

An analysis of the determinative difference between singlets and doublets of *Oxytricha fallax*

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SUMMARY

Doublets of *Oxytricha fallax* possess two complete sets of ciliature. The doublet phenotype is inherited through sexual and asexual reproduction as a cortically determined trait. The trait is also inherited through cystment, independent of cyst size. Prior work shows an absence of all visible cortical organelles, except cell membranes, in the cyst; thus the visible structures are not themselves determinative.

Results from excision experiments performed on encysting doublets indicate that the determinative difference between doublets and singlets is the presence of one or two 'determinative regions' located on the ventral surfaces of the organisms which serve as sites for the initiation of ciliary primordium development. Doublets possess two such areas, but singlets possess only one.

1. INTRODUCTION

The occurrence of a genetically stable doublet form of the ciliate *Oxytricha hymenostoma* was first described by Dawson (1920) and has since been described in numerous ciliate species (Fauré-Fremiet 1945). The doublet ciliate phenotype is inherited as a cytoplasmic trait, without changes of nuclear genotype, thus serving as an example of the role of the existing cell structure in determining the organization and orientations of new structures when they are synthesized in association with cell reproduction (Sonneborn, 1963).

Reported here are results of descriptive and experimental studies on doublets of *Oxytricha fallax*. The results indicate, on each 'half' of the doublet cell, a cortical region which is responsible for formation of primordia of the ciliature, but the region's inheritance is not dependent upon the presence of the visible cortical organelles. Such determinative cortical regions have been reported by others (Hanson, 1962; Jerka-Dziadosz, 1964; Sonneborn, 1963), but in no other study has it been demonstrated that the region can be devoid of all visible cortical structures except cell membranes.

2. MATERIALS AND METHODS

Techniques for protargol staining and culture were, in general, those previously described (Grimes, 1972; Sonneborn, 1970). Precise cultural methods were modified occasionally, and will be noted in Results where relevant. Doublet animals

(possessing duplicate sets of cortical structures) arose spontaneously by fusion at conjugation in November 1969, and have been maintained either as cysts or vegetative cells in clonal culture, with periodic mating to prevent clonal ageing. Doublets have never been observed to arise in any other manner.

Excision of portions of encysting doublets was carried out as follows. Encysting cells were transferred from baked lettuce to exhausted cerophyl (Sonneborn, 1970); cells in baked lettuce invariably lyse when cut. Doublets were then placed in a microdrop of culture fluid on a freshly prepared drop of 0.5% agar on a coverslip. Culture fluid is absorbed by the agar resulting in flattening and immobilization of the double animals. The coverslip was then inverted on a small moist chamber, viewed at $\times 100$ in a compound microscope and cut from below using a needle ($\sim 3\text{--}5\ \mu$ diameter) operated by a de Fonbrune micromanipulator. Recovery of cut cells was accomplished by flooding the coverslip with culture fluid and reisolating the cut cells. With the apparatus used, the adoral zone of membranelles and nuclei were clearly visible. However, individual cirri could not be distinguished; thus in all excision experiments the cirri possessed by fragments is not known.

