as at (*d*), was much better.

Note: although both dark induction and growth were suppressed at 1 °C, the cultures remained viable and, if subsequently dark induced, their growth in the light or in the dark was a function of time and temperature.

Thus, there is a dark inductive effect which promotes growth and morphogenesis (especially when the units are small); there is a 'poising' effect, which allows further development to be arrested and this is reversible. Furthermore, the morphogenetic induction, which is brought about even in poised cultures by darkness and is subsequently expressed in the light, requires a minimum temperature for whatever chemical change this induction involves; below this minimum temperature darkness is morphogenetically ineffective (cf. figure 35*a*, *b*), although the low temperature did not impair the viability of the cells or preclude a later dark induction at 25 °C (figures 35*c*, *d*).

The combined inductive effects of darkness and of the treatments which poise, or arrest, development may be sustained for long periods of time. Sparsely inoculated cultures that had not received a dark inductive treatment have been maintained in this non-induced, arrested, state for as long as one month, during which period they could be successfully induced by the appropriate dark treatment and then released for development (figure 34).

3. Aspects of growth and form in carrot cultures

(*a*) *Various responses to treatments*

Plate 5, figures 36–52, with their descriptions, summarizes the range of responses induced by the specified treatments. Cultures were sparsely inoculated in liquid media, in rotated tubes or culture flasks, or after inocula were distributed within a thin-layer of semi-solid agar medium. As shown, growth and morphogenesis ensue both in liquid and on semi-solid agar culture media, granted that the cultures receive and are capable of responding to the appropriate stimuli.

Inocula from uniformly graded (fraction no. 7) stocks could be so sparse that the cultures as inoculated seemed free of viable material (figures 36, 38); nevertheless they produced visible crops in 28 days (figures 37 and 39). As this occurred the small, uniform cell clusters

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Description of plate 5

The effects of specified treatments on growth and morphogenesis: a summary.

Figures 36 and 37. These photographs show a single tube, 3 cm in diameter, which has been inoculated in 10 ml of preconditioned liquid medium with units of fraction 7 (figure 36) and 28 days later (figure 37) after growth in the dark.

Figures 38 and 39. The effect of the same treatments as for figures 36 and 37 carried out concurrently on a semi-solid agar medium in petri dishes 5 cm in diameter.

Figures 40–43. These figures show, microscopically, the units that relate to the treatments of figures 36–39.

Figure 40 shows units of the initial inoculum and figures 41–43 show their subsequent embryonic development as in the crops seen in figures 37 and 39.

Figure 40, calibration: 250 μm; figure 41, calibration: 1 mm; figures 42 and 43, calibrations: 0.5 mm.

Figures 44–47. Details of initial globular units (cf. figure 40) and of their living cells as seen at higher magnification and at various phase-optical sections.

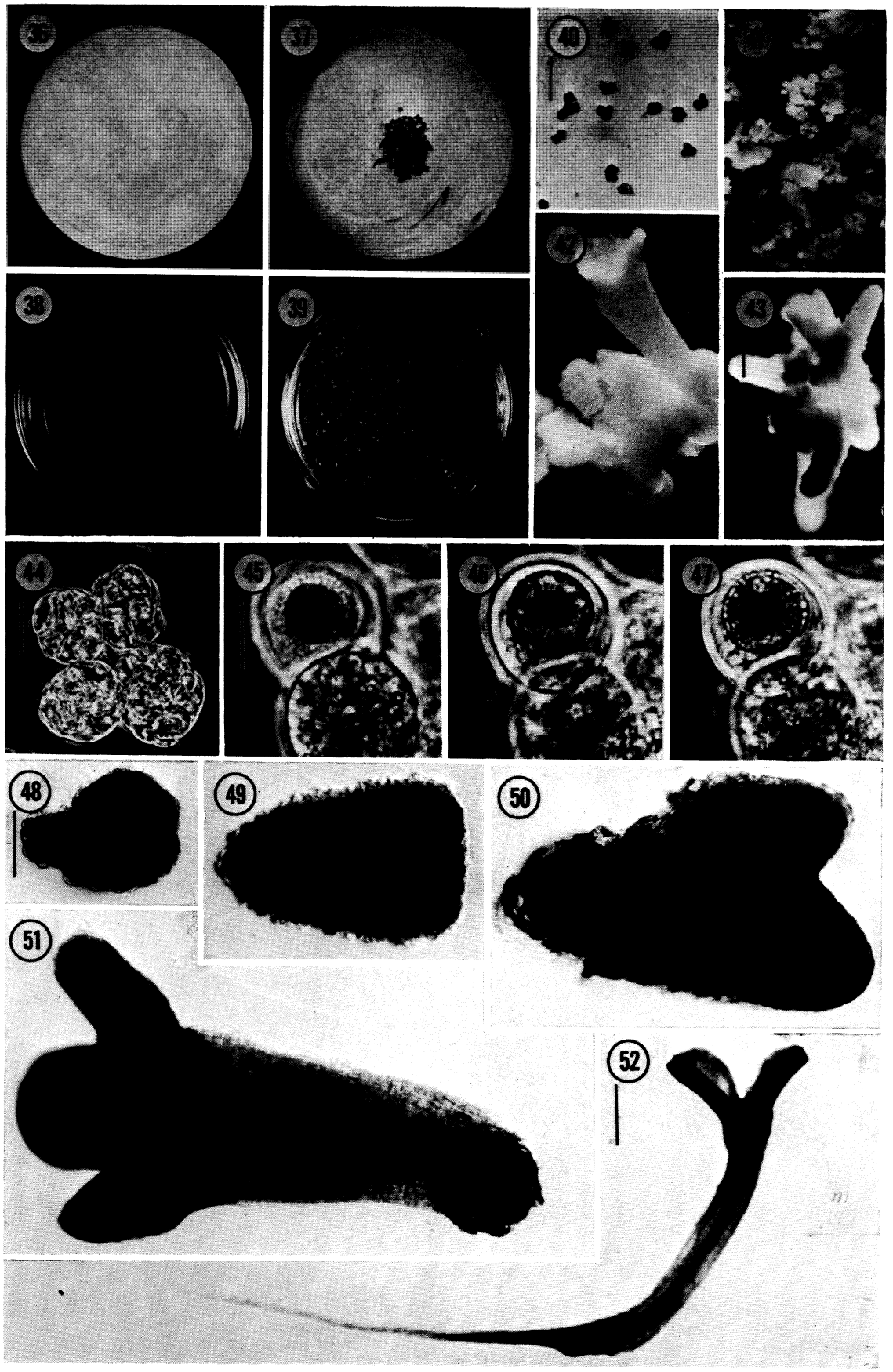
Figure 44 shows a cluster of 4 spherical pro-embryonic globules;

Figures 45–47 show successive phase-optical sections of small spherical, densely protoplasmic, 'egg-like' cells.

Figure 44, calibration: 35 μm; figures 45–47, calibration: 10 μm.

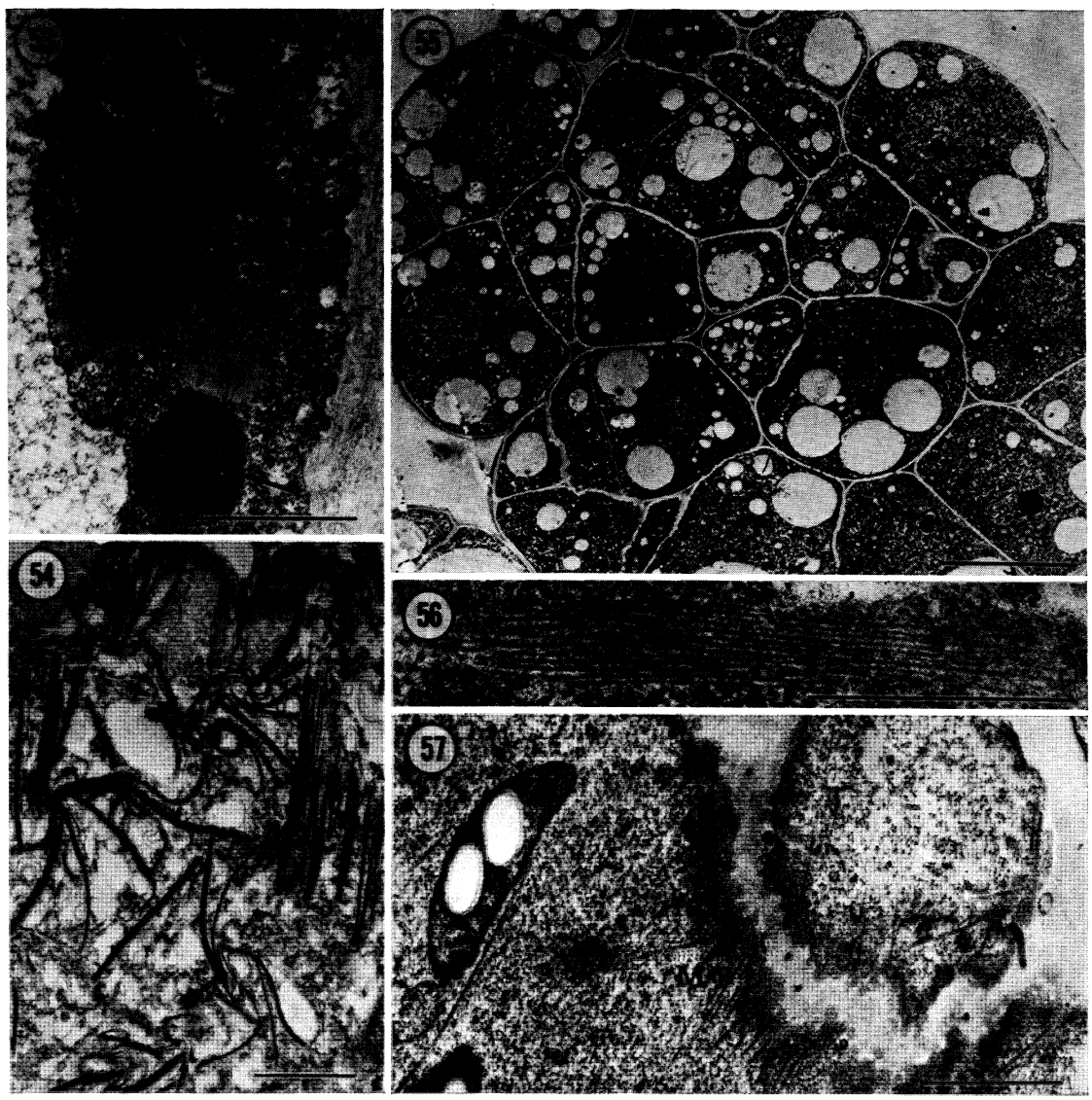
Figures 48–52. Embryonic growth forms, traceable to the globular units seen at figure 44 and which developed under the conditions that have been described.

Figures 48–51, calibration: 0.1 mm; figure 52, calibration: 0.5 mm.



FIGURES 36–52. For description see opposite

(*Facing p.* 44)



FIGURES 53–57. For description see opposite

or aggregates (figure 40) gave rise to groups of variously organized forms (figures 41–43). These clusters remained loosely attached on a semi-solid medium but they tended to separate and develop independently in the slowly rotated liquid cultures. The small pro-embryonic globules, which developed especially well in conditioned media after dark induction, are illustrated in figure 44 and their densely cytoplasmic, thin-walled, relatively unvacuolated cells with their organelles (which in life exhibited a characteristic condition of activity best described as one of great 'agitation') are also illustrated in figures 45–47. The subsequent progress of such pro-embryonic globules, through stages of development that parallel the familiar growth forms of zygotic embryos, may be seen in figures 48–52. Moreover, it has been shown that such forms may be produced in great abundance and more or less continuously (Steward 1970*b*). Cultures in which this occurs may respond to their environment and nutrition by normal or abnormal growth forms (Ammirato & Steward 1971) or by steps that are analogous to premature germination as they produce plantlets with expanded adult leaves directly (cf. Steward *et al.* 1970, Fig. 2) without the prior formation of 'cotyledonary' embryos or of juvenile leaf forms.

(*b*) *Characteristics of cells in relation to morphogenesis*

The cultured cells (cf. figures 45–47) which best lead to a simulated pro-embryogeny have departed very far from the mature form and composition of those in the plant body. In fact they resemble primordial cells more than parenchyma. A curious but interesting aspect of this contrast between normal differentiated somatic cells and their pro-embryonic counterparts is in their respective responses to osmotic stress. Table 2 shows that the former, i.e. the vacuolated cells of growing but unorganized carrot explants, maintain their high internal osmotic values despite wide differences in the concentration of their ambient media. By contrast, the pro-embryonic units maintain an internal osmotic value which corresponds closely to that of the external medium.

But the question arises whether there are other visible signs in the protoplasm that distinguish the cells that have acquired the status of cells that are freely capable of morphogenesis. Earlier reference has been made to the fine structural characteristics of cells induced to grow and divide as at stage (i); it would be helpful if there were discernible

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Description of plate 6

The fine structure of cultured carrot cells under treatments conducive to growth and morphogenesis.

Figure 53. Cells as cultured on a basal medium ($B_{M.S.}$) showing cratile (grating-like) inclusions in the cytoplasm. Calibration: 1 μm.

Figure 54. Cells cultured on a basal medium (B_W) supplemented by IAA, zeatin and inositol showing the abundant rotate (pin-wheel-like) inclusions in the cytoplasm. Calibration: 1 μm.

Figure 55. Cells of a pro-embryonic globule as at table 1 stage (ivb) (cf. figure 44). Calibration: 10 μm.

Figure 56. Detail of multifibrillar bundles as seen in the cytoplasm of a cell of a pro-embryonic globule (cf. figure 55). Calibration: 0.5 μm.

Figure 57. Portions of cells developing embryonically, as at table 1, stages (iv)–(v), showing abundant microtubules in cytoplasm free of the other special inclusions (cf. figures 53, 54, 56). Calibration: 1 μm.

Note: figures 53–57 show, at the fine structural level, the varied use that cultured cells may make of the same genetic information and that this may be modulated by growth regulatory substances and environmental factors in ways that control both complex synthesis and resultant forms.

characteristics of cells as at stages (iv) and (v). Other work from this laboratory (Wilson, Israel & Steward 1974) has addressed itself to this problem.

Experience shows that the cells which are stimulated to grow and to develop into plantlets via pro-embryonic globules (i.e. as at stage (iv b) are as free from unusual cytological inclusions as embryonic cells normally are of stored secondary plant products. This is shown by figure 55, plate 6. If, however, the carrot cells receive a more partial and imbalanced stimulus to grow, e.g. by the use of IAA + zeatin + inositol additional to a basal medium (B_W), they may show a rich array of inclusions other than the well-known organelles which have their known functions (figure 54, plate 6). In carrot tissue these special inclusions may take various forms. The relevant inclusions may consist of grid-like (cratile) crystalline inclusions (figure 53), of rotate (pin-wheel-like) inclusions borne on 'stalks' or filaments (figure 54) or of multifibrillar bundles (figure 56); the latter may be present in activated cells awaiting, but not yet released

TABLE 2. A COMPARISON OF THE OSMOTIC VALUES OF UNORGANIZED AND OF POTENTIALLY EMBRYONIC CARROT CULTURES IN RESPONSE TO DIFFERENT TREATMENTS

		osmotic values (mosmol/l)	
cells	culture media	media	cellular fluids†
of explants	B_W + CM	180	459
	$B_{M.S.}$ + CM	320	452
of pro-embryonic units	$B_{M.S.}$ + NAA + sorbitol	152	170
	$B_{M.S.}$ + NAA + sorbitol	256	286
	$B_{M.S.}$ + NAA + sorbitol	393	388

† These fluids are made up of both inorganic (K^+, Na^+, Cl^-) and organic solutes (sugars, organic acids and soluble-N compounds, etc.), the latter range from about 70–80 % of the total solutes.

DESCRIPTION OF PLATE 7

The appearance and behaviour of free protoplasts isolated from pro-embryonic globules as seen under the phase and electron microscopes. Figures 58–63, phase microscope; figures 64–67, electron microscope.

FIGURE 58. Representative free protoplasts as they emerged from the pro-embryonic globules (cf. figure 44) after 4 hr of contact with a cell wall digesting enzyme in a medium of low osmotic value (50 mosmol/l). Calibration: 20 μm.

FIGURE 59. A representative, uninucleate, unvacuolated, free protoplast (cf. figure 58 and the cells of figures 45–47) maintained in a medium with added mannitol. Calibration: 10 μm.

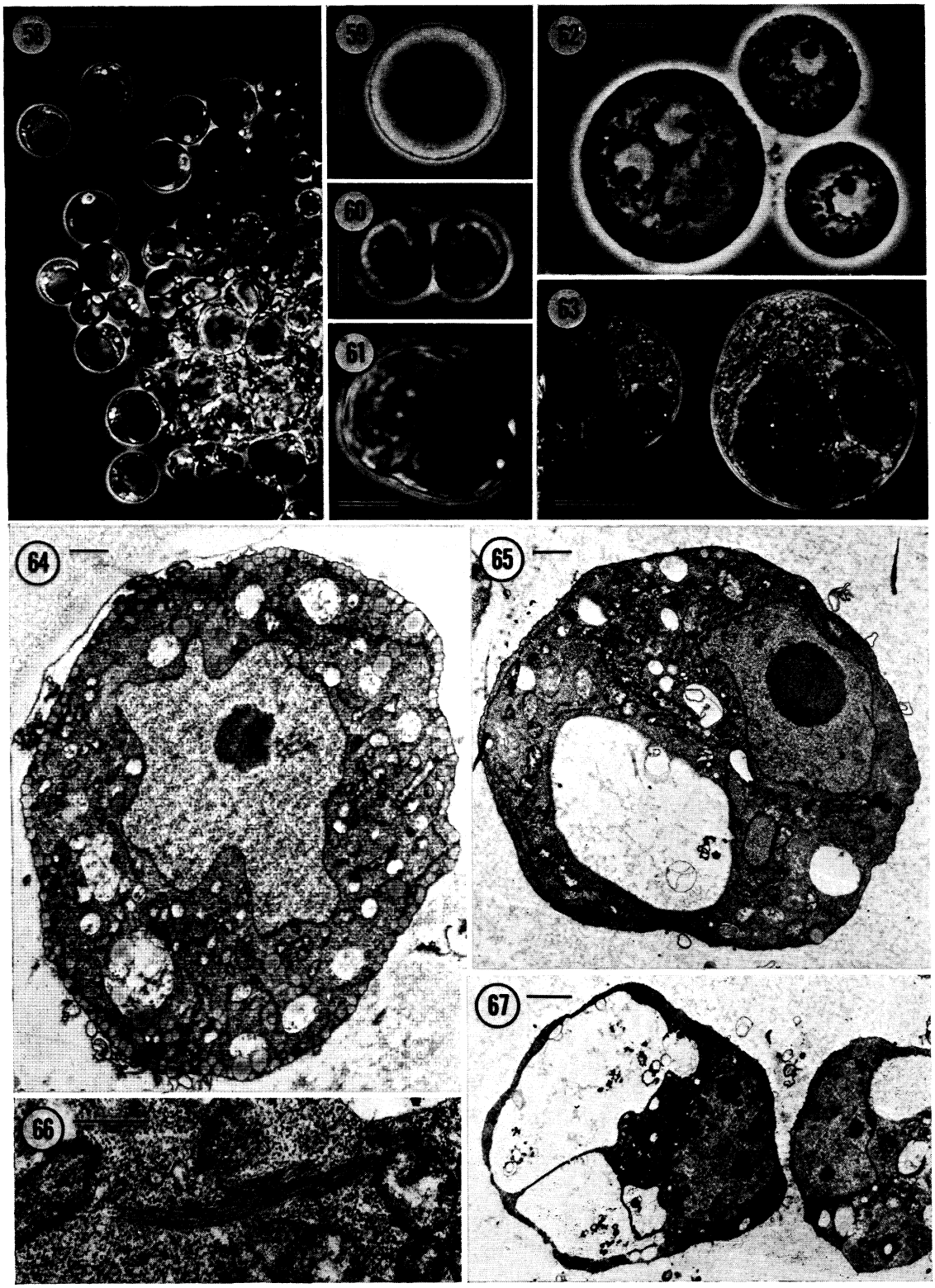
FIGURES 60 and 61. Show divided protoplasts existing in a medium of relatively high osmotic value. Calibrations: 10 μm.

FIGURES 62–3. Show degrees of aggregation of protoplasts by evident fusion.
Figure 62 shows single and fused protoplasts in the same suspension. Calibration: 10 μm.
Figure 63 shows colonies by multiple fusion of protoplasts. Calibration: 20 μm.

FIGURES 64–65. Representative 'normal' protoplasts from a preparation made from globules as shown in figures 44 and 55. Calibration: 1 μm (× 6000). Note the near spherical shape, the single nucleus with prominent nucleolus and the small vacuoles.

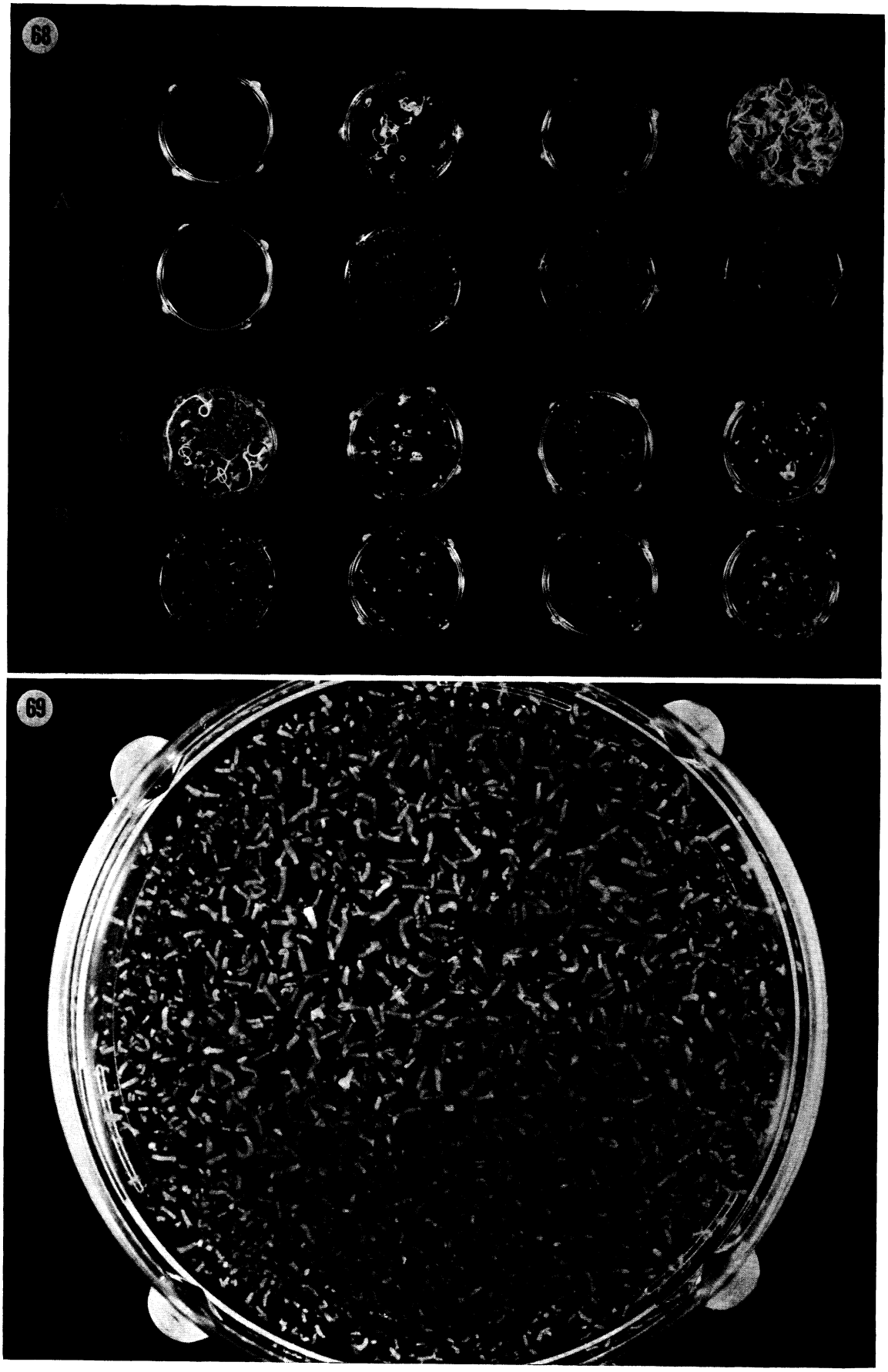
FIGURE 66. A portion, enlarged, of typical cytoplasm in protoplasts as at figures 64 and 65, showing some residual presence of multifibrillar bundles. Calibration: 0.25 μm (× 50000).

