J. Cell Sci. 39, 299–312 (1979)
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MITOCHONDRIAL ASSOCIATIONS WITH SPECIFIC MICROTUBULAR COMPONENTS OF THE CORTEX OF *TETRAHYMENA THERMOPHILA*

I. CORTICAL PATTERNING OF MITOCHONDRIA

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SUMMARY

Many of the mitochondria of *Tetrahymena thermophila* are localized in the cell cortex in regular, identifiable patterns. Two different mitochondrial patterns are seen; whether a cell expresses one or the other type apparently depends upon nutrient conditions in the culture and not upon other factors tested. Consistent associations between cortical mitochondria and certain of the cortical microtubular bands are seen at the light-microscopic level. Electron-microscopic observations confirm this and, furthermore, identify ultrastructural associations between cortical microtubules and the cortically located mitochondria. It appears that the cortical microtubular arrays serve as a guide for the localization (and thus patterning) of the cortical mitochondria.

INTRODUCTION

Several reports have noted that mitochondria are capable of associating with cytoplasmic microtubules (e.g. Smith, Järlfors & Cayer, 1977; Heggeness, Simon & Singer, 1978). Associations between mitochondria and the cilia or flagella of many different organisms have also been observed (Woolley, 1970; Tokuyasu, 1974; Simpson, 1972; Anderson & Ellis, 1965). In ciliate protozoa, several workers have reported associations between mitochondria and the cell cortex (Fauré-Fremiet, 1910; Horning, 1927; Elliott & Bak, 1964; Parsons & Rustad, 1968; Peck, 1978), and some have noted a spatial regularity in these associations (Chatton & Brachon, 1935*a*, *b*). This paper confirms the observations of earlier workers that mitochondria are indeed associated in a regular way with the ciliate cortex, and extends those observations to identify specific microtubular components in the cortex as structural correlates of mitochondrial localization. The analysis of the phenomenon of cortical mitochondria was aided, in part, by the unique staining of both the microtubular components of the cortex and also the mitochondria of the same cell by a batch of protargol from E. Merck. Preliminary results have been published in abstract form (Aufderheide, 1978).

MATERIALS AND METHODS

Cells of *Tetrahymena thermophila* (neé *T. pyriformis*, syngen 1 (Nanney & McCoy, 1976)), stock B1975, mating type III, were cultured axenically in 1 % proteose-peptone, 0·1 % yeast extract (PPY) medium, or monoxenically in PPY inoculated with a non-pathogenic strain of *Klebsiella pneumoniae*, and diluted 1:70 in distilled water. In some situations, axenic cells were washed and resuspended in Dryl's (1959) solution, either full strength or diluted 1:25 with distilled water. Unless otherwise specified, incubation temperature was 27 °C.

Phase-contrast observations of living cells were made by immobilizing them in a Schaeffer rotocompressor (Spoon, 1978) and observing them under oil-immersion optics. Only the minimum pressure to hold the cells was used, except in certain situations.

Cells were stained with protargol, following the technique of Ng & Nelsen (1977), using the protargol available from E. Merck, Darmstadt (Strong-silver protein, order no. 7447, lot no. 6950443). The addition of up to 4 % Na_2CO_3 to the hydroquinone-sodium sulphite developer solution allowed differential staining of cortical elements (Aufderheide, unpublished results). Without Na_2CO_3, both cortical microtubular arrays and mitochondria were made visible. When Na_2CO_3 was added to the developer following impregnation with this lot of protargol, only the cortical microtubules, but not mitochondria, were stained.

Preparation of cells for transmission electron microscopy followed standard procedures with minor modifications (Allen, 1967; Jaeckel-Williams, 1978). Cells were fixed in 1·25 % glutaraldehyde in 0·1 M cacodylate and 1 mM $MgSO_4$, pH 7·0, for 20 min at room temperature. The cells were then washed and postfixed in 1 % OsO_4 in the cacodylate buffer for 30 min. Dehydration in acetone was followed by embedment in Spurr's medium (Spurr, 1969). Sections were stained with uranyl acetate and lead citrate (Venable & Coggeshall, 1965) and were examined with a Philips 300 microscope.