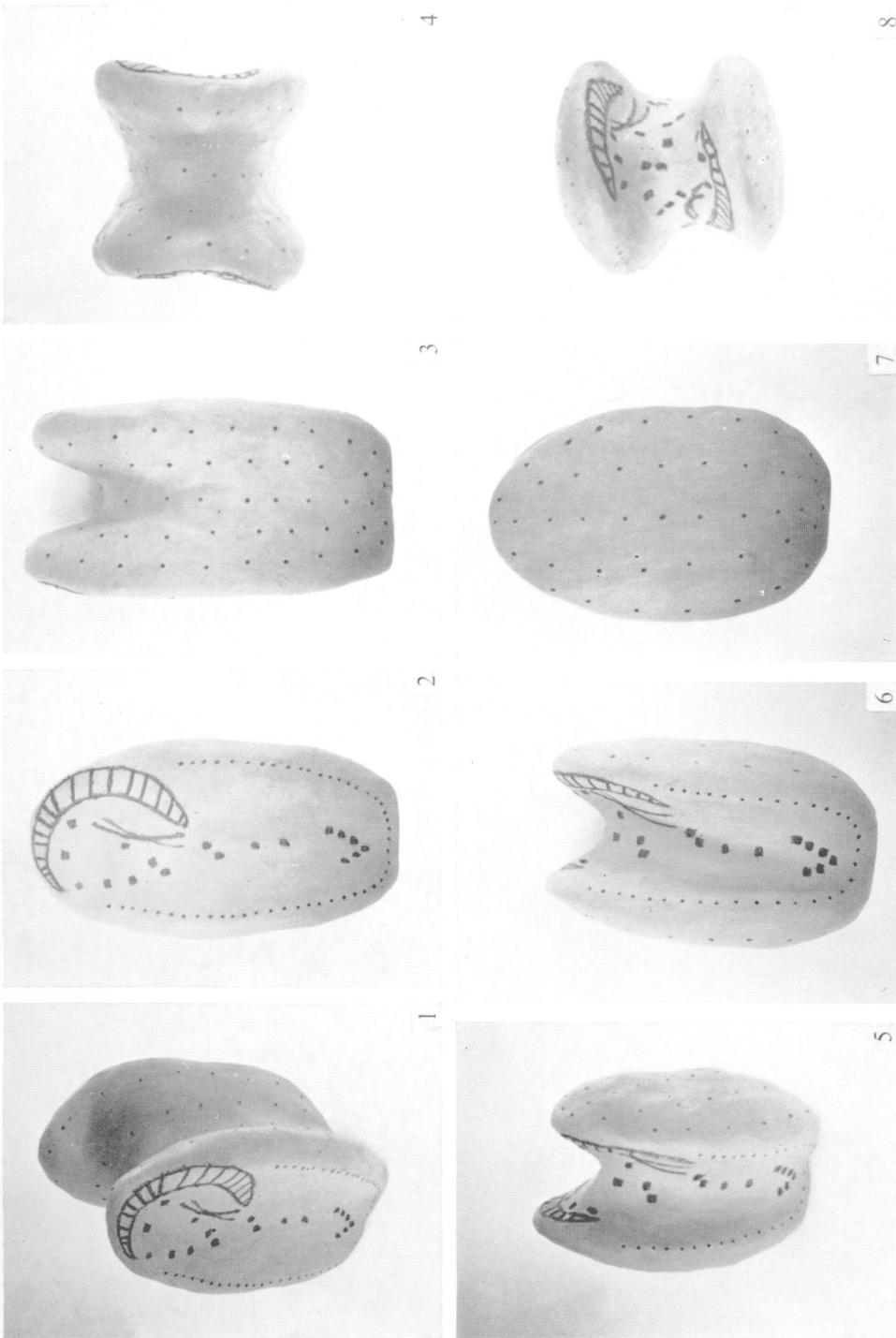
3. RESULTS

(i) *The doublet types*

Doublets within any one line of descent possess two distinct arrangements of cortical structures. Type I is illustrated in Figs. 1–4 and conforms to the doublets described in *Oxytricha hymenostoma* by Dawson (1920). Type I doublets are interpreted as two single animals fused along their mid-dorsal lines, slightly separated at the anterior end but firmly united posteriorly. Observations of cycloisis in living cells and electron microscopy of doublets indicate complete cytoplasmic continuity between the ‘halves’ of the doublets.

Type II doublets are illustrated in Figs. 4–8. The spatial relations of the two posterior ventral ciliatures are similar to those described for Type I in that they are 180° apart and facing outward; but the two anterior ventral ciliatures face each other on the inner surface of the anterior cleft between the two halves of the doublet. The inner surface of the cleft is thus not covered by dorsal bristles as in Type I, but by frontal cirri, undulating membranes, and the two adoral zones of membranelles.