FIGURE 67. Representative 'abnormal' protoplasts from the same preparation as that which yielded the 'normal' globules of figures 64–65. Calibration: 2 μm (× 36000). *Note*: such abnormal features as the irregular shape, larger vacuoles, fragmented nucleoli, are also to be seen in multinucleate fusion products as shown in figure 63.



FIGURES 58–67. For description see opposite

(*Facing p.* 46)



FIGURES 68 AND 69. For description see opposite

for, morphogenesis, and they tend to disappear when that release occurs (figure 57). Thus, from cells in the carrot root, or on a basal medium not conducive to rapid growth, there may be a progression of special inclusions depending upon the medium in question and the degree to which growth and development are stimulated. However, the most characteristic feature seems to be that the cells most capable of, and engaged in, morphogenesis are rich in microtubules (figure 57), but they are then very free from other special cytoplasmic inclusions that can otherwise occur. While these observations are indicative of interesting changes in cytoplasmic organization that accompany growth induction and morphogenesis, it is difficult at this point to assign causal significance to them; this requires further work and understanding.

(*c*) *Behaviour of free protoplasts from pro-embryos*

An even closer zygotic parallel would obtain if the pro-embryogenic cultured cells stripped of their thin cellulose walls could re-commence their development, like fertilized eggs, as freely suspended naked protoplasts. Indeed, it is interesting that other investigations of the authors have shown that the pro-embryogenic globules (as at stage (iv) of table 1, and as seen in figures 44–47, plate 5) may produce small, free spherical protoplasts in large numbers (figure 58, plate 7). These protoplasts were successfully obtained from the cultured cells by following the method communicated to us by Professor E. C. Cocking (cf. Cocking & Evans 1973). The protoplasts, like the cells of the pro-embryonic units from which they were derived and unlike the proliferated cells of explants, were unexpectedly resistant to osmotic stress (table 2), for they seemed to exist isotonically in media of a wide range of osmotic concentrations; moreover, they remained viable in liquid and in or on agar media for long periods of time. However, despite their stability and longevity, these protoplasts (figure 58, plate 7, *et seq.*) have not yet responded to the conditions which suffice for the growth and morphogenesis of the cells from which they were derived. Although occasional protoplasts (figures 60 and 61, plate 7) have shown signs that they may have divided, or re-formed some wall, their frequency is so low that it is even open to question whether the walls of these particular cells had been completely removed. Moreover, the infrequent presence of two nuclei (cf. figure 62) in the protoplasts may be explained by the fusion of protoplasts which readily occurs (figure 63). However, until one can induce further growth and development from the free protoplasts

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Description of plate 8

Growth and morphogenesis in cultures of carrot cells derived from seedlings: responses of cultivars and effects of environment.

Figure 68. Behaviour of cells from cultures of different cultivars, shown in columns 1–4 (1, Imperator Long; 2, Gold Pak; 3, Royal Chantenay; 4, Scarlet Nantes), which were originated from seedlings pre-grown in the light (A) or in darkness (B) and as subsequently cultured in darkness, rows (a) or in the light, rows (b), in 50 mm diameter plates sparsely inoculated with small units. All the cells were grown initially in liquid ($B_{M.S.}$ + CM 10 % + NAA 2 p.p.m.) and were then plated into an agar medium containing $B_{M.S.}$ + CM 10 %.

Note: light-grown seedlings of Imperator Long yielded cells with minimum ability to grow in agar whether in the light or in darkness, whereas dark-grown seedlings yielded units that could grow in the light though they responded even better in darkness. Cultures established from the other cultivars showed intermediate degrees of response to the environment.

Figure 69. Shows a large number of young somatic embryos after they had developed in darkness, at a high plating efficiency, from small units and within a shallow layer of agar medium, in a dish of 50 mm diameter during 19 days. Note the profuse crop of relatively uniform embryos.

as consistently as in the cells from which they are derived, problems will remain to be solved before such systems will be as useful as it has been claimed (Colloques International CNRS. No. 212, 1973). It is true that reports have been made (Takebe, Labib & Melchers 1971; Nitsch & Ohyama 1971; Grambow, Kao, Miller & Gamborg 1972; Kameya & Uchimiya 1972) that some plants can be reared from naked protoplasts isolated from certain somatic cells (e.g. of tobacco and of carrot), and even that somatic hybrids via protoplasts may be made (Carlson 1973; Melchers & Labib 1974). But in most of these situations (see, for example, Grambow *et al.* 1972; Kameya & Uchimiya 1972) a copious production of living protoplasts gives rise to but very few organized derivatives. Moreover, the latter, whether as embryos or plantlets, form *after* the free protoplasts have re-formed walls and have produced proliferations within which the organized structures form adventitiously. In other words, a direct somatic embryogenesis does not occur.

The outstanding and unexpected feature of work with isolated protoplasts from otherwise 'morphologically competent' cells is the extremely low frequency of their subsequent development. An idea was that the inhibited development of these protoplasts might be associated with adverse accumulations of the kinds of inclusions shown in figures 53 and 54. However, fine structural studies on populations of protoplasts prepared from pro-embryonic globules (cf. figure 44) showed (figures 64–66) good nuclei, with prominent and intact nucleoli, with few and small vacuoles and without the aberrant inclusions (cf. figure 55). Nevertheless other protoplasts in the same preparation did show features (such as increased vacuolation, irregular non-spherical shapes, and prominently fragmented nucleoli as shown in figure 67). However, the relative frequencies of the seemingly 'normal' protoplasts (as in figures 64 and 65) and the more 'abnormal' protoplasts (as in figure 67) were of the same general order. Therefore, the very low frequency of development in protoplast populations, in comparison with populations of free cells or of the cells *in situ* in pro-embryonic globules, cannot yet be attributed to any known and visible features of their fine structure.

4. Totipotency, its release and restriction: concluding remarks

(*a*) *Somatic cells and zygotes*

This paper re-emphasizes that morphogenetic propensities in zygotes may remain intact even in mature quiescent cells of angiosperms. This inherent totipotency has now been demonstrated by many somatic recapitulations of normal zygotic embryogeny. Nevertheless, zygotes in their embryo sacs and ovules develop with a degree of infallibility which cannot yet be emulated, even in the most favourable cases, in cultures of somatic cells. Moreover, there are many plants which yield cultured cells that are still morphogenetically recalcitrant.

The reversible arrest of the early development of somatic carrot cells into embryo-like forms, here called 'poising', is reminiscent of the frequently observed dormancy or arrested development of zygotic embryos *in situ*. Such arrested development may occur when an embryo is morphologically immature (as in the coconut) and it must await a later stimulus prior to germination; or it may be a feature of embryos of many semi-parasitic flowering plants which are often shed in a relatively undeveloped state (cf. Figures 2.7, 2.8, 2.9, 4.5 of Steward 1968). Similarly, the inductive effects of darkness in the morphogenetic development of free cells are noteworthy because zygotes in ovules normally develop out of direct contact with light.

The dark induction of the growth of suspended carrot cells and small units recalls Yeoman's

work on the light-inhibition of cell division in Jerusalem artichoke explants (Fraser, Loening & Yeoman 1967; Yeoman & Davidson 1971). This inhibition seems, however, to be observable only when the induction of that growth is brought about by 2,4-D rather than by more complex stimuli such as coconut milk and, in the artichoke system, the dark response occurs more promptly (i.e. after 30–56 h).

Yeoman found that the growth of artichoke explants was suppressed if they were removed from the tuber in the light, although this did not occur if this operation was performed in the 'physiological darkness' of a green or appropriately 'safe' light. However, artichoke explants isolated and cultured in this laboratory in the light, on a medium ($B_W + CM$) and under conditions similar to those used for carrot explants, have consistently grown vigorously; thus, the light inhibition of artichoke cells seemed surprising. It is, of course, possible that a light inhibition to which attached carrot cells of explants may be subject may have passed unnoticed under the culture conditions used, because it could have been overcome on this medium ($B_W + CM$) during the so-called 'lag period' of 4 days. The lag period produces growing cells *en masse* in the explant and permits them to develop, metabolically, the conditions or circumstances that sustain their later growth and, in so doing, any light-induced inhibition of the surface cells, that might otherwise occur, could be overcome. But the free cells and small units that lack organic contact with this activated mass of cultured tissue are demonstrably and easily light suppressed, so that they need to be dark-induced, or released to grow, by the contributions which larger cultured units, aided by suitably conditioned media, can contribute.

While many morphogenetic effects of light are familiar, the formative effects due to short days are often attributable to long uninterrupted daily periods of darkness. There is also a precedent for morphogenetic induction by darkness in the work of Werckmeister (1970, 1971) on orchid cultures. In these situations, it is possible that long exposure to darkness may inactivate light-induced inhibitors such as xanthoxin, or even the substance heliangine, which, like light, suppresses growth by elongation (for references see Steward & Krikorian 1971, pp. 99–101). Xanthoxin is a powerful inhibitor, which resembles abscisic acid which, in turn, is known to interact with the growth factors of coconut milk and with gibberellic acid (cf. Fig. 8 of Steward 1970*b*) in their effects upon carrot cells.

Even so, the dark effect presents a paradox. After the primordial organs of embryos have developed normally as in ovules, without direct contact with light, though ultimately nourished by the parent sporophyte in the light, their *further* development obviously does require light for the formation of new leaf primordia and the minimal development of those pre-formed in the embryo. The paradox, therefore, is that *early* embryogenesis, as from carrot cells *in culture*, is fostered by darkness, although the later heterotrophic development of the pre-formed embryos specifically requires light.

(*b*) *Totipotency versus diversification*

Our ability to evoke in culture any feasible feature of the normal growth, form and composition of cells *in situ* is still the measure of our ability to apply external stimuli that successfully emulate the signals that cells must receive during normal development. These complex interactions have involved multiple growth regulators in synergistic and sequential combinations; the composition and total concentration of the basal medium with respect to trace elements (e.g. Fe), sources of nitrogen (NO_3^-, NH_4^+ or organic nitrogen) and environmental factors

such as those of light, darkness and temperature in diurnal periodicities. The possible combinations are here too numerous for the optimum conditions in any given case to be arbitrarily selected. One senses, intuitively, that if one could recapitulate all the characteristics of the embryo sac and ovule in the environment of cultured, free, totipotent cells, the problems of somatic embryogenesis would be fully solved. The devices which have been described are but empirical means to this end.

It has long been known that embryos in immature seeds, as of carrot (Steward *et al.* 1963), are effective sources of totipotent cells – even more readily so than the parenchyma of mature organs. It is, therefore, relevant here that the most lavish production of somatic embryos and plantlets, with least demands for inductive stimuli, has occurred in suspension cultures grown from tissue segments cut from aseptically germinated seedlings. Graded preparations from such cultures, containing only very small units, dispersed within agar media have given rise, with very high plating efficiency, to carrot embryos and plantlets. This has been repeated despite the complications of selecting active strains from a different range of cultivars and working in a different laboratory setting with different personnel (figures 68 and 69, plate 8). However, the ease in the use of seedlings or embryos for clonal propagation of individual plants that have desirable features is lost where seeds are not produced and is defeated in those cases in which undesirable segregation occurs. Therefore, one should still face and overcome the greater difficulties encountered when isolated cells or tissue explants from *mature* organs are the sources from which pro-embryonic suspension cultures are to be derived.

Particular stages or events that occur normally in cells *in situ* are not yet reproducible *at will* in cultures of free cells, although the quiescent tissues and cells may be progressively restored to physiological activity in culture and the restored activities of the cultured cells may even be directed along a course that recapitulates normal embryogeny. But the progress of these events may be reversibly arrested and restored by suitable chemical control of the medium, even as their course may be caused to bypass certain otherwise normal steps in development or to accentuate them abnormally (cf. Fig. 2 of Steward *et al.* 1970).

Thus, cell cultures which are at present recalcitrant, in which growth is sluggish or in which organization does not occur, may eventually respond to the appropriate balance and sequence of factors and to the appropriate environments to induce growth and to control morphogenesis. In due course, no doubt, chemical alternatives to 'conditioned media' will be found, even as the paradoxical dark induction of morphogenesis in the smallest cultured units may be resolved.

Perfected and generally applicable procedures for somatic embryogenesis from cells in culture would provide systems for the investigation of development and, in selected cases, be a valuable means of clonal micropropagation at the cell level. Even so the ability to control, at will, any selected aspect of metabolism or biosynthesis in cultured cells, without the prior formation of plantlets, may still prove to be an even more difficult objective (cf. Krikorian & Steward 1969; Israel, Mapes & Steward 1969; Steward 1970*a*). However, if this art is developed it could be exploited in the industrial use of angiosperm cells and so emulate the rôle of micro-organisms. The observations here recorded may be a step toward these ends, even though they stress the number of interacting factors (nutritional, environmental, growth regulatory) which may need to be controlled in synergistic and sequential combinations to bring about an 'ontogeny' from somatic cells to plants or a fully controlled 'biochemical differentiation'.

Along the developmental route, gene actions regulate all the minute unit steps, within an already committed plan, and determine the detail of the system that emerges; this is especially so inasmuch as the formation of complex molecules is regulated. But the dilemma is to know how the more complex organizations are regulated for great blocks of inherited information seem to be called simultaneously into play as cells in multicellular units, or in organs, respond to multiple combinations of interacting exogenous stimuli that determine the course of metabolism development and morphogenesis. Neither the location, or the operation, of these integrated controls are presently understood but, obviously, this is an area of enquiry in which 'reductionism' below the cellular level may tell what is biochemically feasible but not what is morphologically practicable. Hence the controlled development from isolated cells or protoplasts to whole organisms, in virtually 100% yield, is still an ambitious and important goal.