RESULTS

Patterning of cortical mitochondria

Cells of *T. thermophila*, whether grown axenically or in bacterized medium, display arrays of mitochondria positioned immediately under the surface in the cell cortex, as well as mitochondria distributed throughout the endoplasm. Endoplasmic mitochondria do not show any obvious patterning or individual localization, although their distribution in the cell does not appear to be random. However, the mitochondria

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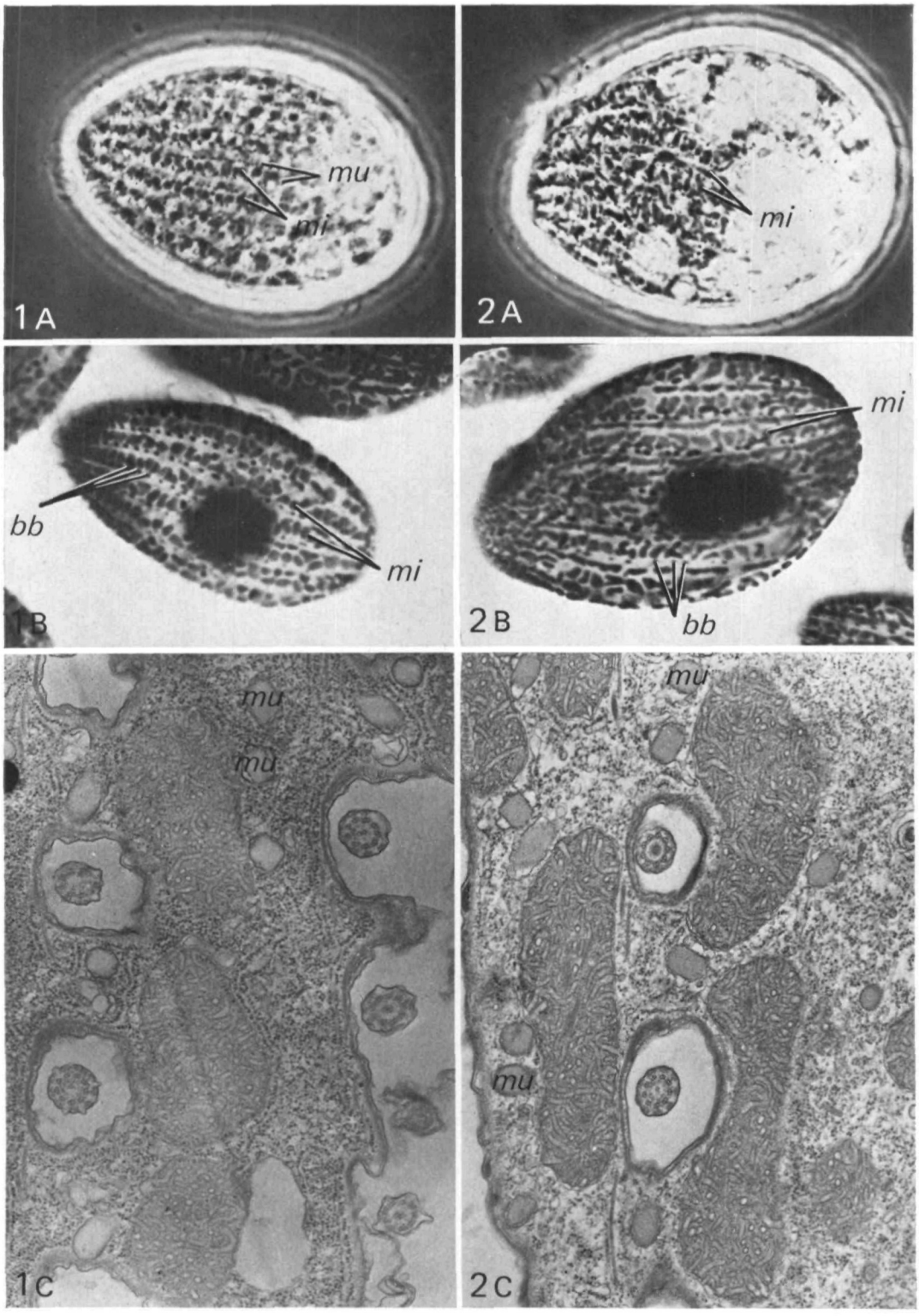
Figs. 1, 2. Appearance of single (Fig. 1) and double (Fig. 2) cortical mitochondrial patterns in different preparations. Cortical mitochondria (*mi*) can be identified in phase-contrast observations of living, rotocompressed cells (1A, 2A). Basal bodies are not visible; the small phase-dark dots are mucocysts (*mu*). The protargol staining technique (1B, 2B) stains basal bodies (*bb*) and reveals their organization into anterior-posterior rows (kineties). The