Type I doublets do not reproduce exactly true-to-type; Type II doublets arise sometimes from Type I doublets. Whether Type II always gives Type II progeny is not clear; Type II cannot be unmistakably recognized without staining. However, single cell isolates are always observed to give rise to clones containing both Type I and Type II doublets. This clearly implies that both types are interconvertible, assuming the doublet type isolated was random. Because of the large shifts necessary to convert one type to the other, the two types are not apparently interconvertible by normal flexibility of the vegetative cell. The presence of a dichotomous population with intermediates lacking also supports this statement. Exactly when and how the conversion from one doublet type to the other occurs



Figs. 1-8. Photographs of clay models illustrating spatial relations of ciliatures in Type I (Figs. 1-4) and Type II (Figs. 5-8) doublets. Figs. 1 and 5: lateral view. Figs. 2 and 6: ventral view. Figs. 3 and 7: view of dorsal ciliature. Figs. 4 and 8: anterior view.

remains unknown. However, a plausible hypothesis can be imagined for the conversion occurring in the opisthe at division, but details of the hypothesis must await confirmation by observation.

Both types of doublets give rise to singlets, by two related processes. The cleft in some doublets can be deepened to the point where, at cell division, the anterior fission products are two singlets, while the opisthe is a doublet. If the cleft is not quite as deep as the fission plane, the proter is a doublet fused only at a small posterior region. The halves of such a doublet eventually pull apart, forming two singlets. The spontaneous formation of singlets from doublets has never been observed by any means other than separation of the halves in one or the other of these two ways. Singlets which are derived from doublets in either of these manners are then capable of producing viable clones, all cells of which possess one set of cortical structures. These observations on the separation of the halves of doublets agree with those of Dawson (1920).

Because of the tendency for halves to separate, constant selection of tight doublets must be performed in order to maintain a clone of doublets, even though doublets usually give rise to two doublets at cell division. During pre-fission morphogenesis, development of two sets of primordia proceeds synchronously, yielding two complete sets of ciliature in daughter cells. By imposing constant selection, the doublets have been maintained for hundreds of cell generations.

Doublets are also capable of conjugation, but whether both Types I and II can conjugate is unclear. Sexual unions may occur between two doublets, or between doublets and singlets. However, the asexual (fission) products of exconjugants always maintain the cortical characteristic (i.e. singlet *v.* doublet) of the cytoplasmic parent. Genetic markers are not available to ascertain if true conjugation, i.e. cross-fertilization, has occurred, but macronuclear anlage formation and nuclear differentiation appear normal. The conclusion of genetic equality between doublets and singlets implied by these observations is also supported by the following observations. (1) Doublets arise by fusion of singlets during conjugation. (2) Since *O. fallax* is a selfer, the doublets arise within a clone. (3) Singlets derived from doublets reproduce true-to-type.

Instead of the two oblong macronuclei and two micronuclei possessed by singlets, doublets contain 2–4 irregularly shaped macronuclei of variable size and up to six micronuclei. Although not measured, the macronuclear complement of doublets appears obviously to have a larger total nuclear volume than singlets. The two most common arrangements of macronuclei for both Types I and II doublets are illustrated schematically in Figs. 9 and 10. Typically, the posterior macronucleus has a broad saucer-shape, with only a shallow depression on its anterior face. Whether one or two macronuclei are located anteriorly appears to be correlated with the extent of separation between the anterior regions of the doublets. If the cleft is deep, then two smaller, more oblong macronuclei, one just internal to each cytostome, are common. When the cleft is shallow, then one macronucleus similar in shape to the posterior macronucleus, but inverted, is usually observed. The shape and number of macronuclei thus appear to be

functions of general cell shape. The reorganization bands marking the site of DNA synthesis migrate as in singles; starting at the anterior and posterior margins of the respective nuclei (resulting in broad reorganization bands) and moving toward the cell's middle. All macronuclei fuse at the end of the S period, as they do in singles, and then the fused macronucleus divides amitotically, its products being distributed to daughter cells. Whether the fused macronucleus divides into three lobes at once, or if the anterior macronucleus divides again to form the two anterior macronuclei in deeply cleft doublets is unclear.