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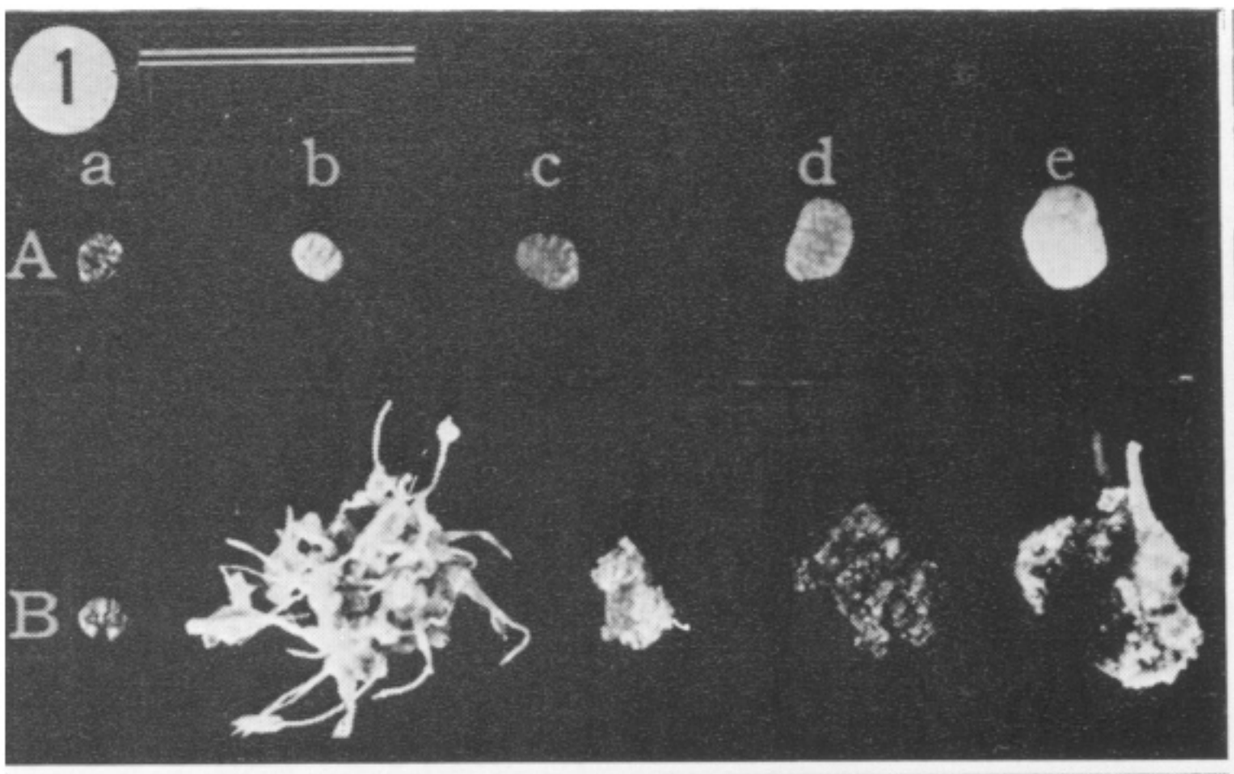
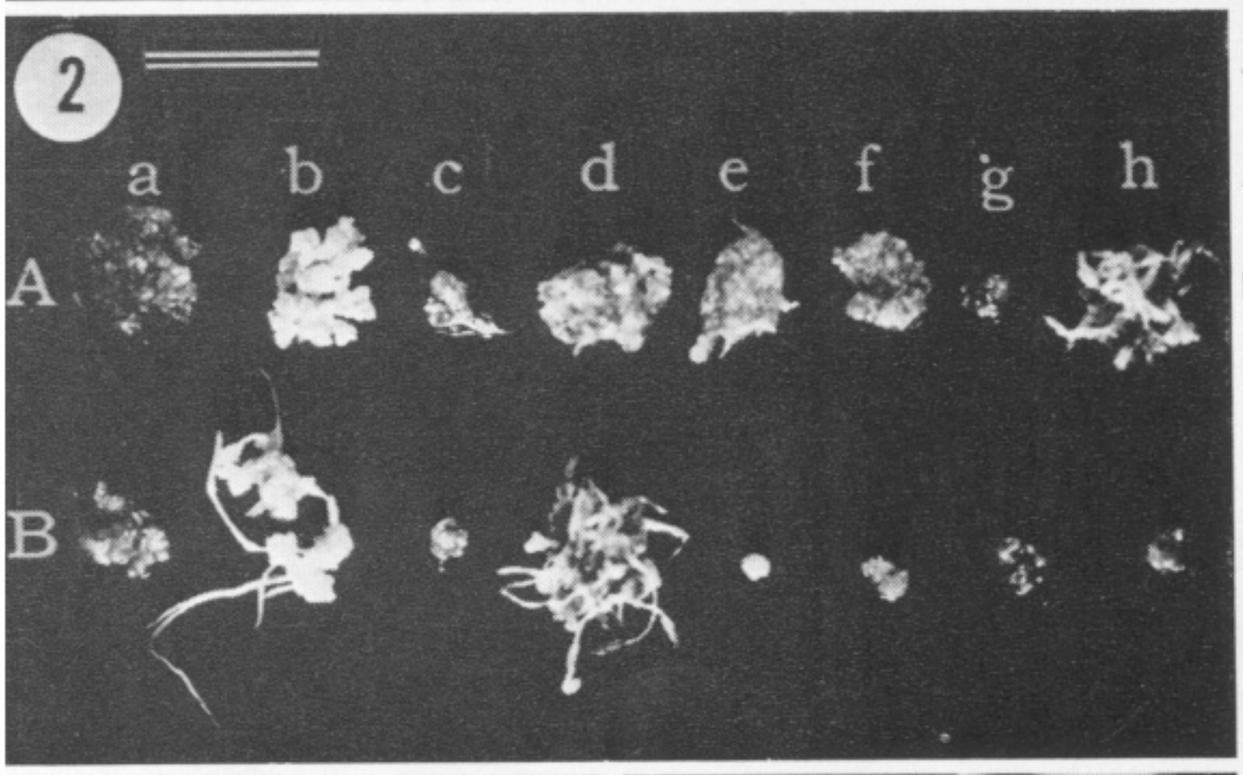
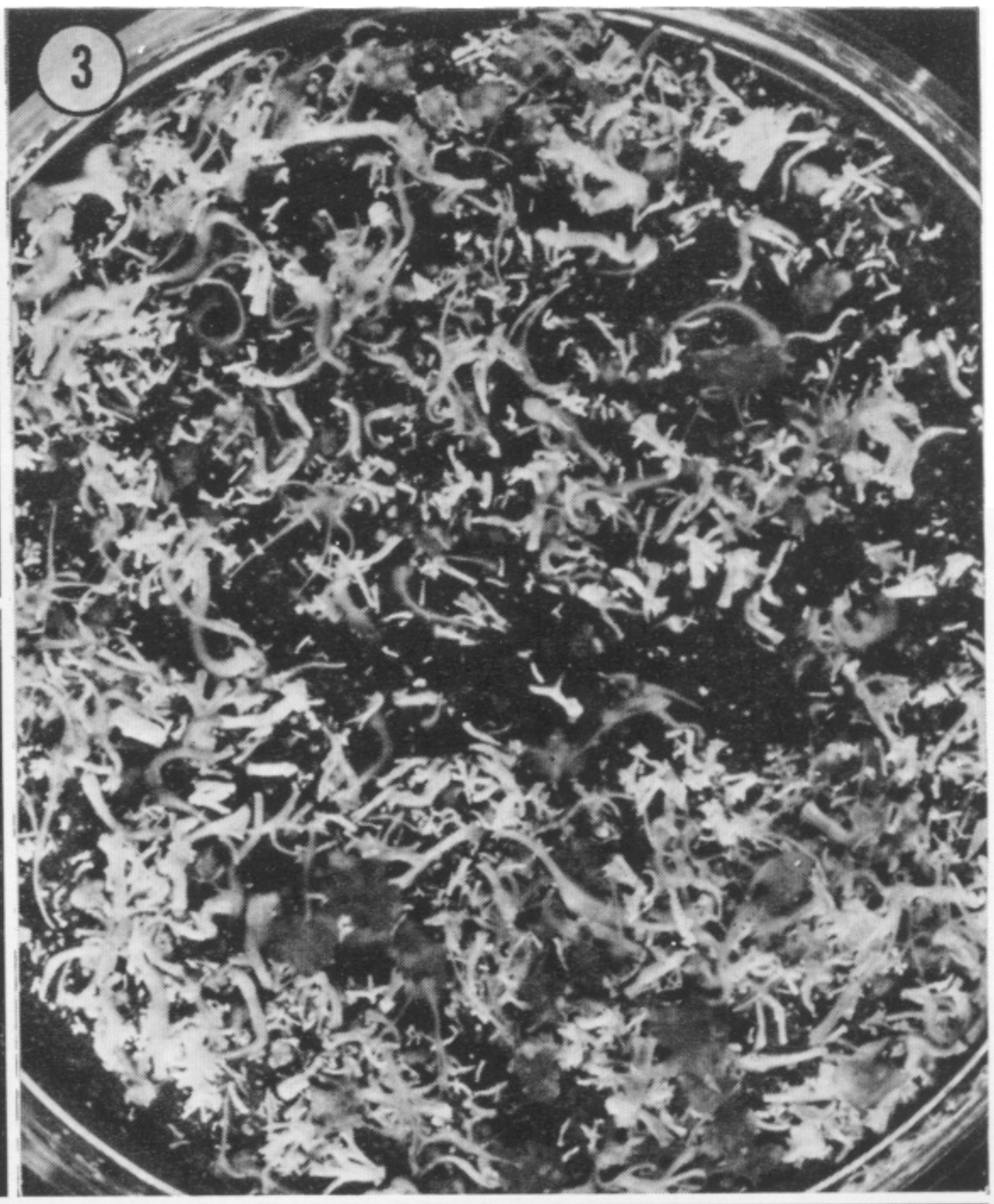
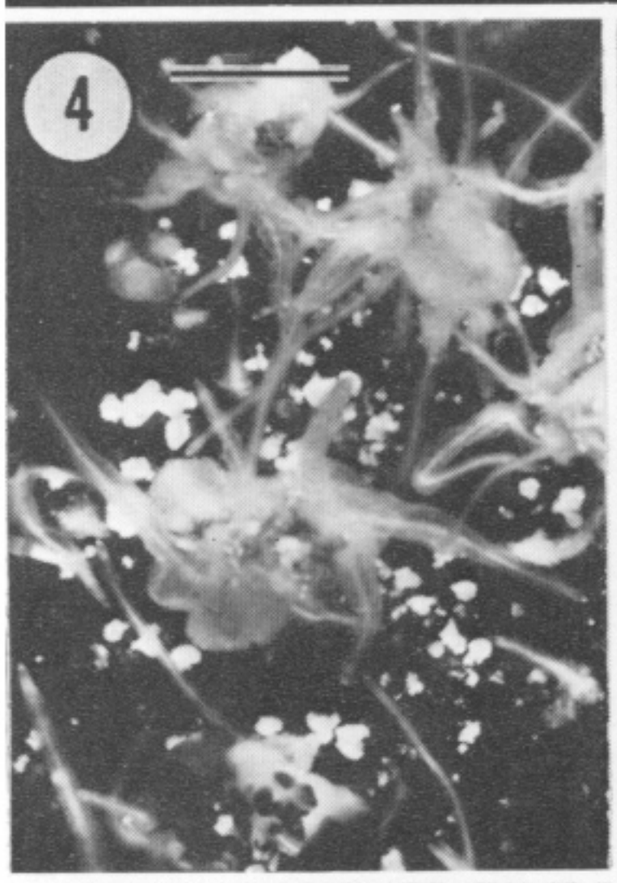
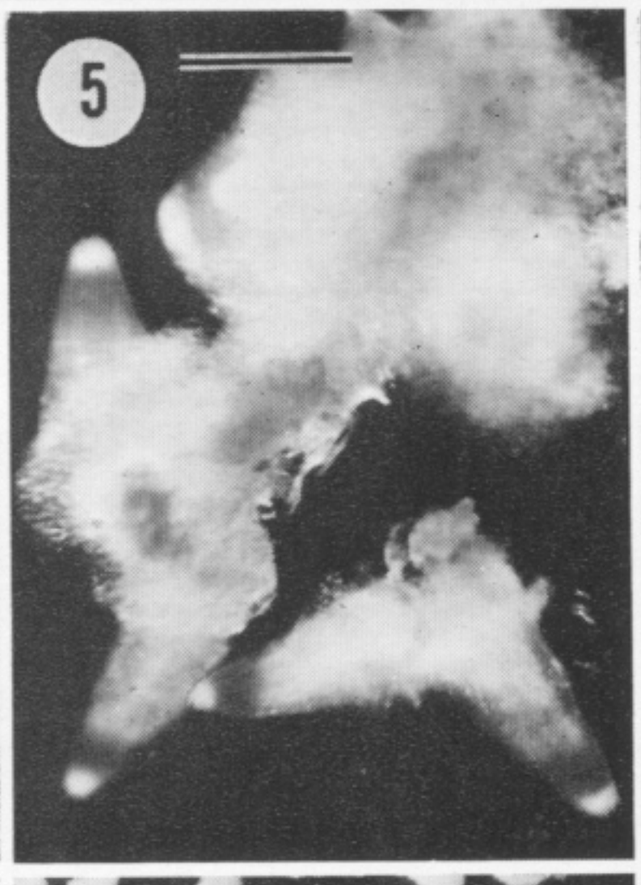
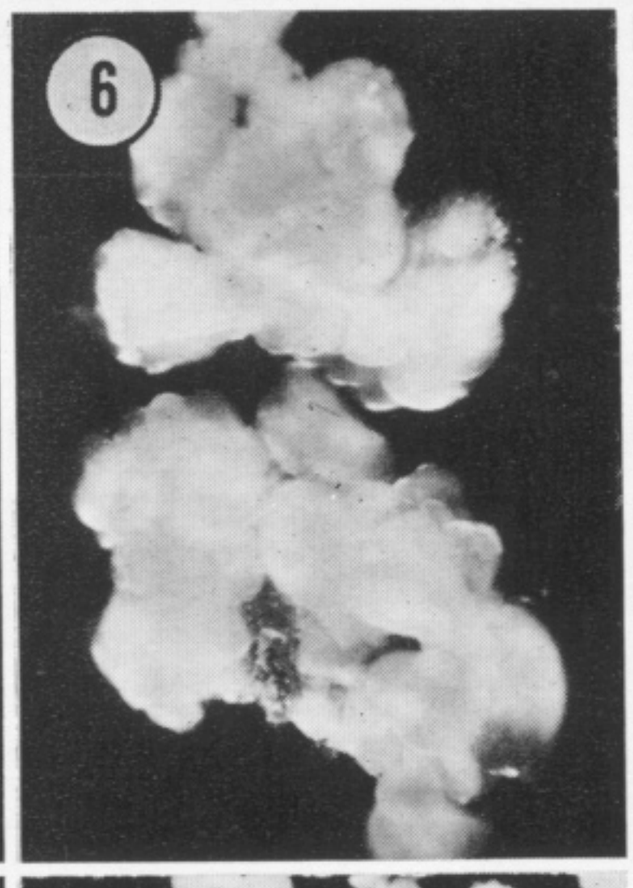