mitochondria also shown by this protargol correspond to those visible by phase-contrast. Grazing thin-section electron micrographs (1C, 2C) of cells show mitochondria localized in the cortex in the same spatial relationship to the cilia and basal bodies as that seen by the light microscope. Fig. 1 shows the single mitochondrial pattern consisting of a row of mitochondria aligned to the cell's left of each kinety. The section in Fig. 1C was cut from a cell in a population known to express predominantly single pattern when fixed. Fig. 2 shows the double mitochondrial pattern. This type has 2 rows of mitochondria, one on each side of each kinety. These mitochondria differ in gross morphology, depending upon whether they are placed in the row to the right or to the left of each kinety, but no obvious ultrastructural differences can be seen between the 2 types (2C). Magnifications: Figs. 1A, B, 2A, B, ×1250; Figs. 1C, 2C, ×26000.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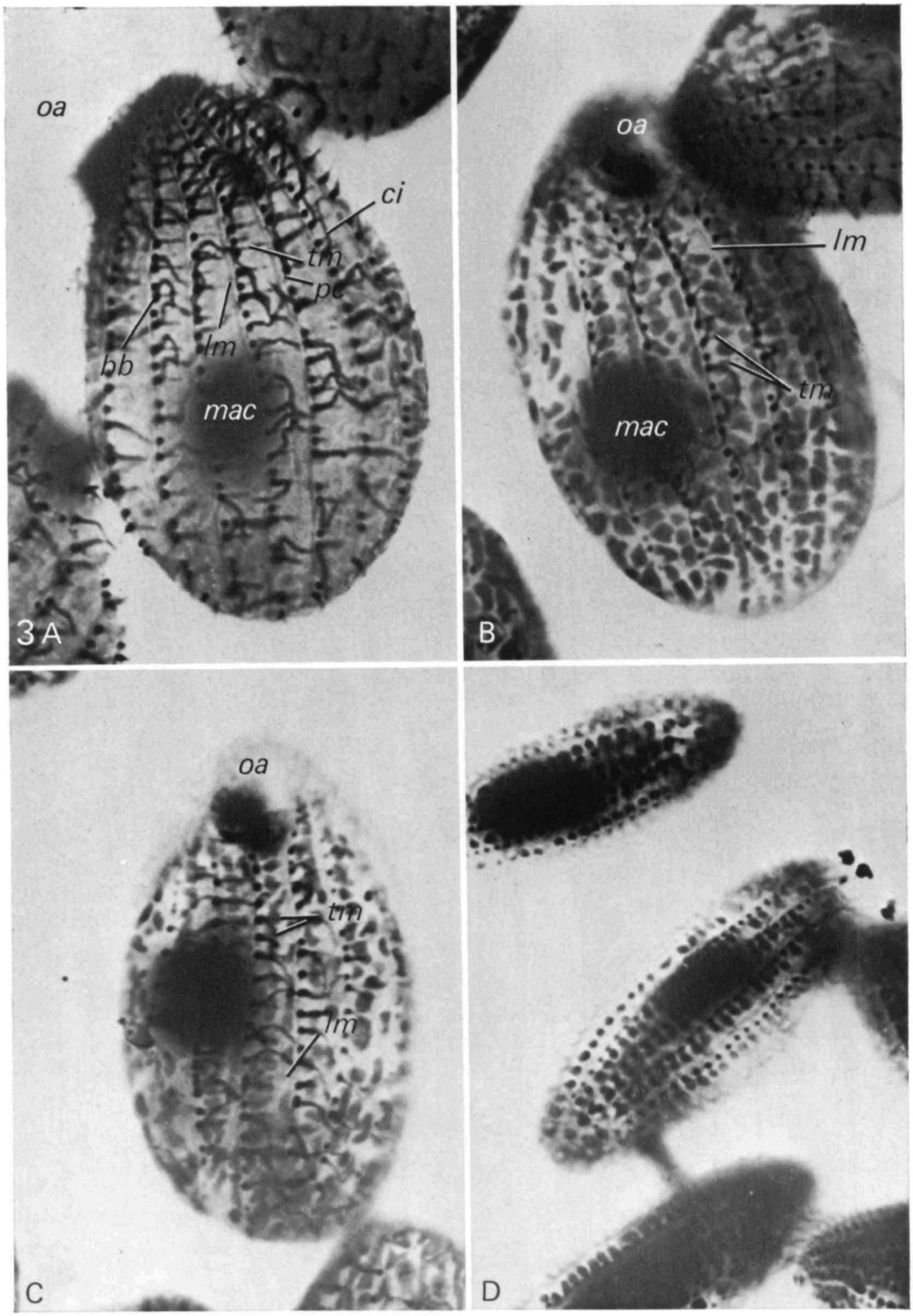