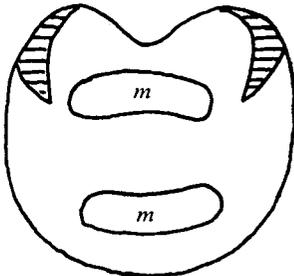


Fig. 9

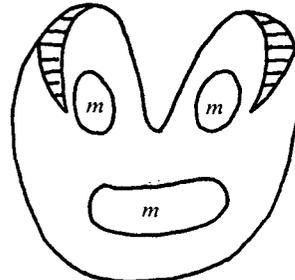


Fig. 10

Figs. 9 and 10. Diagrammatic representations of the two most commonly observed macronuclear arrangements in doublets. *m* = macronucleus.

(ii) *Inheritance of the doublet characteristic during cystment*

Jack Swelstad (unpublished) found that doublets emerge from cysts; however, no study was attempted to ascertain if the doublet characteristic is faithfully maintained through cystment. To answer the question of whether cells always excyst as doublets if they were doublets before encystment, the following experiment was performed. Starving doublets from a clone were concentrated by micropipette. Cells were observed at approximately $\frac{1}{2}$ h intervals during encystment and any singlets resulting from separation of deeply cleft doublets were removed. All non-encysted cells were then removed, and the cysts stored for 3 weeks. At the end of this period excystment of 164 cysts was induced. All which excysted (162) were doublets upon emerging from the cyst wall. Two months later, 235 doublets were excysted from the same group of cysts; no singlets were observed. No singlet has ever been observed to emerge from a cyst that was formed by a doublet animal, unless the encysting cell was experimentally modified (see below). The doublet feature is thus inherited faithfully through cystment. The converse also appears to be true – singlets never emerge from cysts as doublets.

(iii) *Structure and morphogenesis of doublet cysts*

The structure of the doublet cysts appears identical to that described for singlets, both at the light and electron microscope levels of observation (Grimes, 1972). Although the study of cystment in doublets has not been nearly as detailed as for singlets, all processes appear identical, except that two sets of cortical structures per cell dedifferentiate synchronously during encystment and redifferentiate synchronously on opposite sides of the cyst surface during excystment.

(iv) *Cyst size and doublet inheritance*

Cysts of doublets are in general larger than cysts of singlets. A simple hypothesis to explain the reliable inheritance of the doublet feature through cystment is that larger cysts can form two sets of cortical structures during excystment, and smaller cysts can form only one set. An experiment was performed to test this hypothesis by obtaining populations of cysts of equal size from doublets and singlets. The technique made use of the observation that encystment can be induced not only by starvation, but also by crowding cells in the presence of excess food. Being well fed, the cells are larger and make larger cysts than starved cells. In this way it was possible to get cysts of singlets as large or larger than those of

Table 1. *Diameters of cysts obtained by crowding well-fed singlets and starving doublets. Relation of cyst size to cortical type emerging from cyst*

(*R* = range. \bar{X} = mean. *n* = no. of specimens measured.)

Cortical type before encystment	(A) Selected cysts		(B) Non-selected cysts	
	Singlet	Doublet	Singlet	Doublet
	<i>n</i>	36	30	22
<i>R</i> (μ)	21–25	17–20	20–25	19–25
\bar{X} (μ)	22.8	18.5	22.2	22.2
No. of doublets excysted	0	30	—	—
No. of singlets excysted	34*	0	—	—

* Two cells did not excyst.

doublets induced to encyst by starvation. All cells used in the experiment were from one exconjugant clone of doublets; the singlets were derived from one singlet that arose at fission from a deeply cleft doublet in this clone. As before, doublets were concentrated, starved, and allowed to encyst, while making periodic observations and removing all singlets resulting from separation of doublet halves. Singlets were concentrated in depressions and fed. At a certain population density, many of the crowded, well-fed cells encysted. Cysts obtained in these ways were collected and the diameters of cysts in a random sample of each were measured. As appears in Table 1B, the ranges and means of the diameter are indistinguishable for the two populations of cysts.