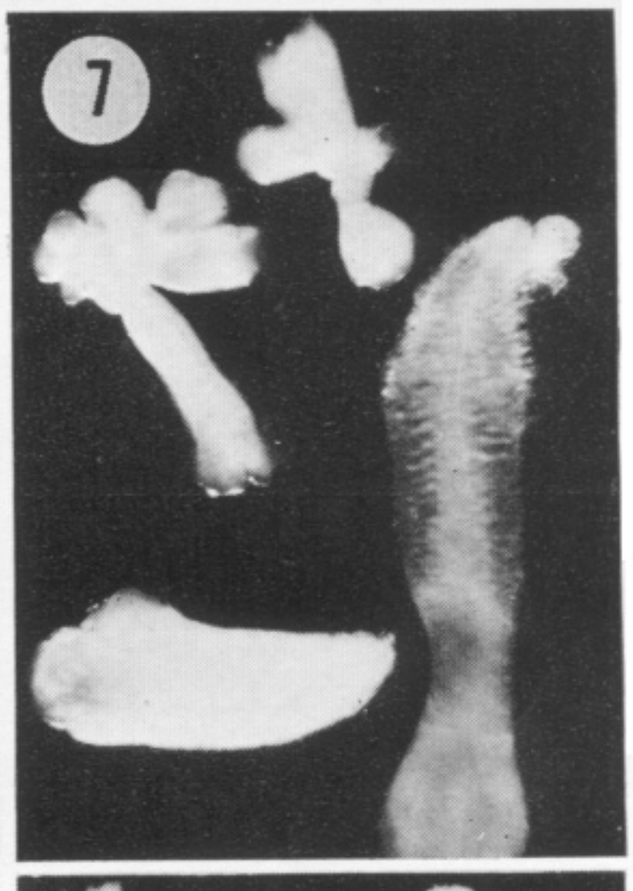


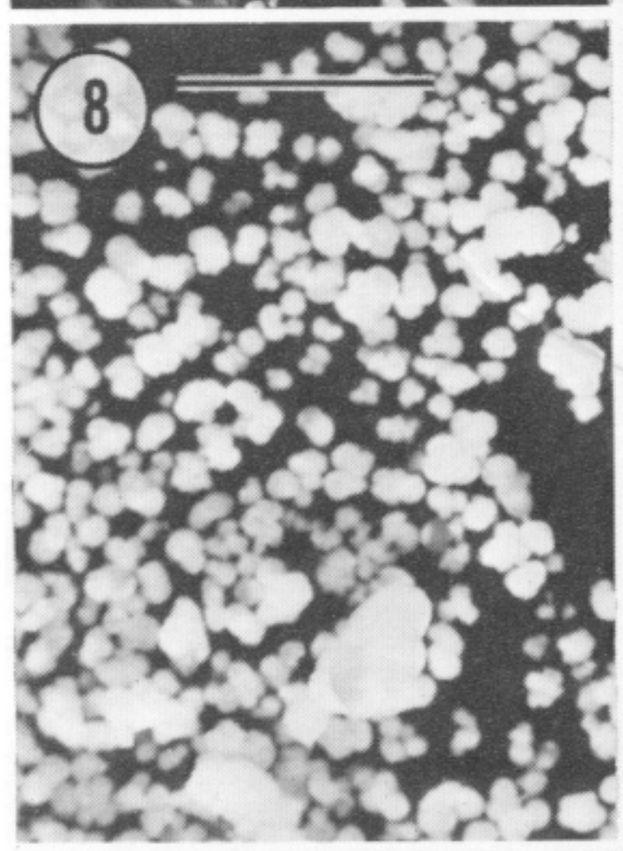


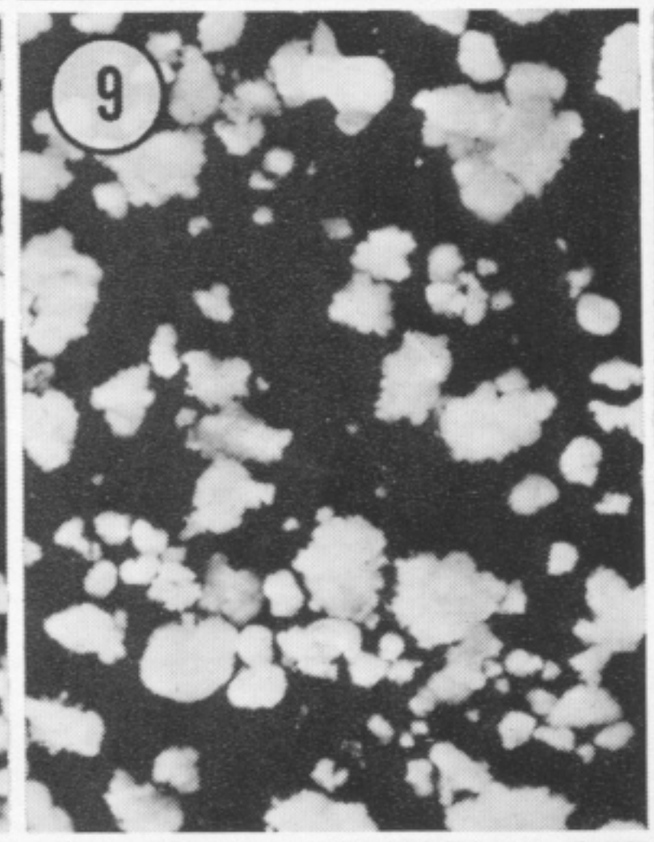


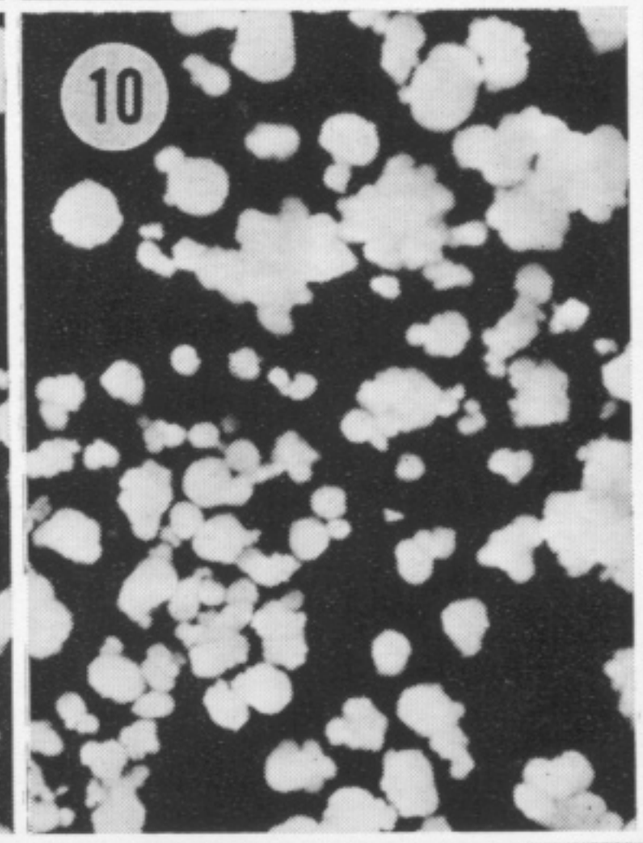


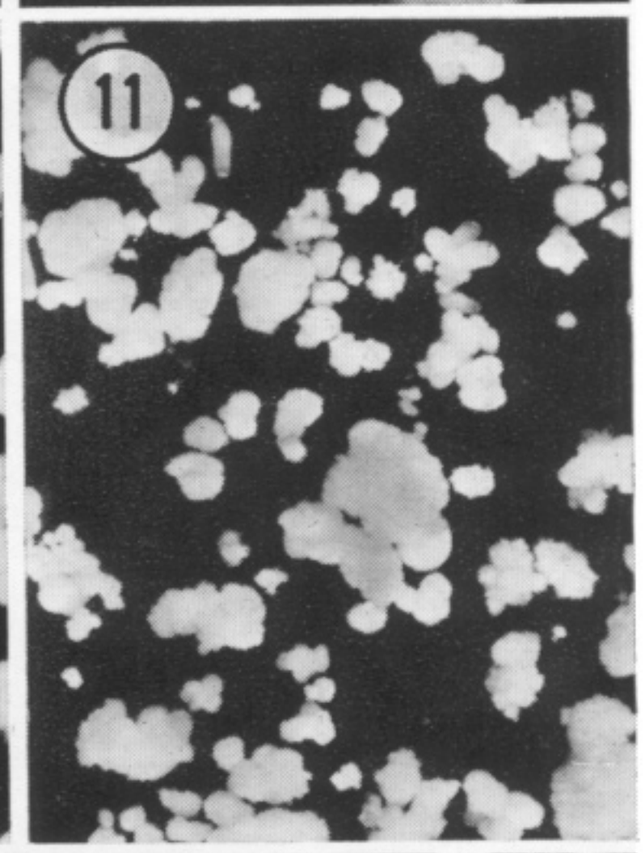


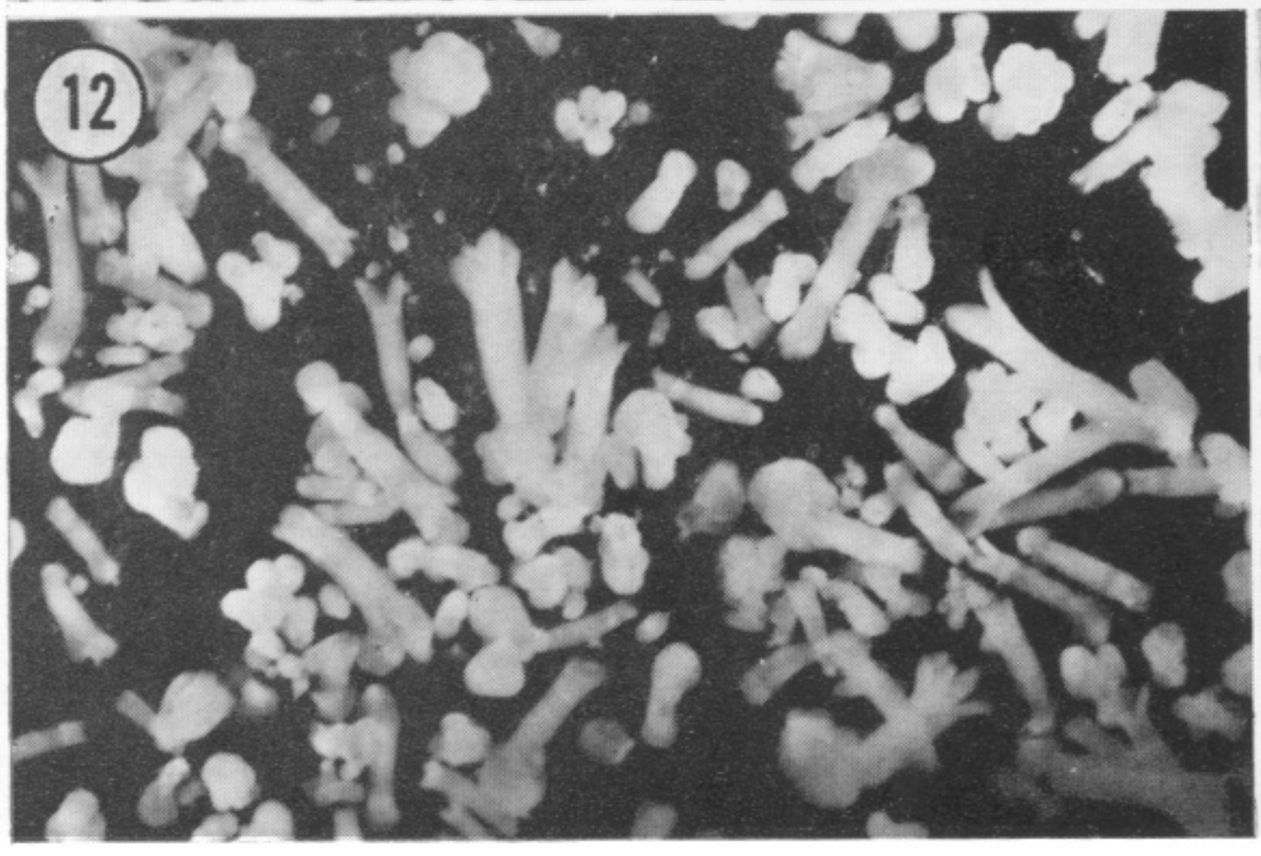




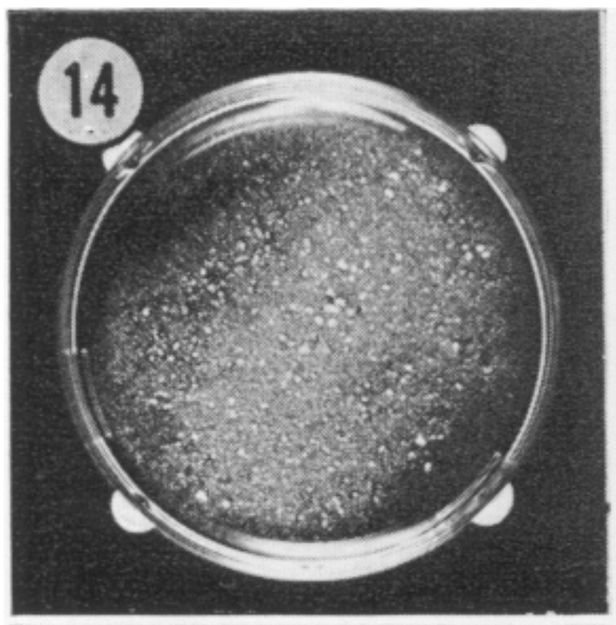


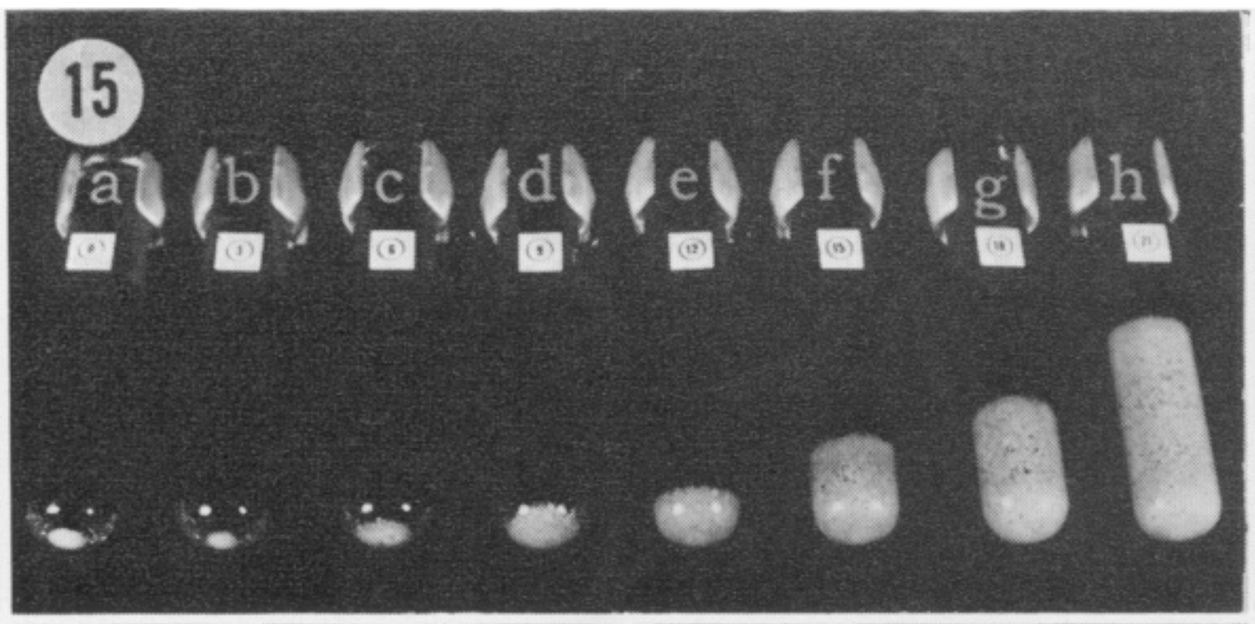


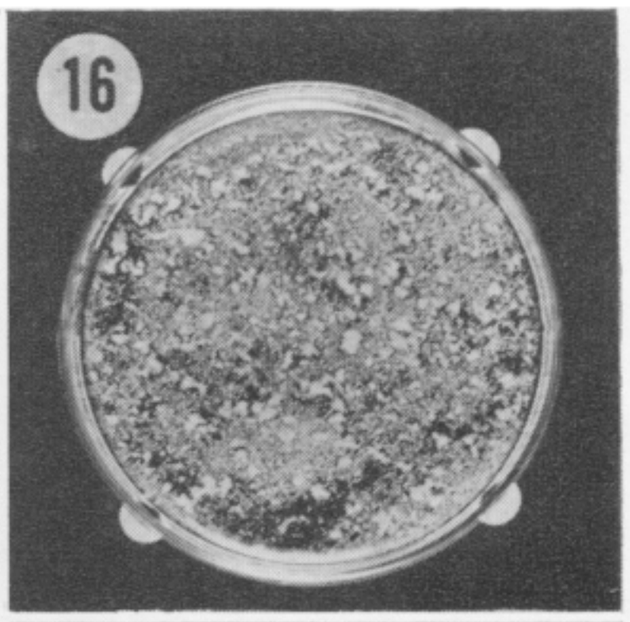


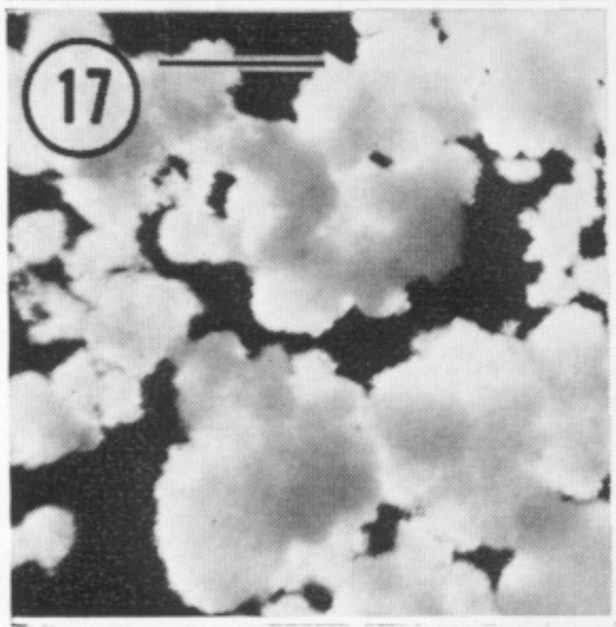


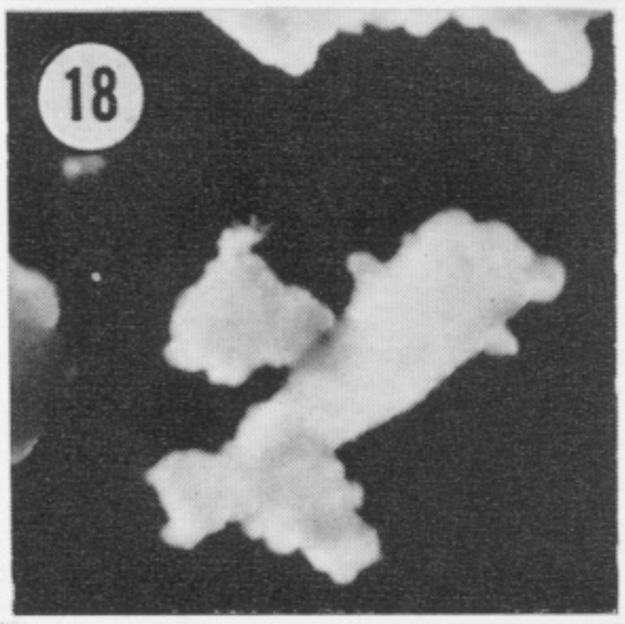
FIGURES 1–13. For description see opposite

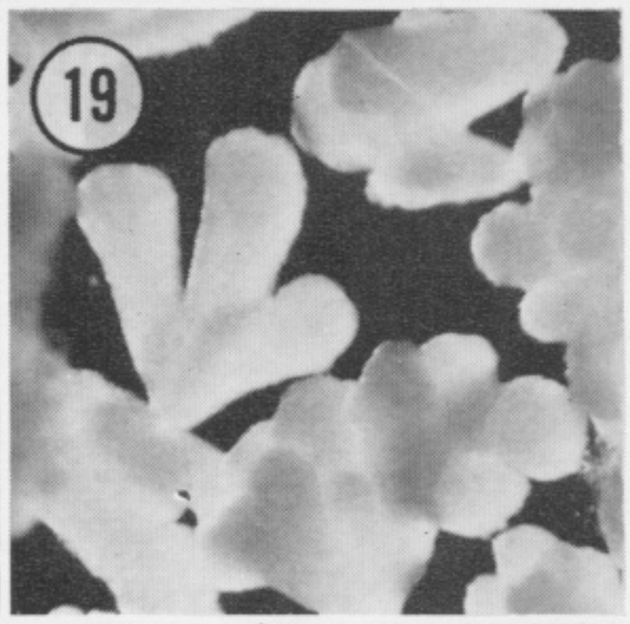


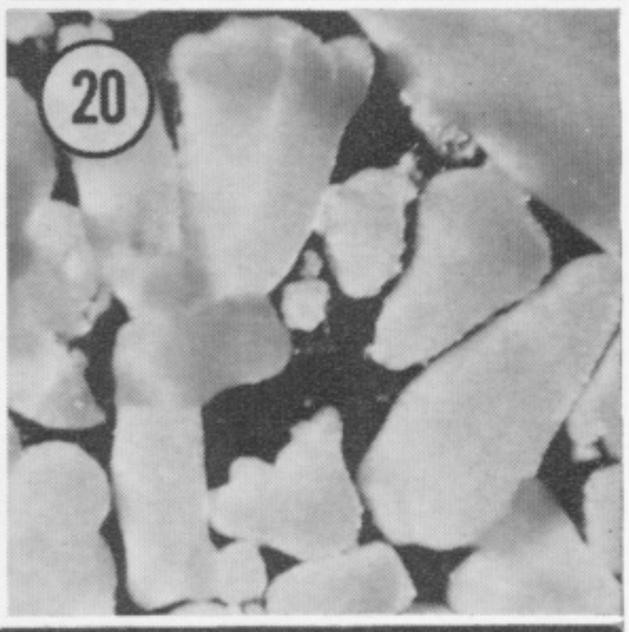


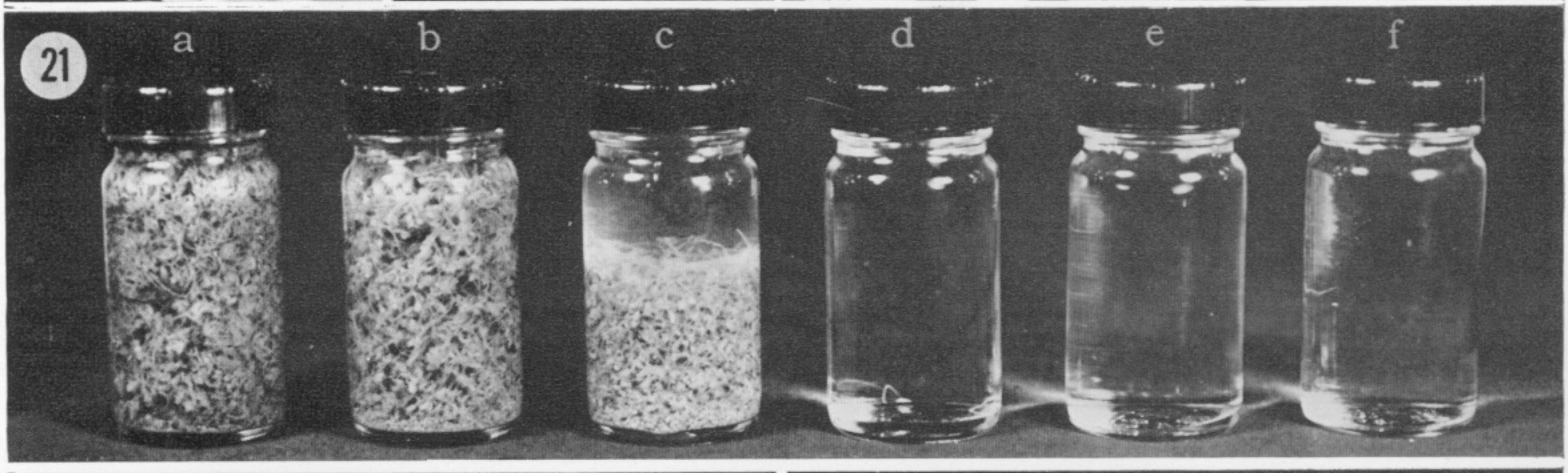


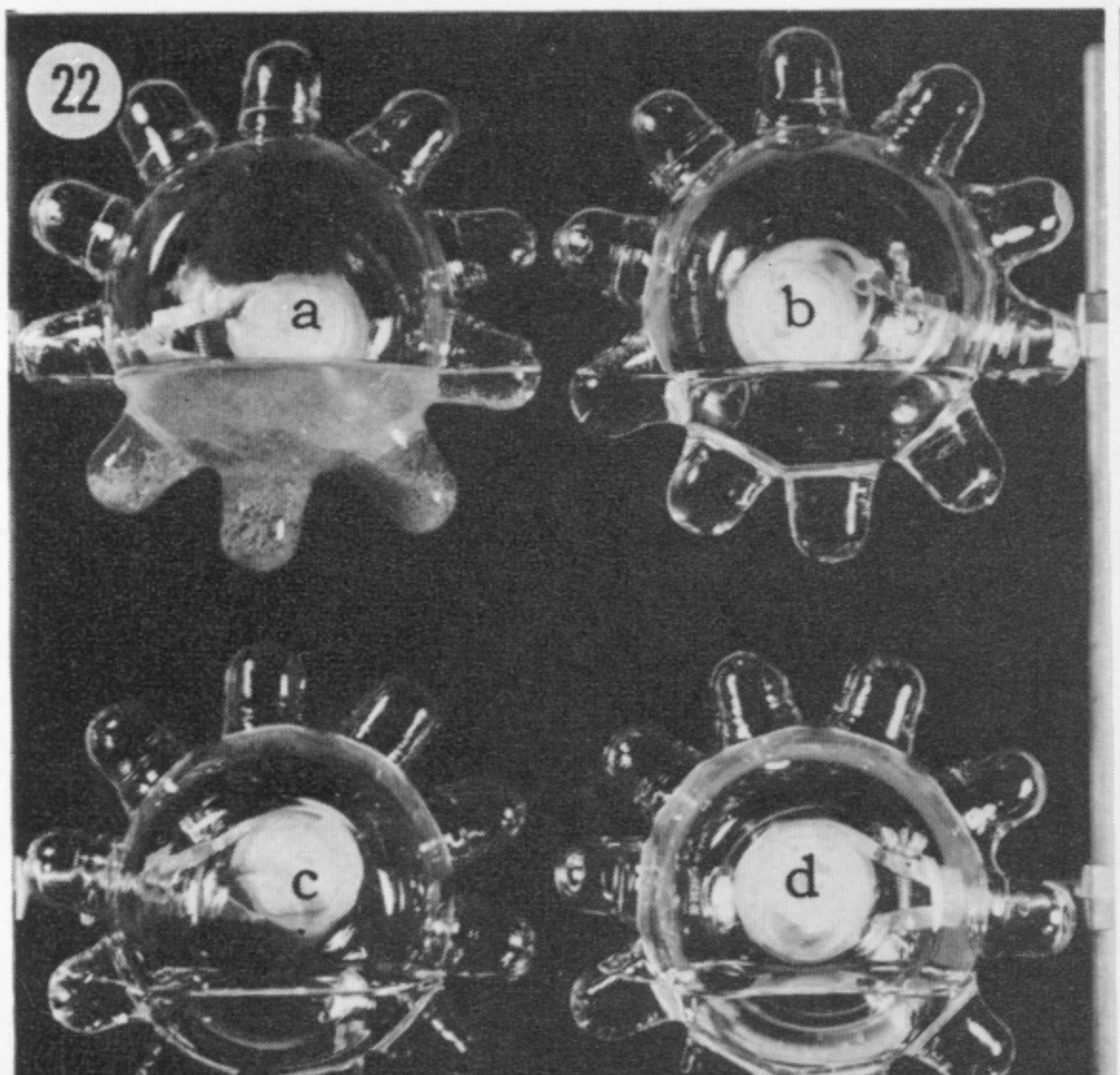


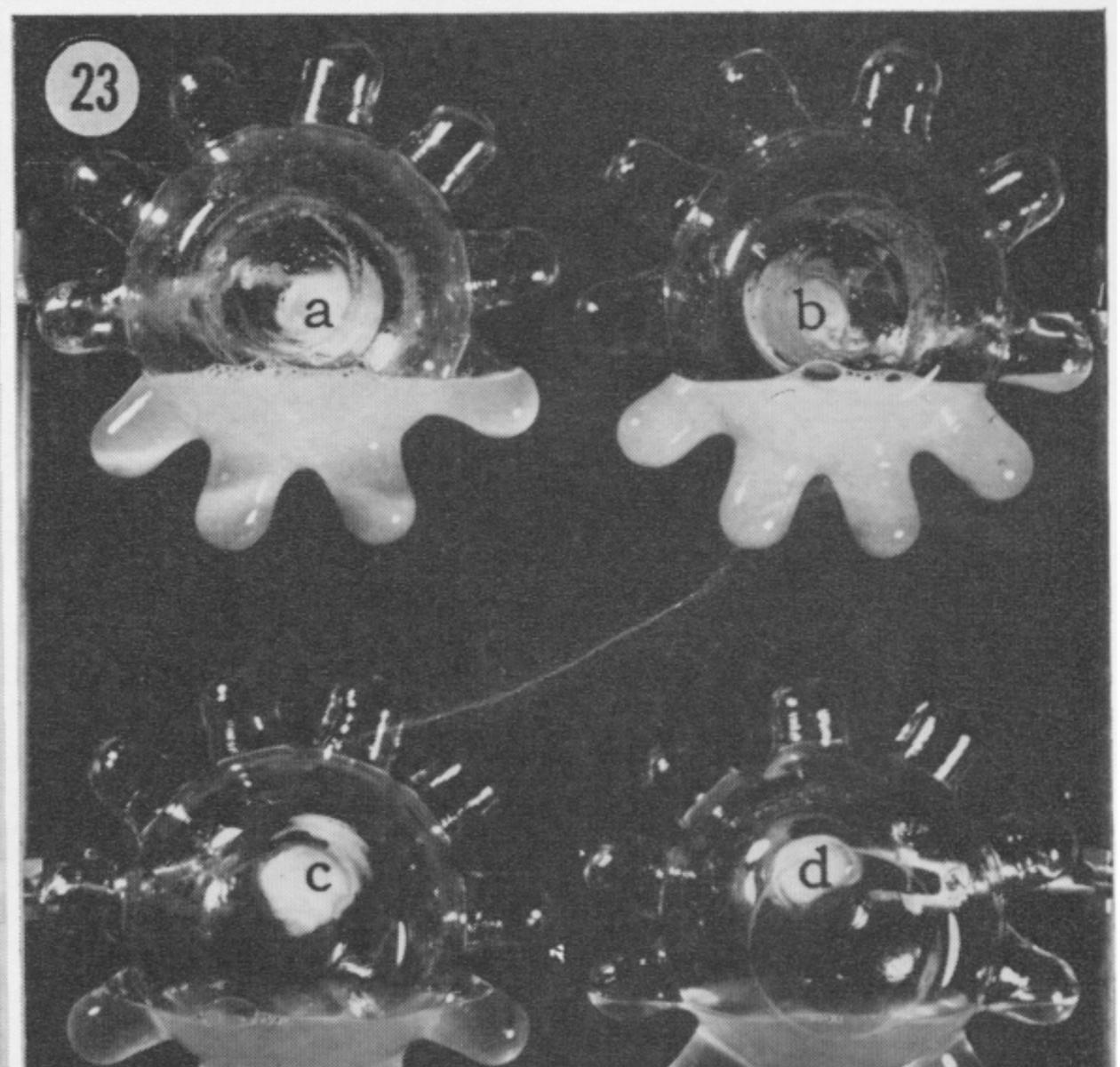


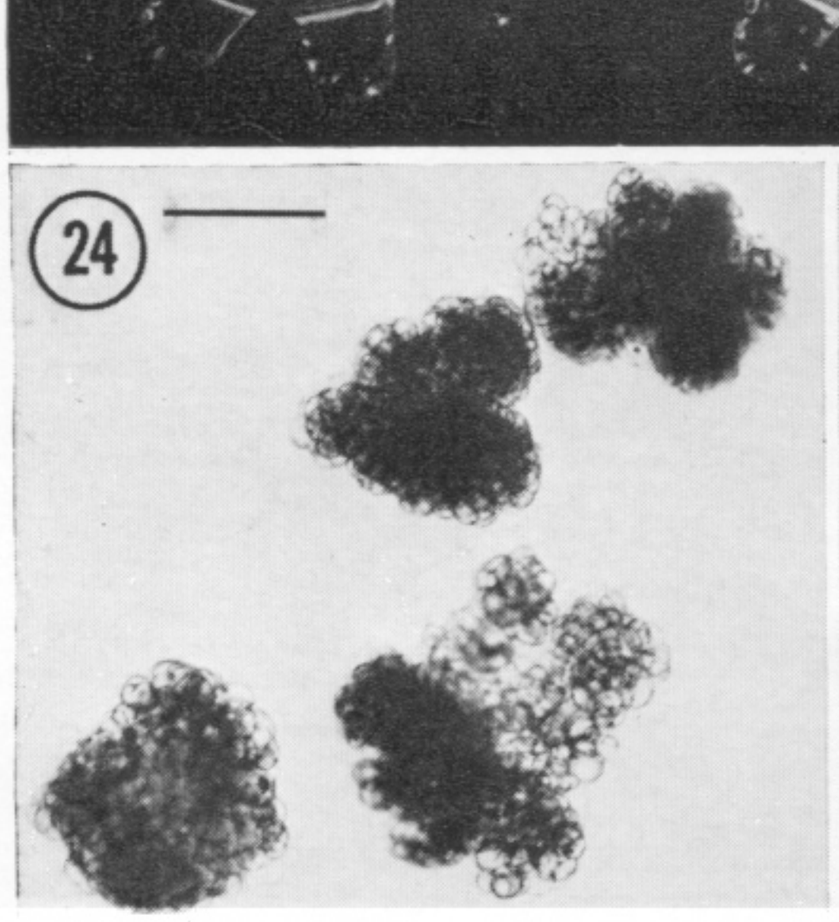


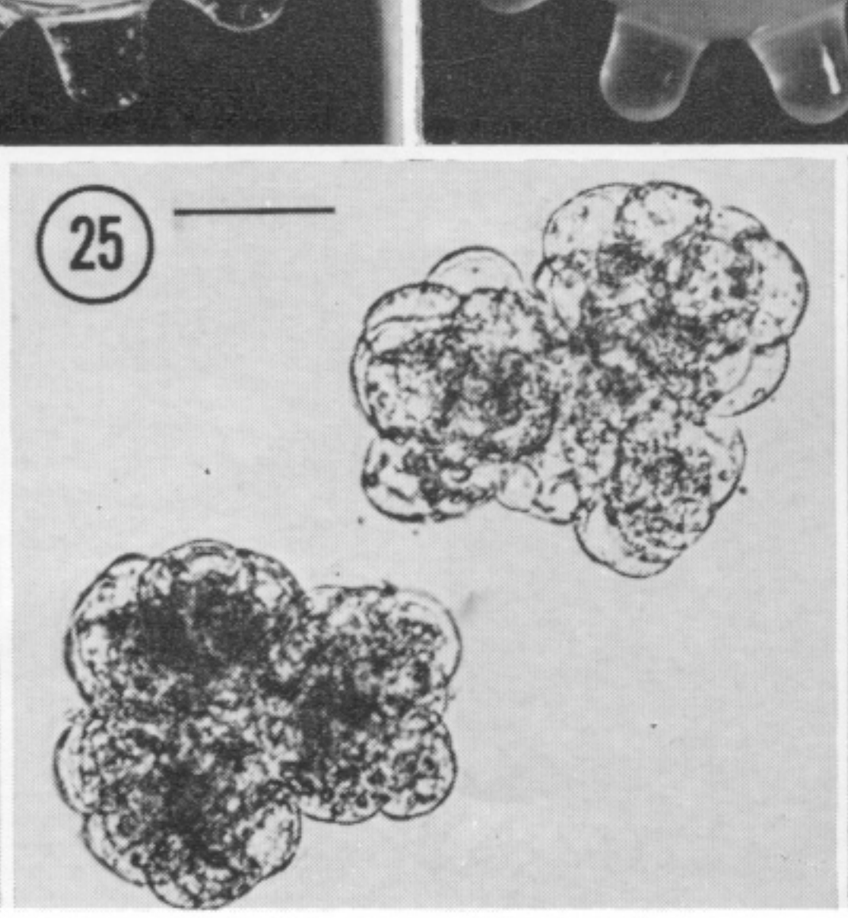


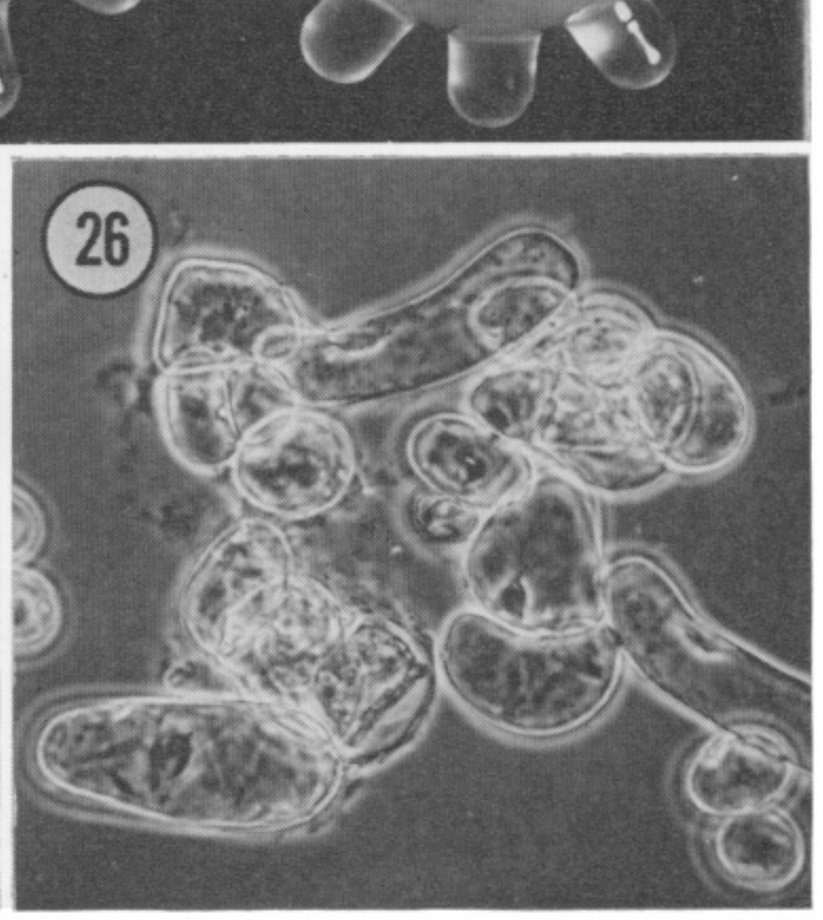












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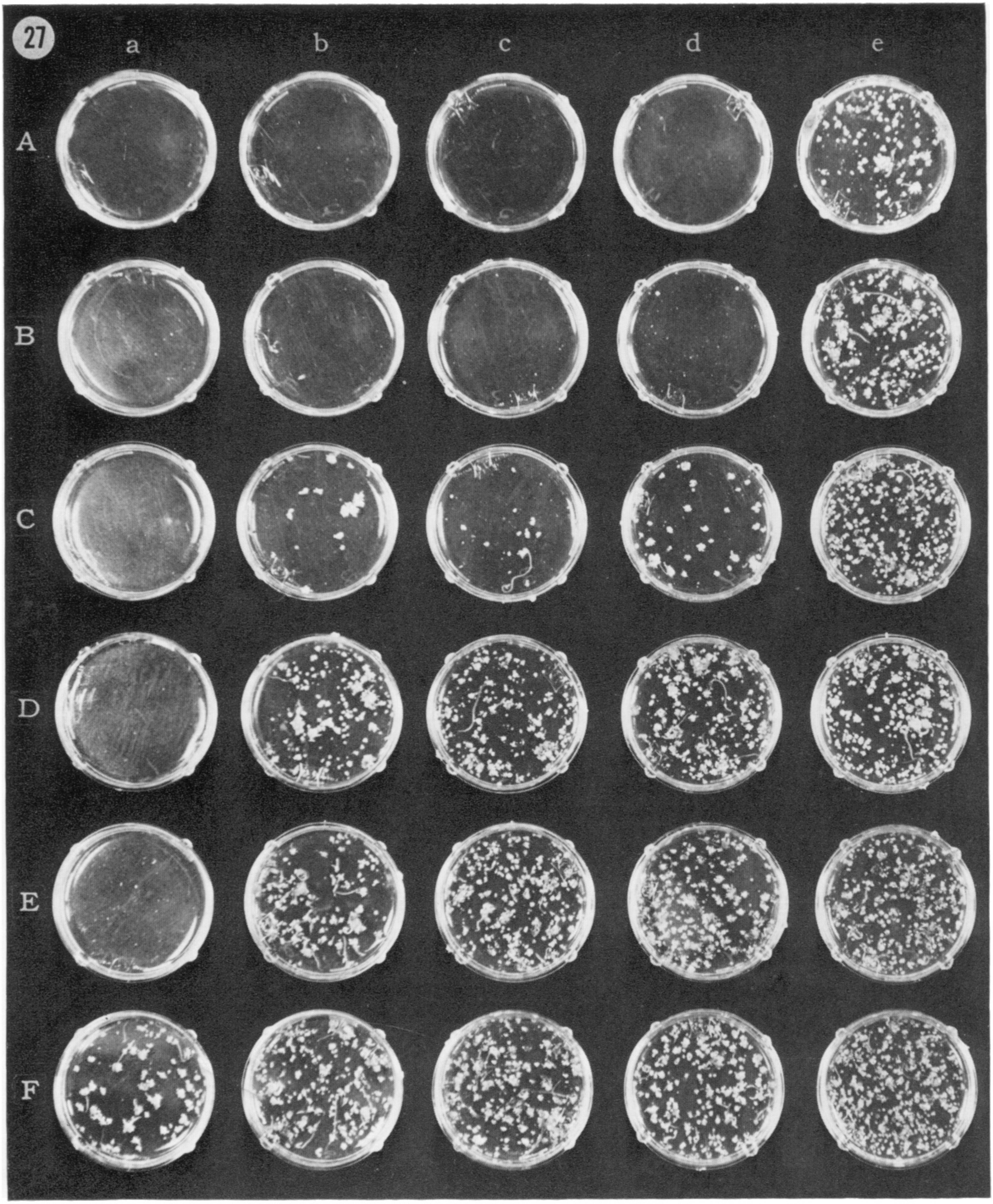

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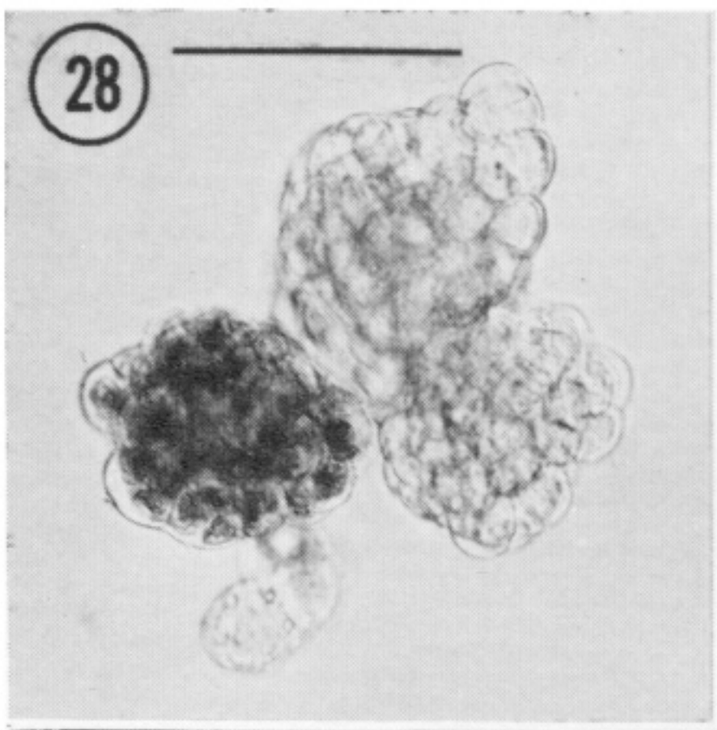
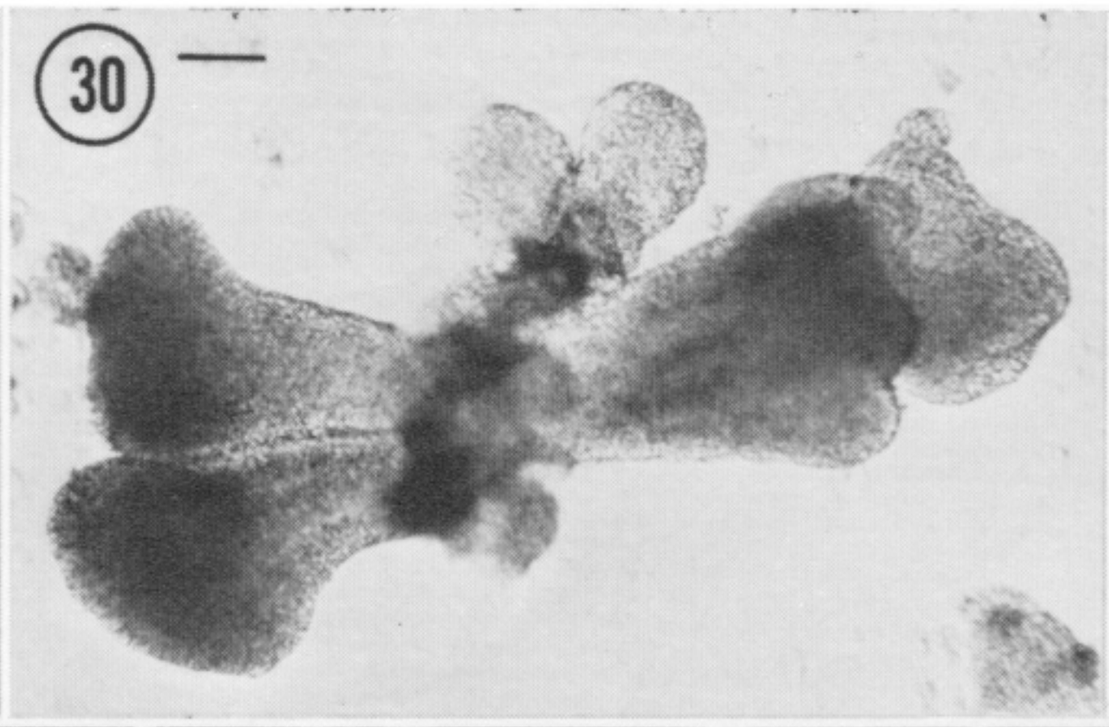
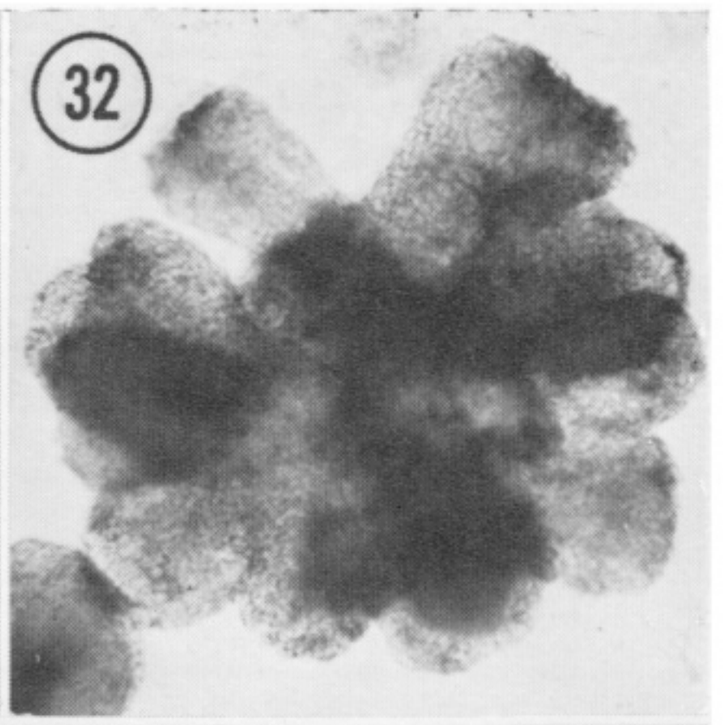
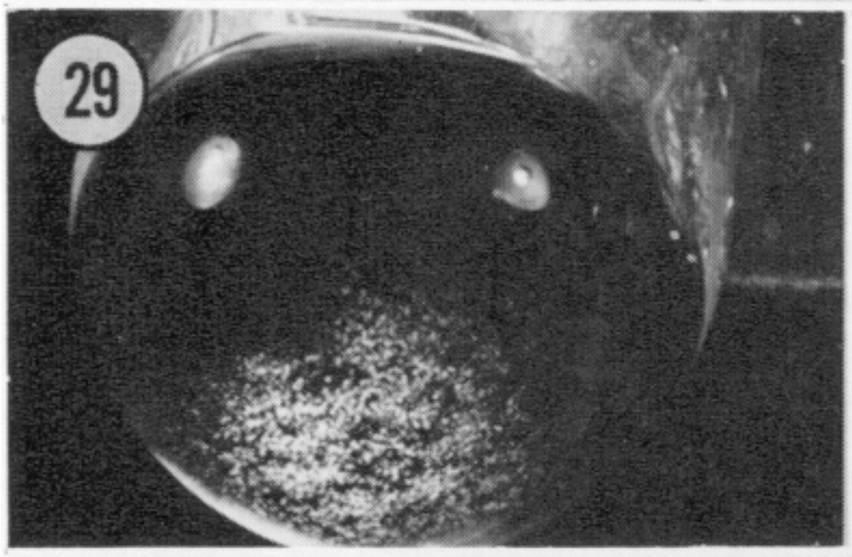
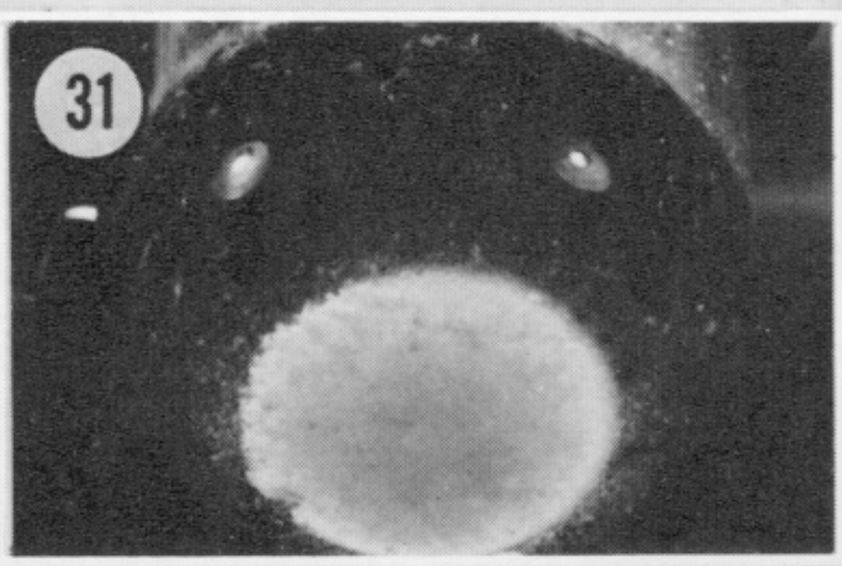
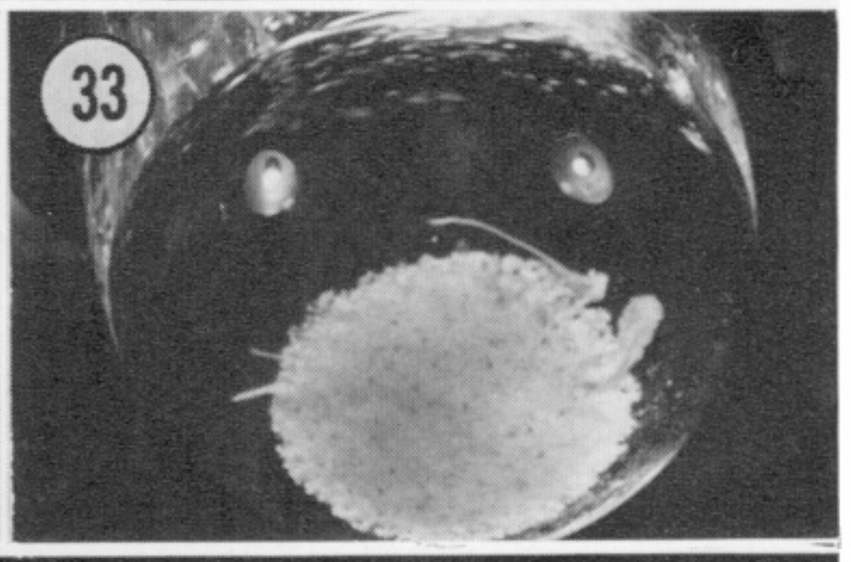