From the two populations, the largest cysts of singlets and smallest cysts of doublets were selected for excystment. The selected cysts were measured, and then excystment was induced, with the results presented in Table 1A. The size ranges did not overlap: all cysts of singlets were larger than any cyst of doublets. Nevertheless, all cysts of doublets excysted as doublets and all cysts of singlets (except two that failed to excyst) excysted as singlets. The inheritance of a doublet *v.* a single set of cortical structures through cystment is thus independent of cyst size. Independence of nuclear genotype is also strongly implied because all cells were from the same clone and therefore presumably completely isogenic.

(v) *Excision experiments on precystic doublets*

Cells were severed in the precystic stage, i.e. after extrusion of cytoplasmic inclusions (crystals, old food vacuoles) and prior to rounding up. This stage lasts for approximately 2 h. Fragments of precystic doublets can do either of two things: regenerate missing organelles or complete encystment, as reported for fragments of singlets by Hashimoto (1962). The option chosen appears to correlate with the length of time the cells have been precystic. Fragments of doublets which recently became precystic regenerate lost structures and do not encyst. Once the ciliature begins to be resorbed, fragments complete encystment. In order to analyse the determination of the singlet *v.* doublet alternative, both regenerating and encysting fragments of doublets were observed.

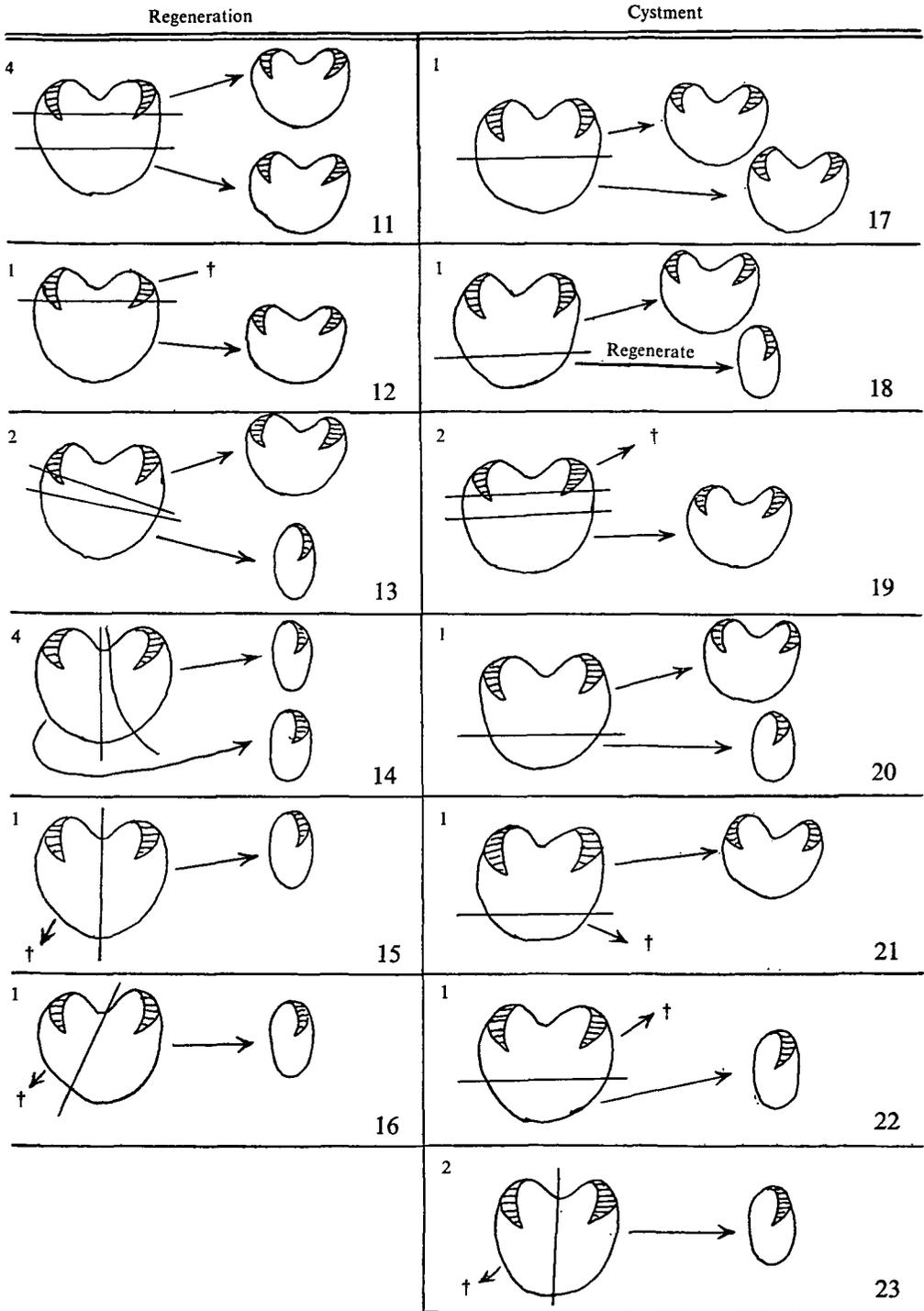
Cortical regeneration involves the production of ciliary primordia and eventual replacement of missing ciliary organelles. If fragments regenerate as doublets, two sets of primordia are formed and develop synchronously. Fragments regenerating as singlets form only one set of primordia.