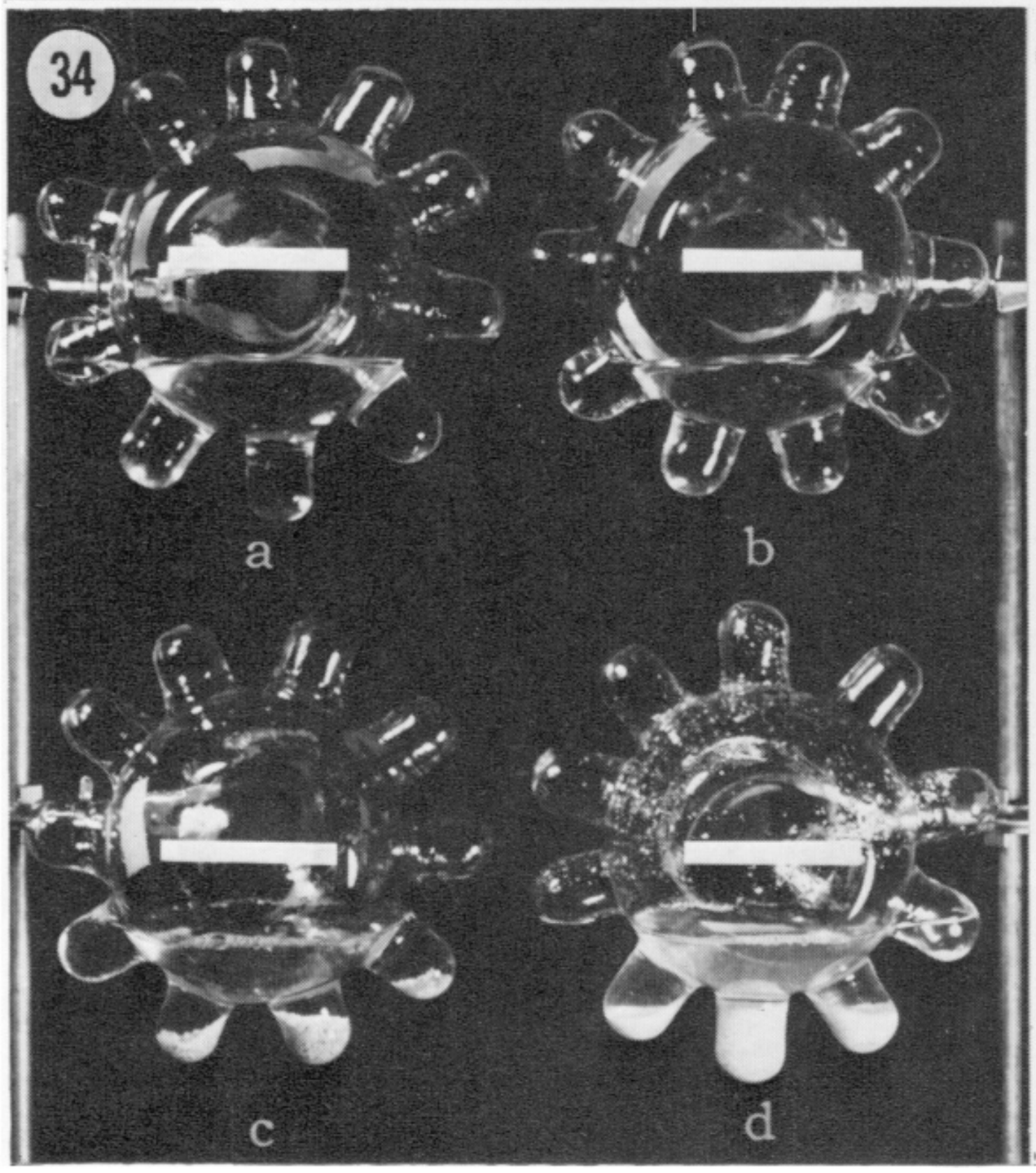


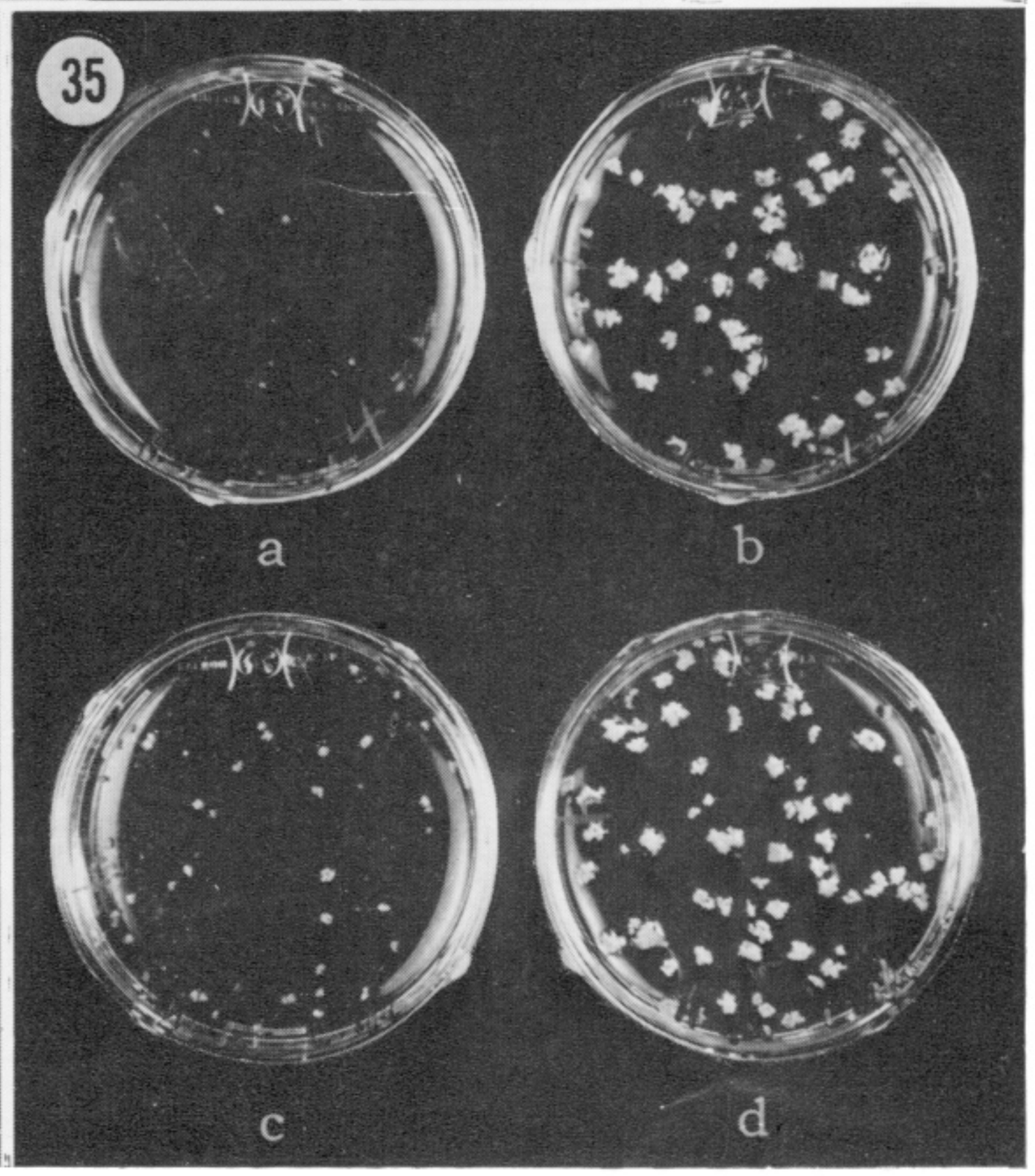




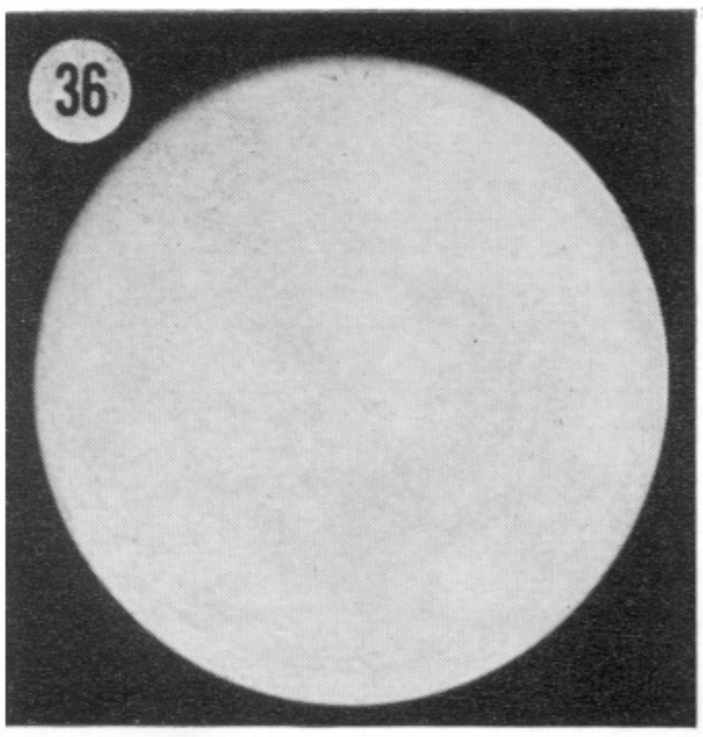


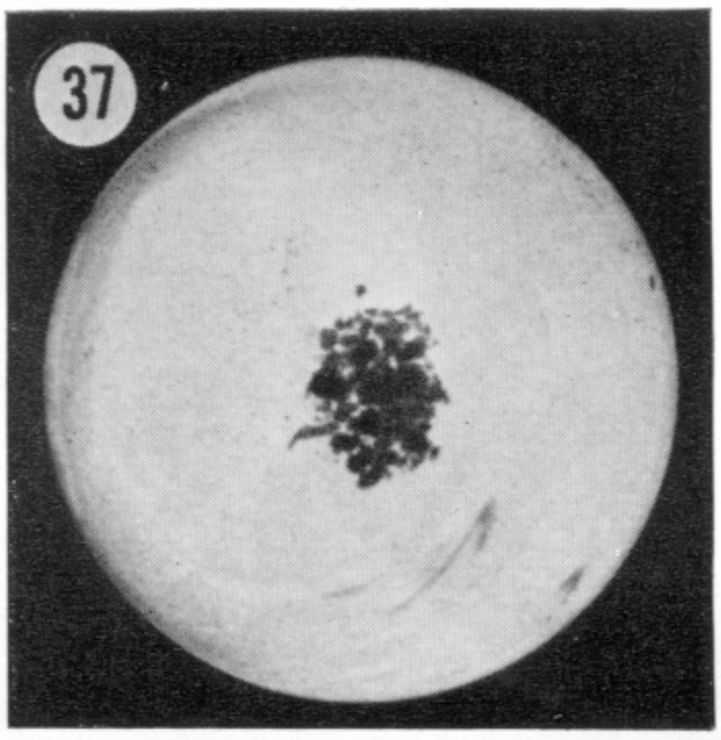


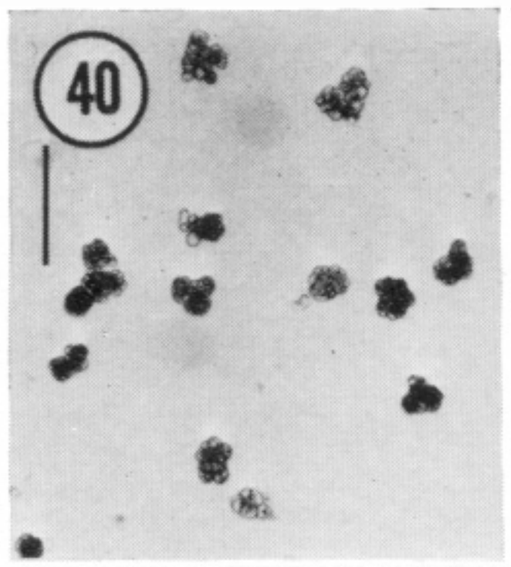


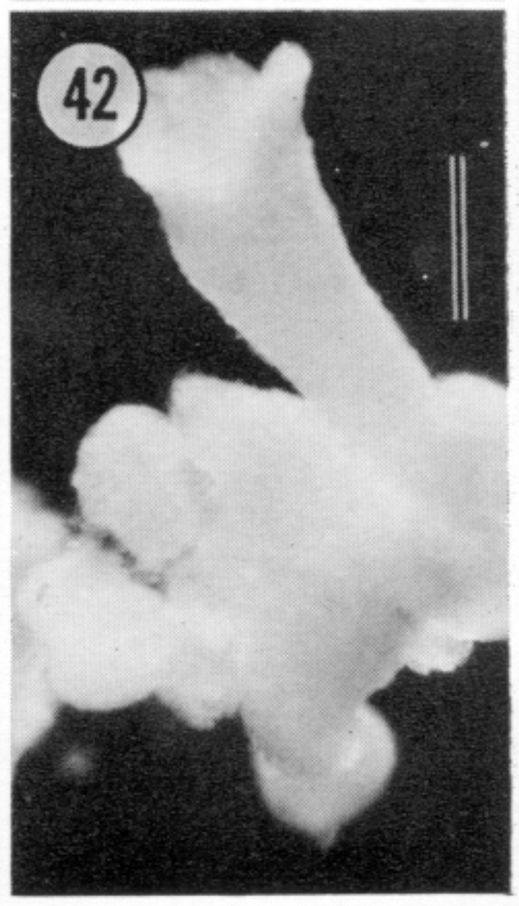


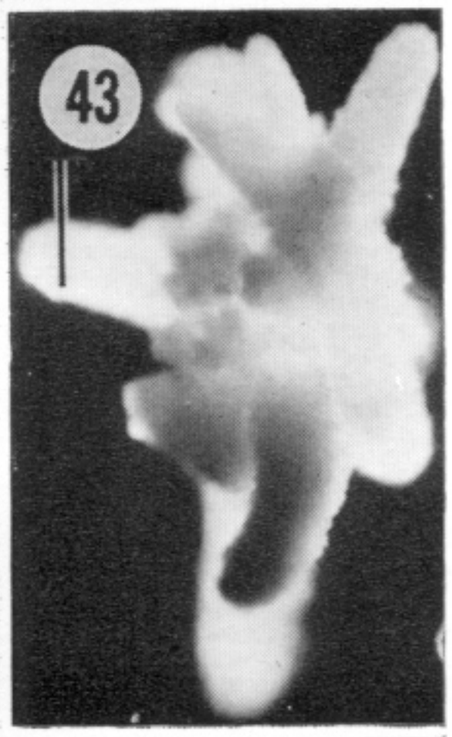
FIGURES 28–35. For description see opposite

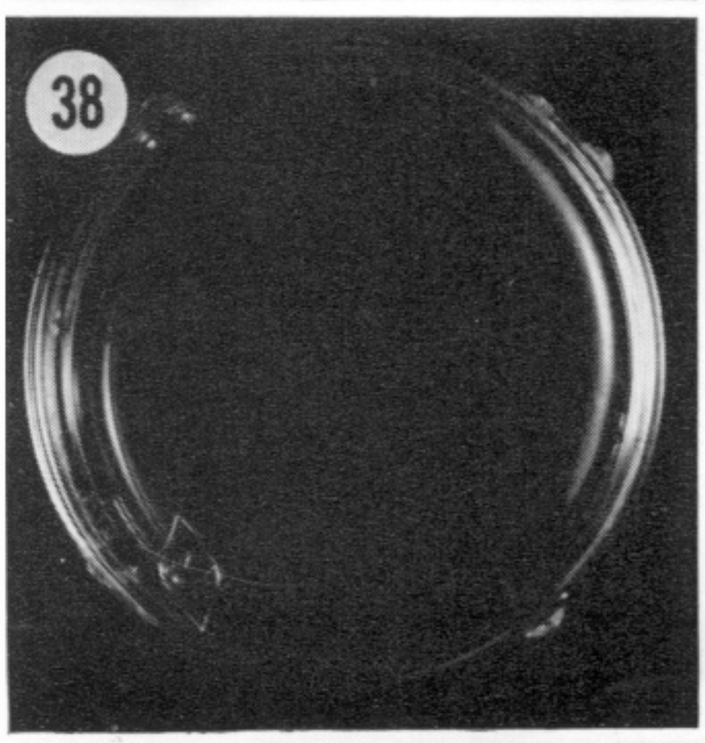


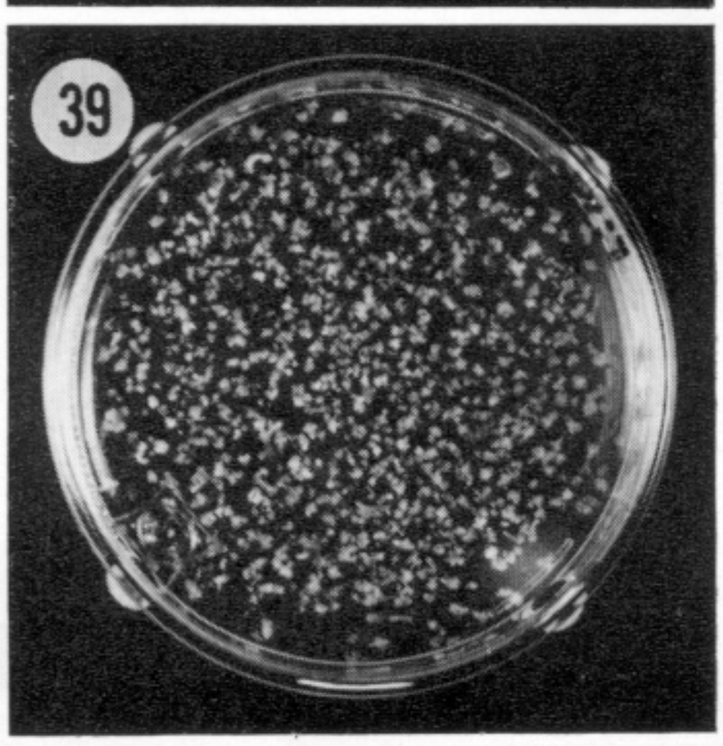


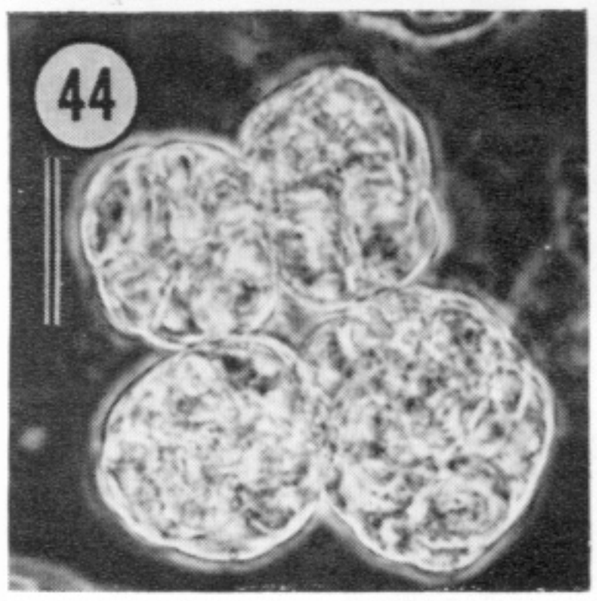


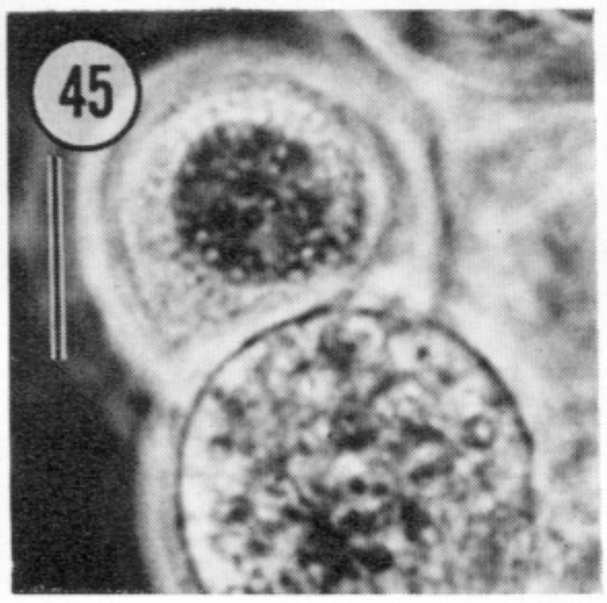


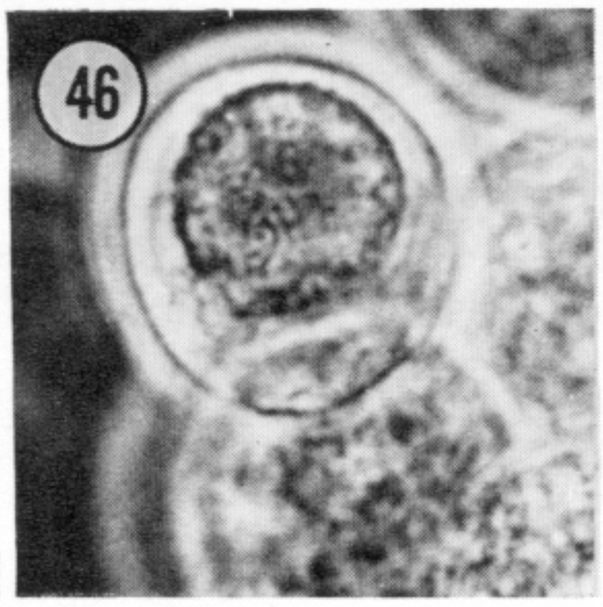


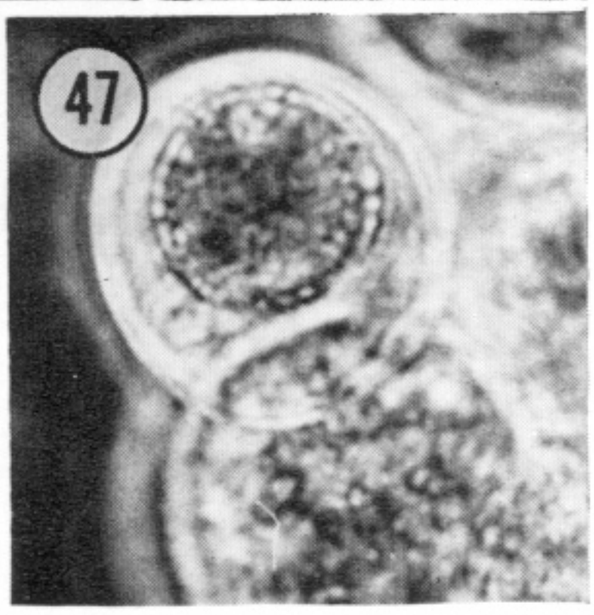


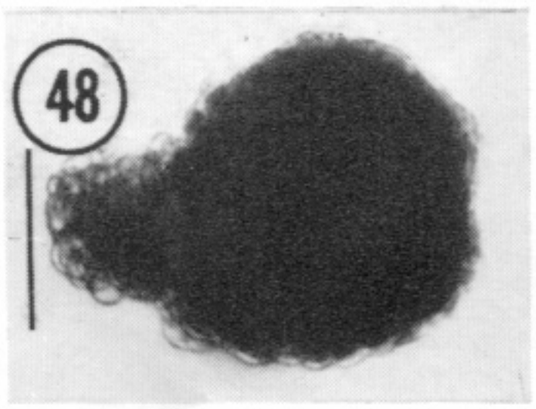


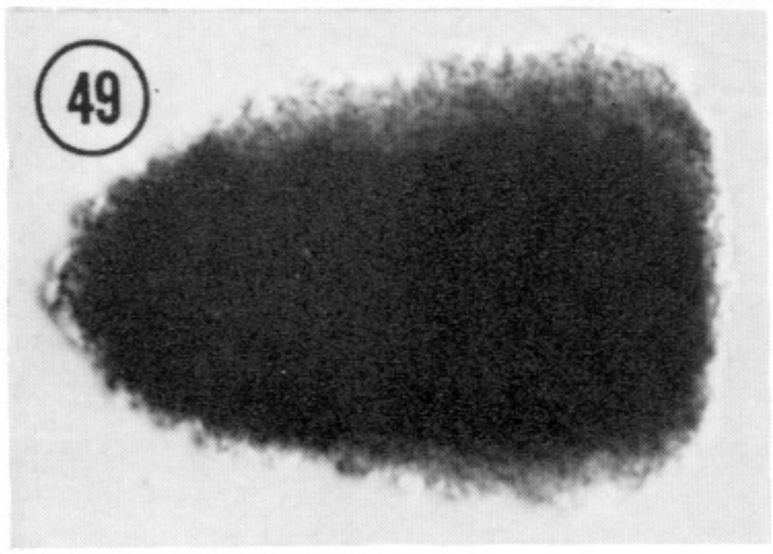


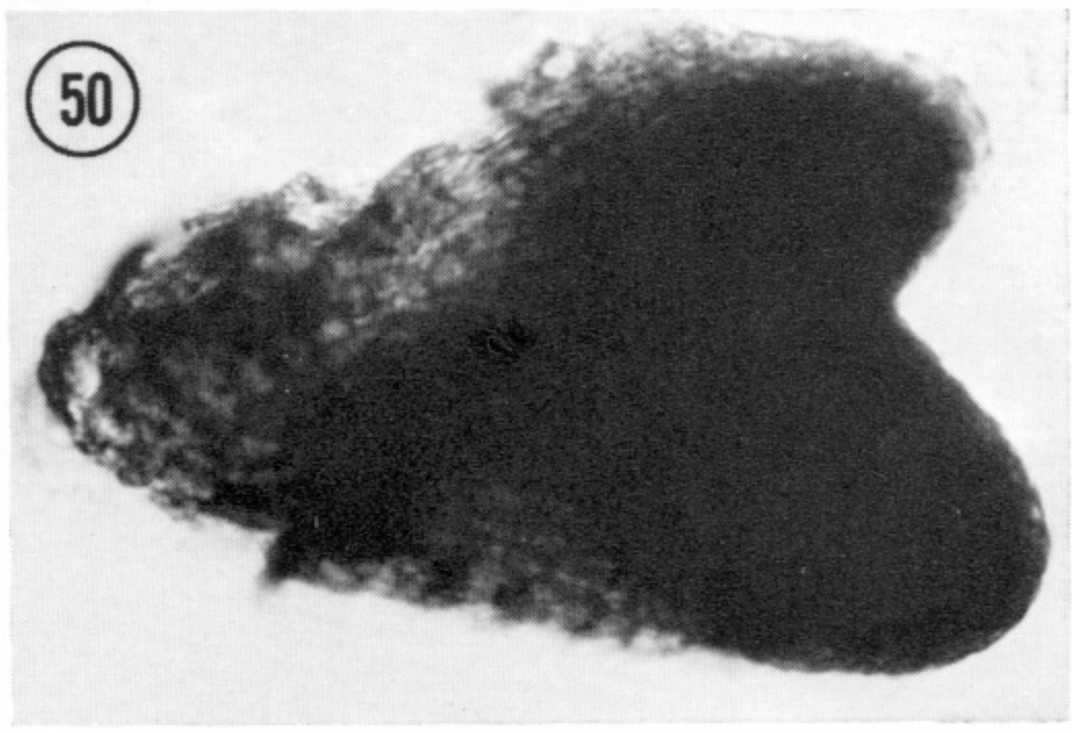


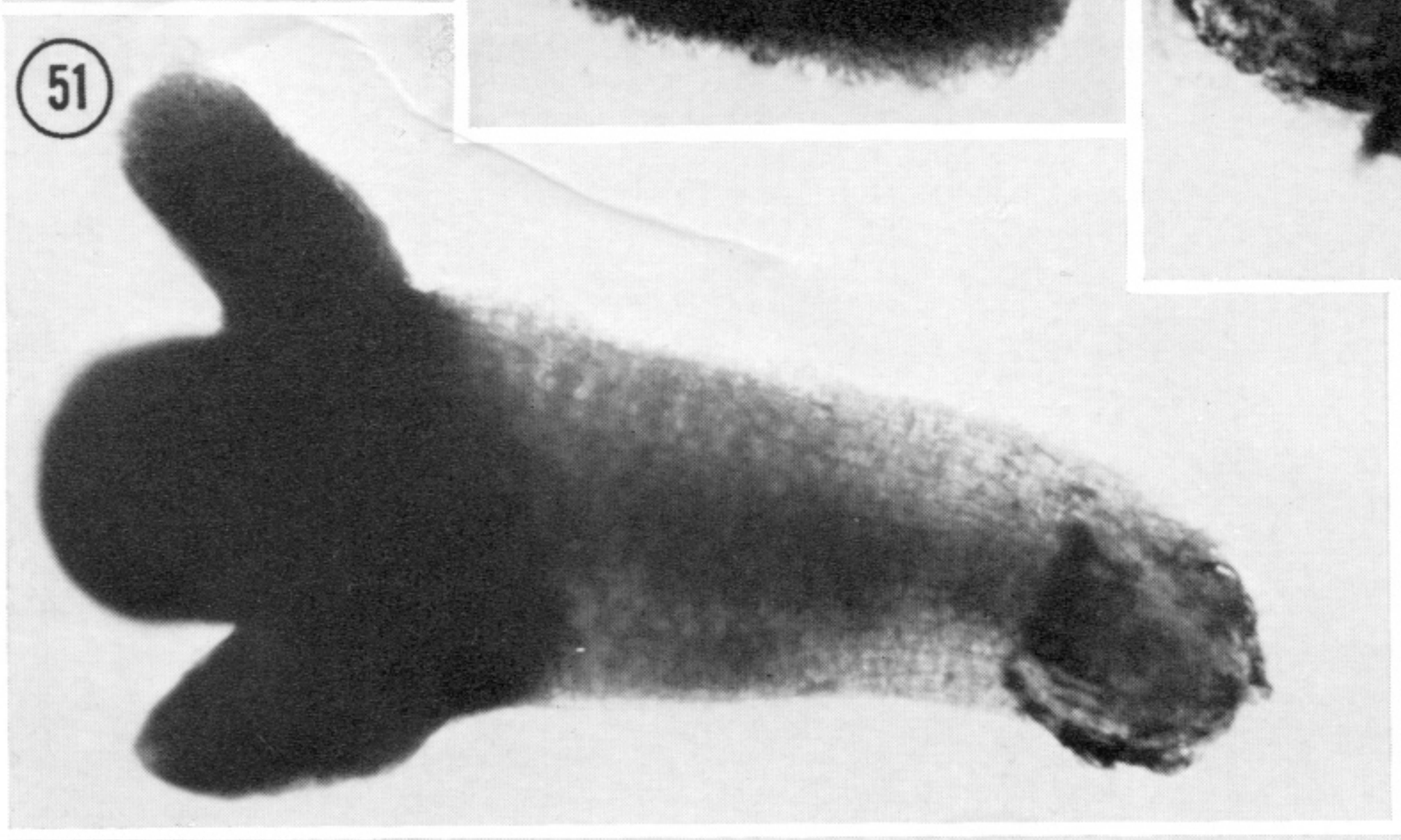




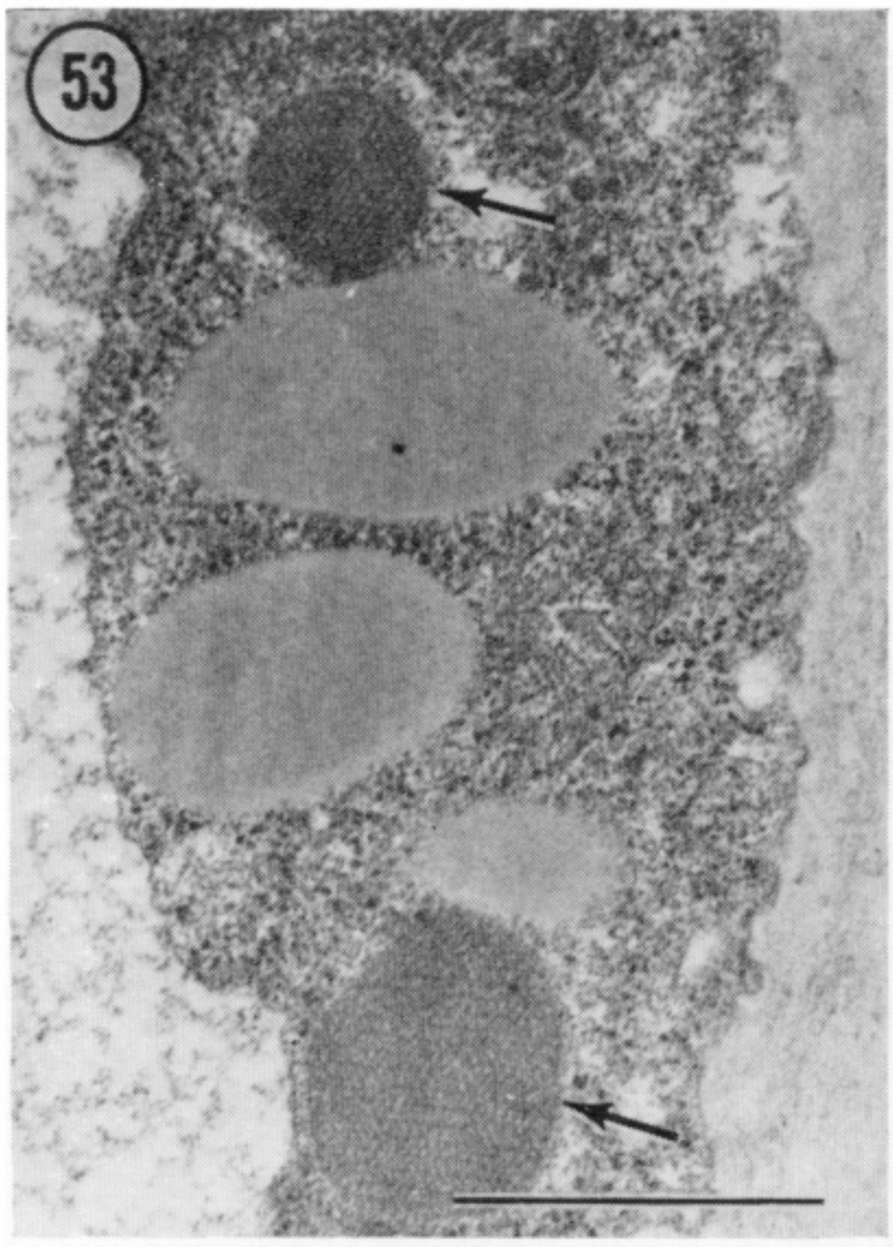


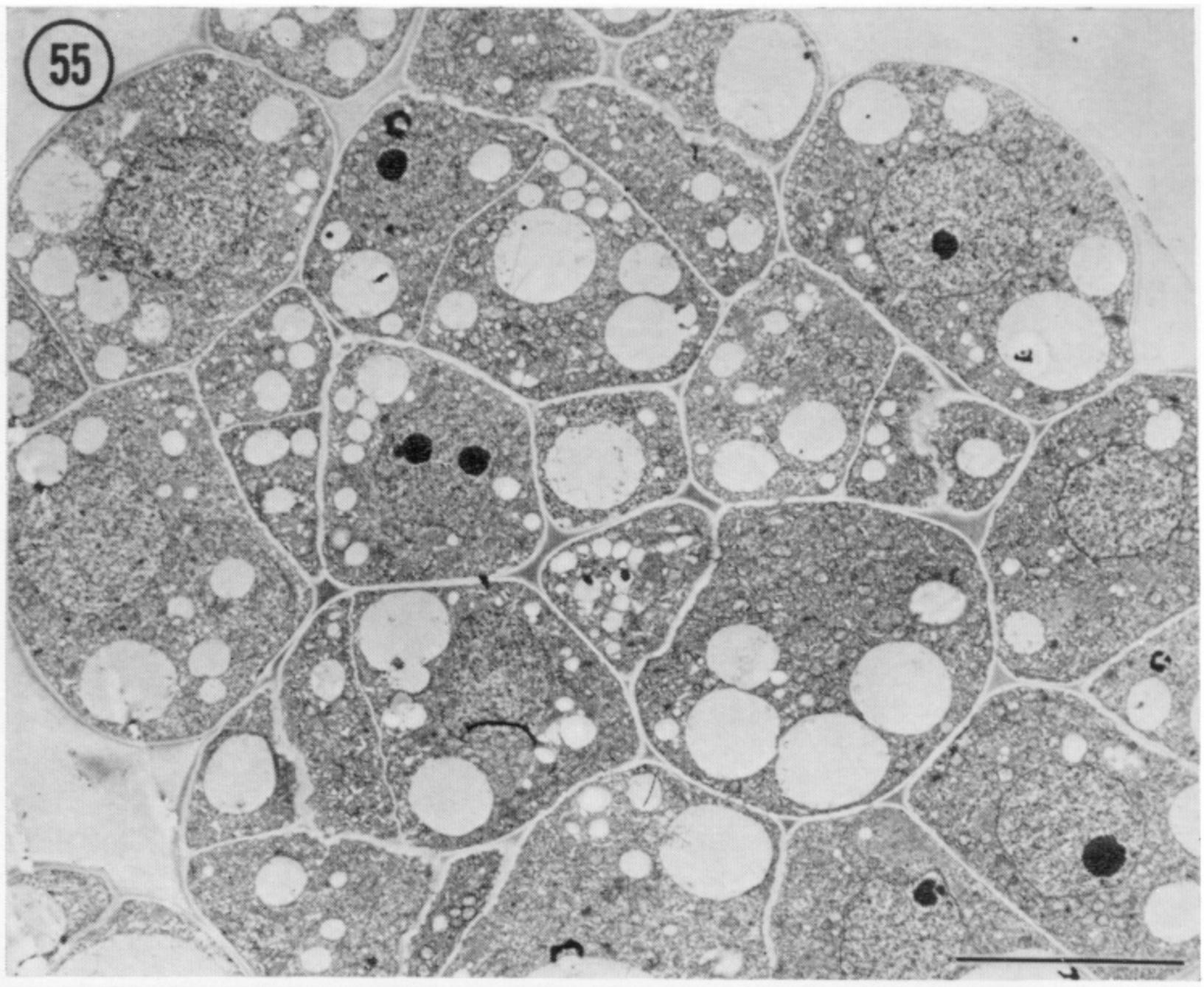


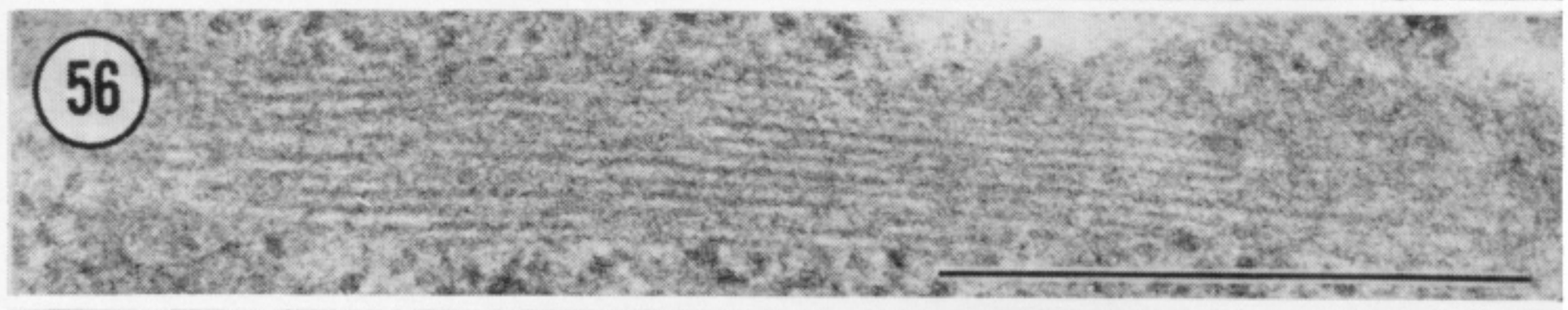


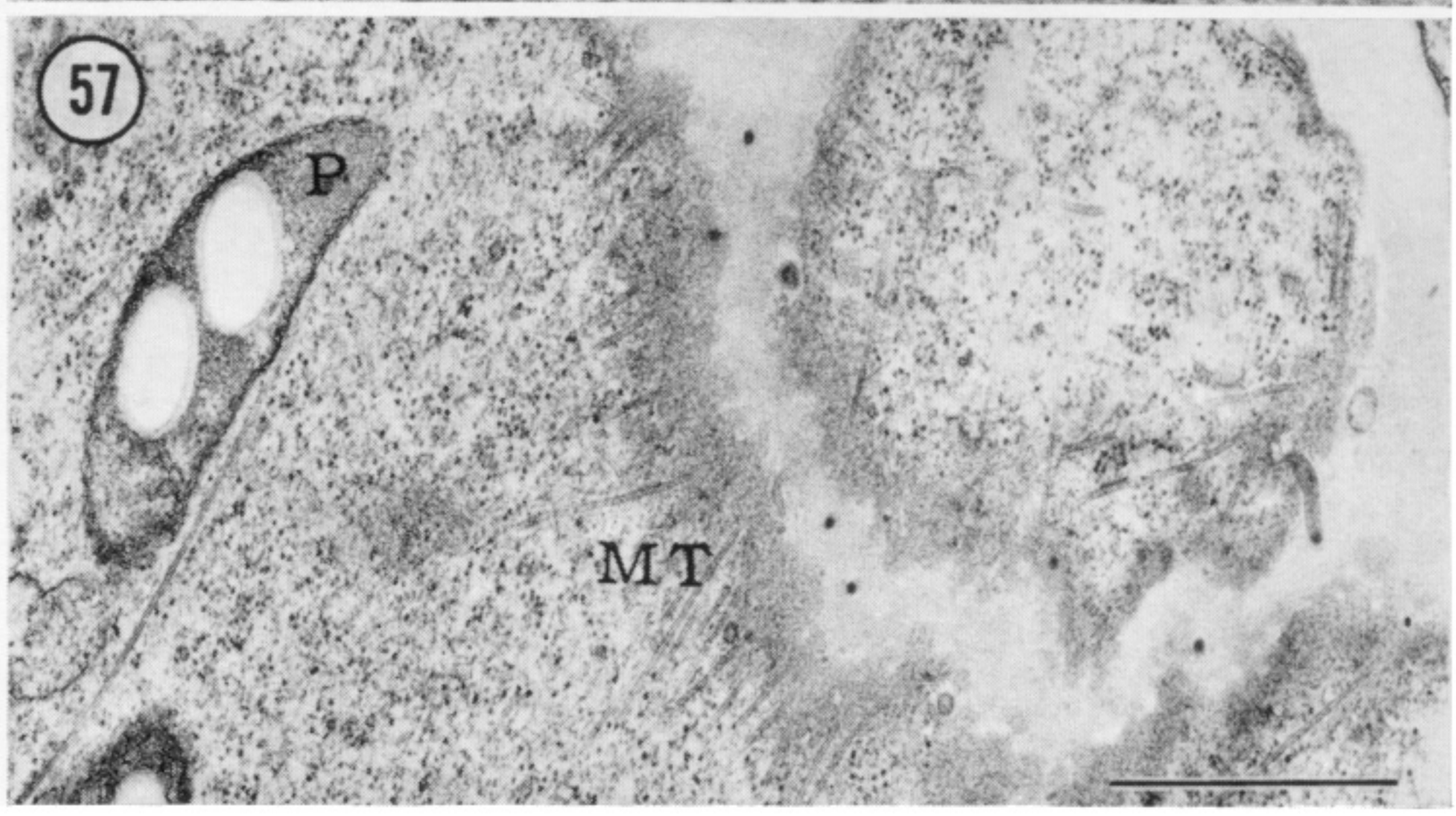


FIGURES 36–52. For description see opposite

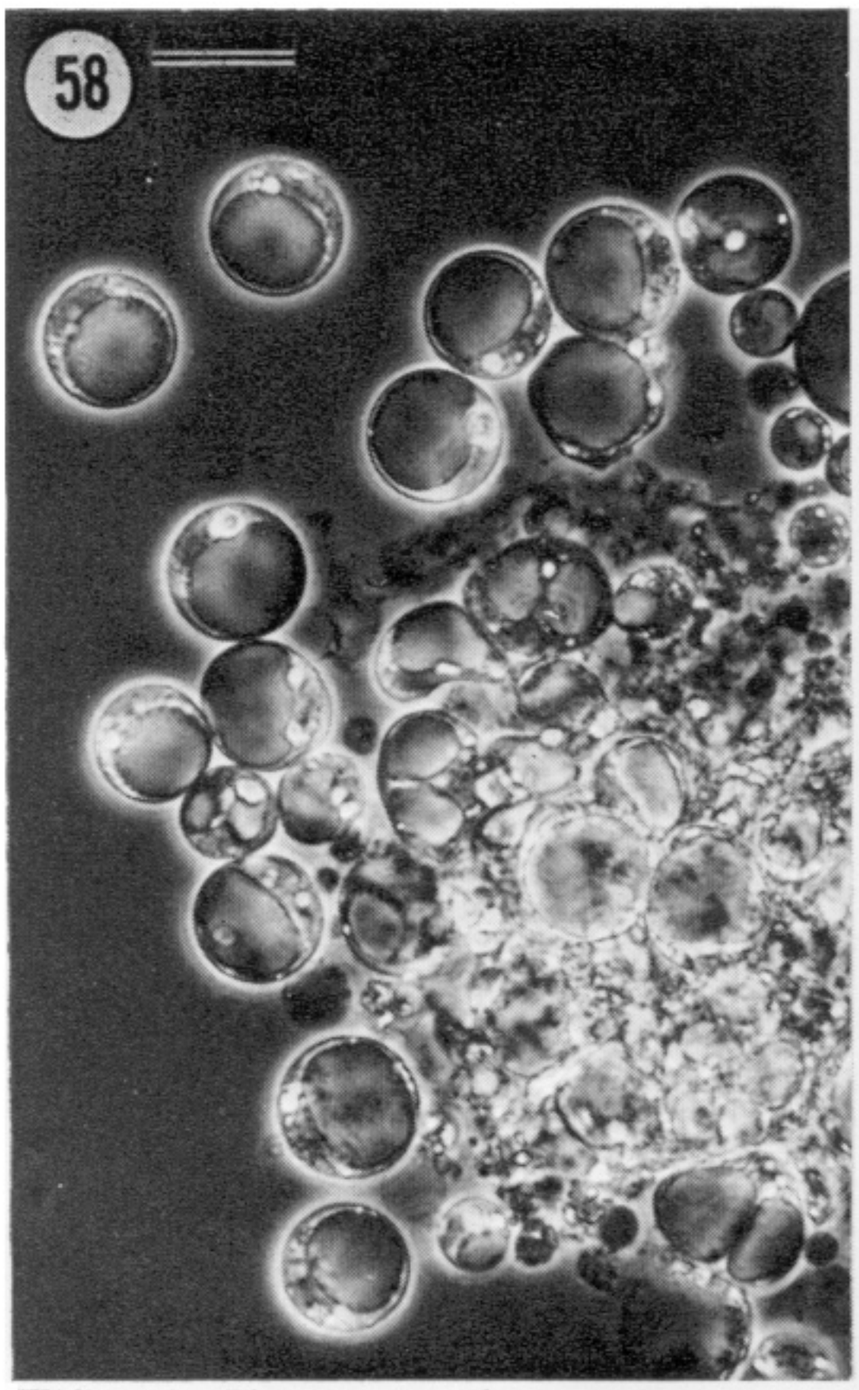


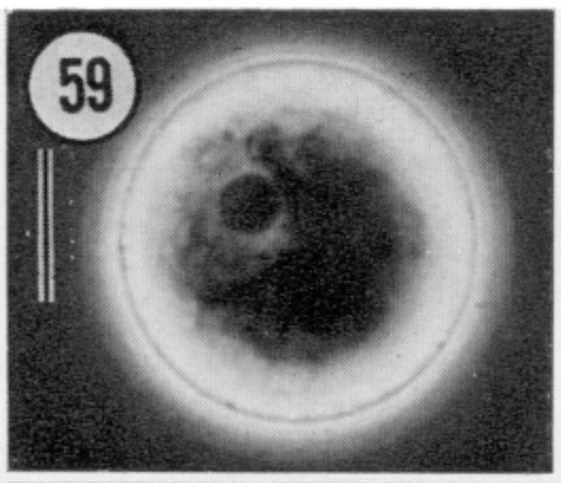


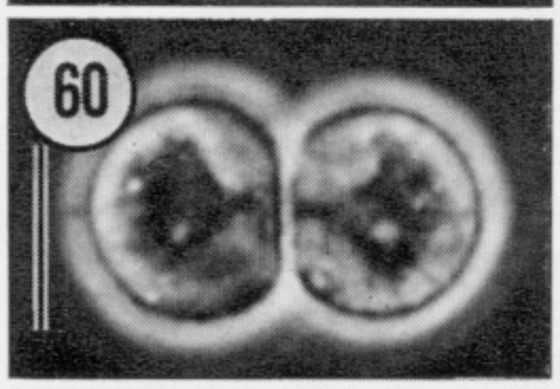


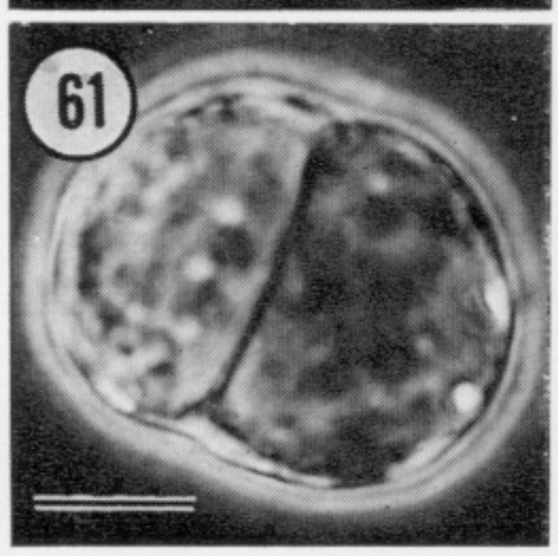


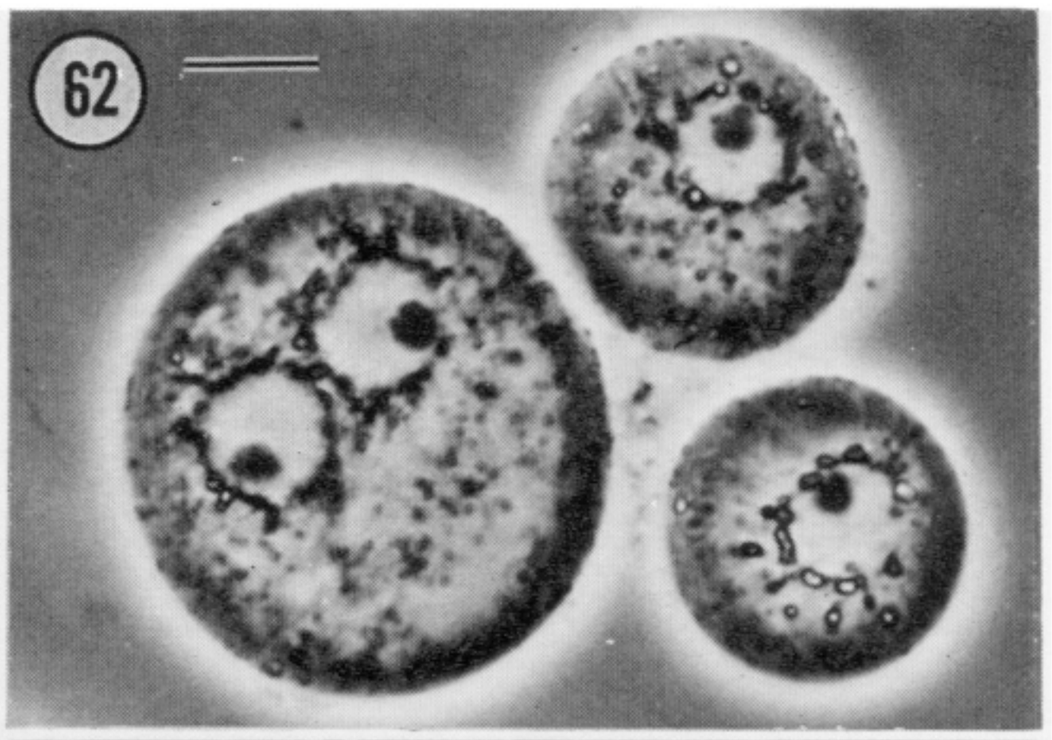
FIGURES 53–57. For description see opposite