Results obtained are only the initial ones in an analysis that is being continued. Here are presented only the results, the interpretation being deferred for the discussion section. No distinction has been made between Types I and II doublets in any of the excision experiments.

(1) Transverse cuts resulting in cortical regeneration. Thus far, only seven doublets have been severed transversely and proceeded to undergo cortical regeneration. The general results are presented in Figs. 11–13. Both the anterior fragment (promer) and posterior fragment (opimer) of four cells recovered and regenerated. All eight fragments regenerated as doublets (Fig. 11). The promer of another cell lysed after severing; the opimer regenerated as a doublet (Fig. 12). The promers of the two remaining cut cells regenerated as doublets; the opimers regenerated as singlets (Fig. 13). In total, 11 fragments resulting from transversely cut precystic doublets regenerated as doublets, and two as singlets.

(2) Longitudinal cuts resulting in cortical regeneration. Six cells were severed more or less parallel to their longitudinal axes such that the two sets of ciliatures were largely separated from each other (Figs. 14–16). Only two fragments lysed when the cells were cut (Figs. 15, 16); the other fragment of these cells and both fragments of the remaining cells were recovered. Although some cells had a little more and others a little less than a complete singlet ciliature, all fragments regenerated as singlets.

(3) Transverse cuts followed by encystment. At least one fragment of seven transversely severed precystic cells continued encystment and formed excystable resting cysts. Both fragments of three cells were recovered (Figs. 17, 18, 20). Of one of these, both the promer and opimer excysted as doublets (Fig. 17). The promer of another cell excysted as a doublet, the opimer as a singlet (Fig. 20). The promer of the remaining cell excysted as a doublet, but the opimer did not encyst; it regenerated a single set of ciliary organelles (Fig. 18). Only the opimer was recovered from three other cut cells (Figs. 19, 22). Two opimers excysted as



Figs. 11–23. Diagrammatic representations of results of excision experiments on precystic doublets. The upper left number indicates the number of cells showing the diagrammed results.

Figs. 11–16. Cells which regenerated after excision. The lines represent the approximate levels of cutting. In Figs. 11 and 14 all cuts were made between the two lines. A † represents death of fragment.

Figs. 17–23. Cells which completed encystment and excysted. Again lines represent approximate levels of cutting, and † represents death of a fragment. The opimer in Fig. 18 regenerated instead of encysting.

doublets (Fig. 19), one as a singlet (Fig. 22). The only fragment, the promer, recovered from the remaining cell excysted as a doublet (Fig. 21).

(4) Longitudinal cuts followed by encystment. The two cells in this category yielded only one viable fragment from each cell. Both of these excysted as singlets (Fig. 23).

4. DISCUSSION

Doublets in *O. fallax* reproduce as doublets through both asexual and sexual reproduction, and encysted doublets excyst as doublets. What is the basis of this genetic stability? Several *a priori* possibilities exist, and will now be discussed in relation to the pertinent evidence.