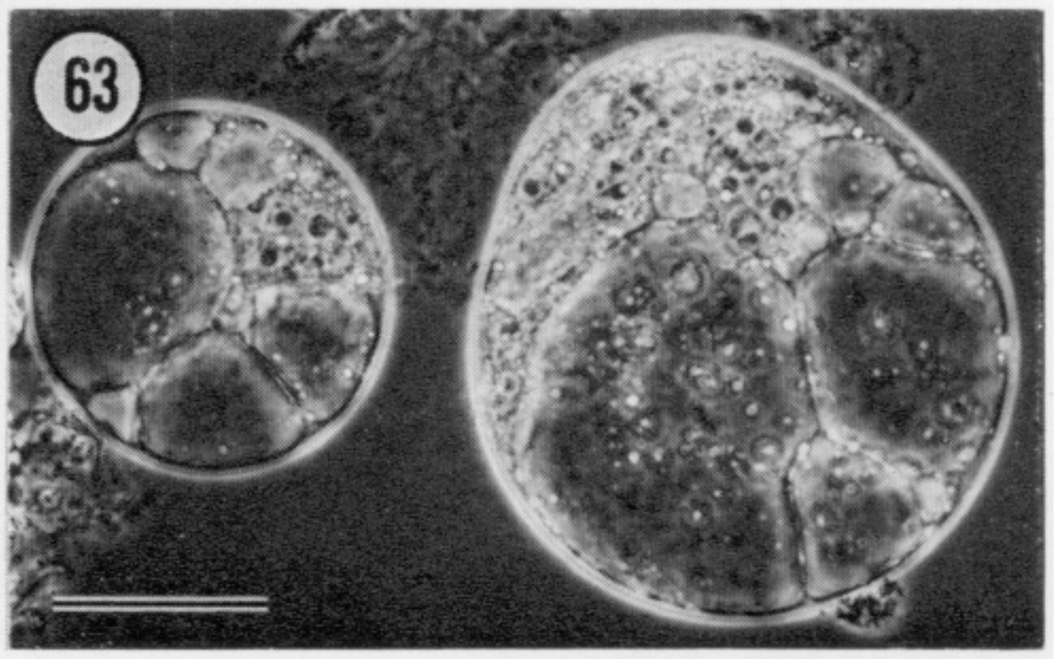


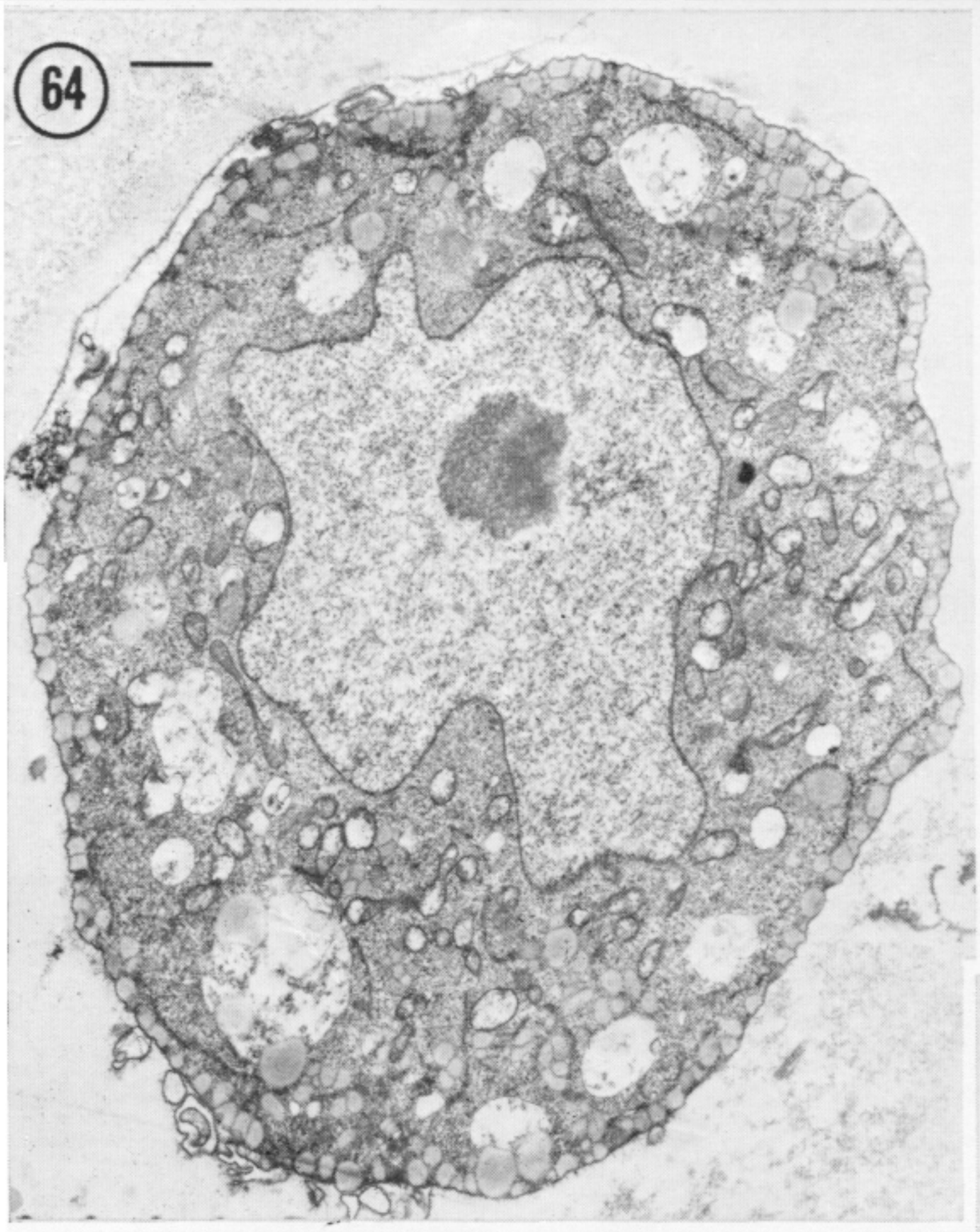


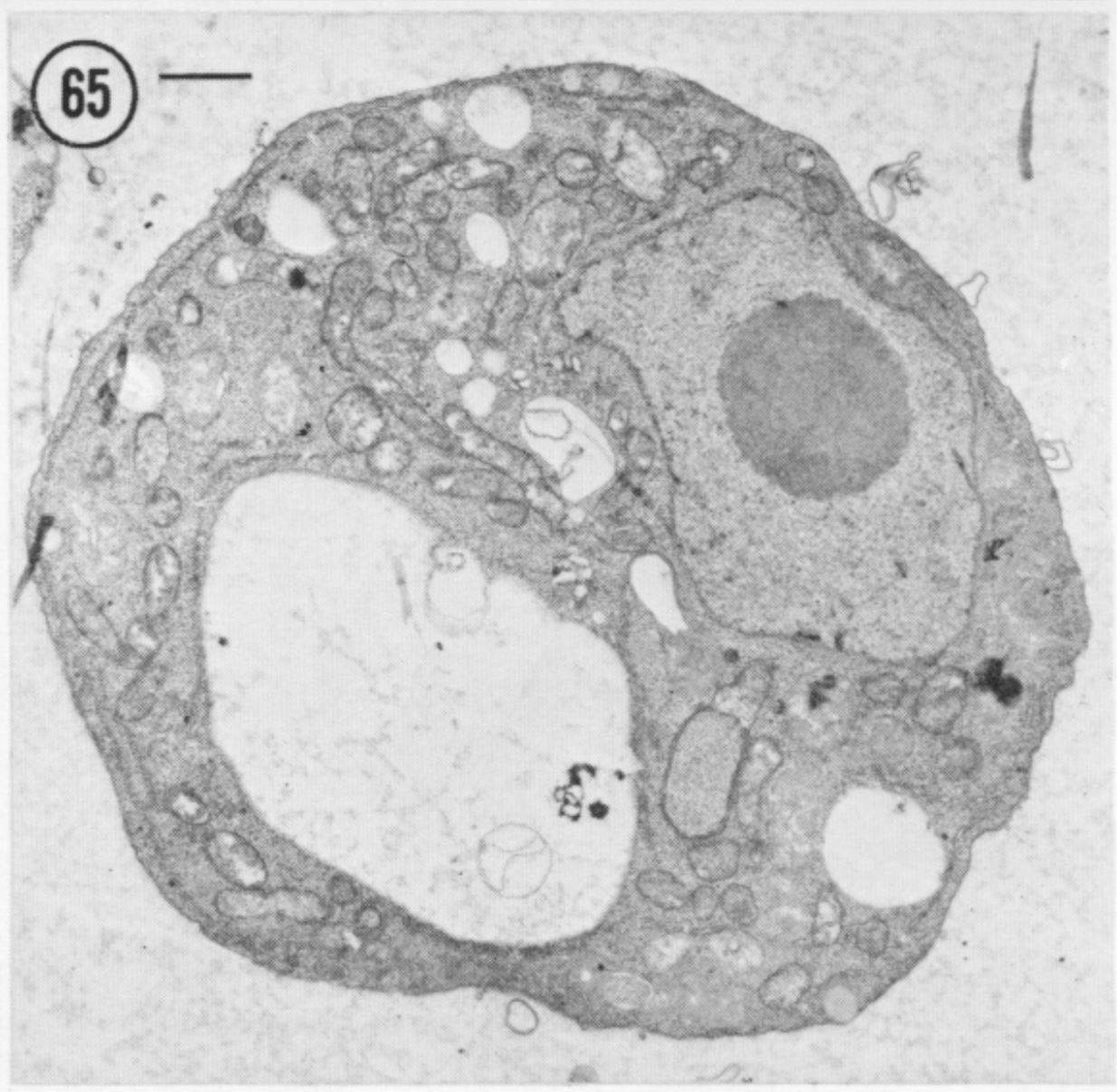


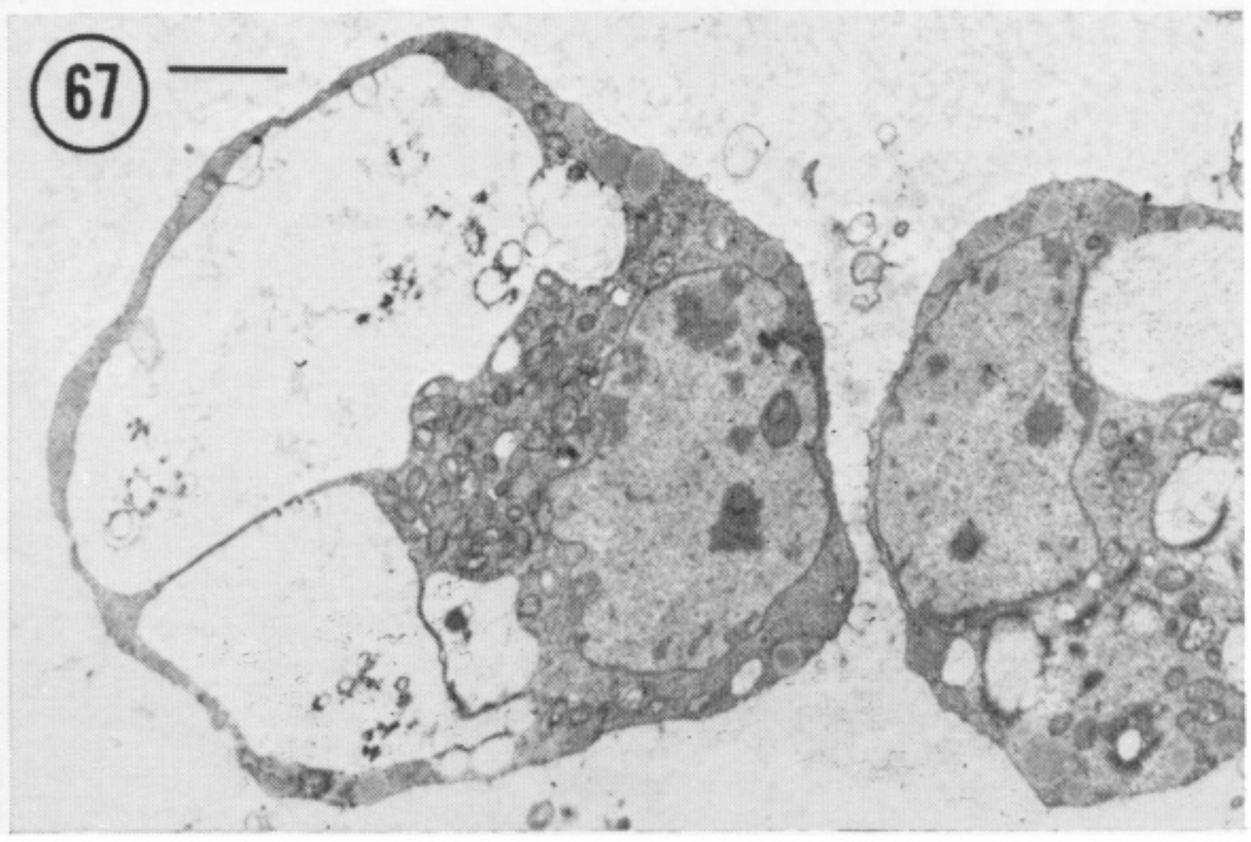


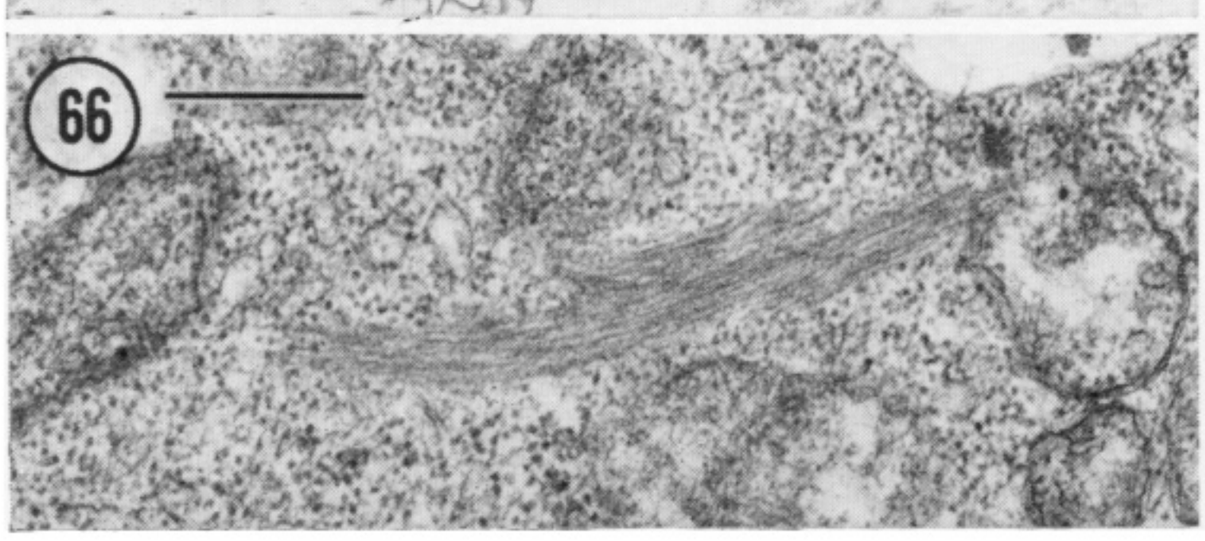




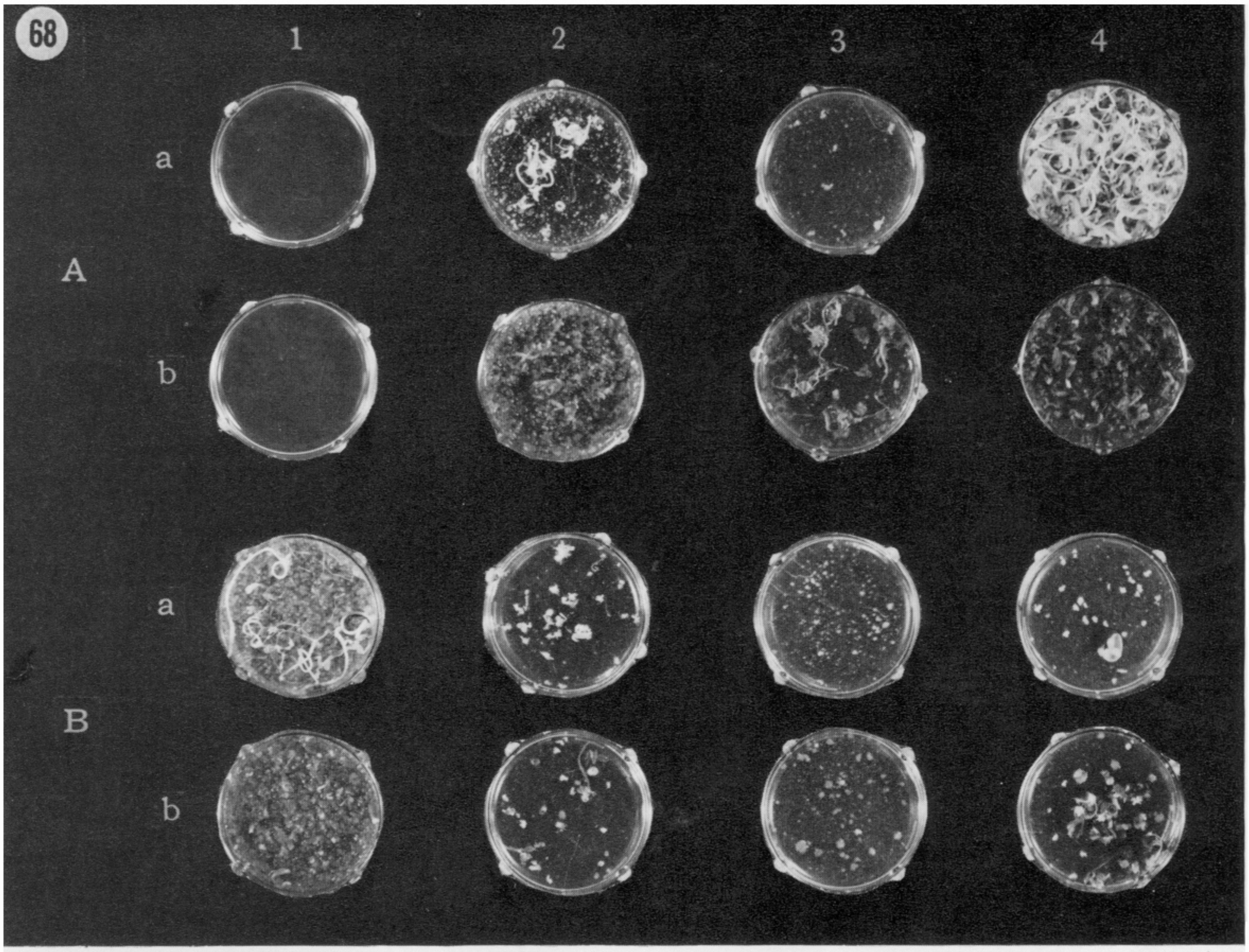


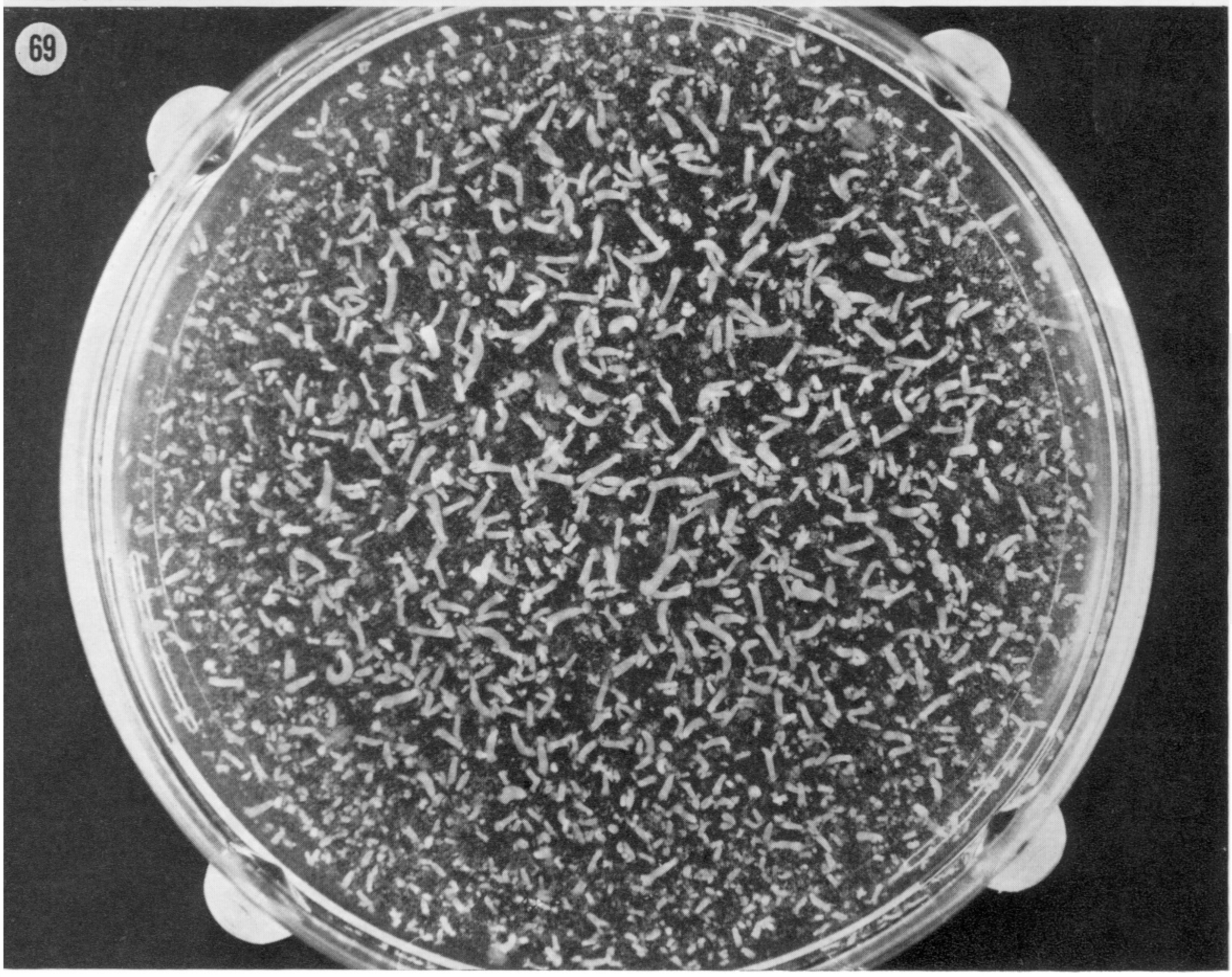






FIGURES 58–67. For description see opposite





FIGURES 68 AND 69. For description see opposite