(1) Is there a genic basis? As mentioned earlier, this hypothesis can be virtually excluded (as it has been in other species; Sonneborn, 1963) by the facts: (a) that the doublets arise by fusion of two singlets of the same clone, and therefore are presumably of the same genotype; and (b) that the singlets derived asexually from doublets always reproduce true-to-type, i.e. as singlets. The same conclusion is indicated by the inheritance of the doublet phenotype in crosses to doublets and singlets: doublet exconjugants regularly produce doublet clones. If a genic basis existed the gene would have to be dominant, but this is inconsistent with the failure of singlet mates of doublets to produce doublet progeny. This kind of evidence was strengthened in other studies in which both genic and cytoplasmic markers were followed in crosses (Sonneborn, 1963).

(2) Is the basis in the size of the macronuclei? This possibility was excluded by results of excisions. (a) Transverse cuts (Figs. 11, 17) across doublets resulted in fragments with amounts of nuclear material similar to the amount in singlets, but most of the fragments regenerated or excysted as doublets. (b) Those fragments which regenerated or excysted as singlets after longitudinal or transverse cuts (Figs. 13, 16, 18, 20, 22) contained about the same amount of macronuclear material as those which regenerated into doublets. In all fragments referred to in (a) and (b) the amount of macronuclear material contained was approximately half of the amount in unaltered doublets. Thus the amount of macronuclear material appears to be a response to cell size.

(3) Is cell size the basis of difference between doublets and singlets? This possibility is excluded by the study of cysts. Although doublets normally form larger cysts than singlets when encystment is induced by starvation, induction of encystment by crowding well-fed (large) singlets yielded some singlet cysts larger than cysts of doublets induced by starvation. Nevertheless, from the larger singlet cysts singlets emerged while doublets emerged from the smaller doublet cysts.

(4) Does the basis lie in the number of ciliatures existing in the cell? Although this seems to be the case in view of the results of asexual and sexual reproduction since each type reproduces its own kind, it is excluded by the observations on cystment. No visible traces of the ciliatures remain in the cyst (Grimes, 1973*a, b*). Nevertheless, at excystment the cell 'remembers' whether it was a doublet or singlet during encystment and emerges true-to-type.

(5) Does the basis lie in some other localized cytoplasmic differentiation? The available data from the excision experiments are indeed consistent with this hypothesis. (a) Longitudinal cuts that separated the doublet into two similar halves always yielded singlets, whether the fragments regenerated or passed through cystment (Figs. 14, 16, 23). This, like the production of two singlets from a deeply cleft doublet, shows that there are two determinative factors in doublets – one in singlets and one in ‘singlet’ fragments formed by longitudinal severing of doublets. (b) The determinative factor is divisible: when doublets are cut transversely so that each singlet half is transected, both anterior (promer) and posterior (opimer) fragments can regenerate or pass through cystment as doublets (Figs. 11, 17). (c) The determinative factor is limited in position and extent because some opimers of doublets have yielded singlets both by regeneration and by cystment (Figs. 13, 18, 20, 22). The posterior limit of the determinative region cannot yet be stated because the level of the cuts is not precisely known. Tentatively, it seems to lie somewhere between the levels of the cytostome and anal cirri. Attempts to identify the exact position and extent of the determinative region are in progress.

Thus far the results in *O. fallax* are similar, but not as extensive as those of Jerka-Dziadosz (1964) on *Urostyla grandis*. She concludes that a specifiable region of the ventral cortex functions as an ‘organization’ area, defined in the same way as ‘determinative region’ above. The area in *U. grandis* also appears to be divisible but limited in extent. The observations of Hashimoto (1962) that both promers and opimers of transected singlets can regenerate or pass through cystment as singlets are also consistent with such a proposed determinative region.

That the determinative region is confined to the cell cortex is not rigorously proved in any case except *Paramecium* (Hanson, 1962; Sonneborn, 1963), although this is usually assumed. In an attempt to solve this problem for *O. fallax*, experiments are now in progress using a laser microbeam to selectively damage, destroy, or remove limited cortical regions.

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