Fig. 3. For legend see facing page.

in the cell cortex do show asymmetrical localizations relative to the basal bodies of the somatic ciliature (Figs. 1, 2). *In vivo* rotocompressor observations of mitochondrial patterns show consistent agreement with the patterns seen in fixed, protargol-stained cells. A cell that is severely distorted in the rotocompressor for 20–30 min will gradually lose its cortical mitochondria as they dissociate from the cortex and drift into the endoplasm. If the pressure is released, and the cell allowed to recover for 30–60 min, then the cortical localization of mitochondria is restored. Whether the same mitochondria return to the cortex, or whether any of the endoplasmic mitochondria can also form associations with the cortex during this period, is unclear. When cells are held under minimum pressure, it is obvious that the cortical mitochondria are not permanently affixed, but rather are seen to drift out of the cortex and mix with the endoplasmic population. The vacated sites in the cortex are occupied by mitochondria from the endoplasmic population. It is not certain whether or not the cortical mitochondria form a permanent sub-population of the cell's chondriome. The above observations suggest that this is unlikely.

Two different patterns of cortical mitochondria can be seen (Figs. 1, 2, 6). One pattern shows a single row of mitochondria located immediately to the cell's left of each row of basal bodies (kinety) (Fig. 1).* These mitochondria are fairly small, and are frequently disk- or spheroid-shaped. Since there is essentially 1 row of mitochondria for each row of basal bodies, this configuration is termed the single cortical mitochondrial pattern. The other kind of pattern has 2 rows of mitochondria for each kinety: 1 immediately to the left and the other immediately to the right of each kinety (Fig. 2). This type is called the double mitochondrial pattern. The mitochondria to the left of each kinety have essentially the same arrangement and gross morphology as those mitochondria in the single pattern type. The mitochondria to the right of each kinety are usually thread- or sausage-shaped, and are often morphologically distinguishable from the smaller mitochondria to the left of the basal bodies. The third possible pattern type, mitochondria located only to the right of

Fig. 3. Protargol-stained cells, showing the cortical organization. A, a cell, impregnated with protargol and developed in Na_2CO_3-buffered developer. Basal bodies (*bb*), transverse microtubule bands (*tm*), postciliary microtubule bands (*pc*) and the longitudinal microtubule bands (*lm*) are all visible. Some basal bodies have cilia (*ci*) attached. The oral apparatus (*oa*) and the macronucleus (*mac*) are also made visible by this technique. B, C, protargol-stained cells, developed without added Na_2CO_3. Mitochondria are also stained, as in Figs. 1B and 2B. Both figures show double pattern cells. The association between transverse mitochondria and transverse microtubule bands, and between longitudinal mitochondria and the longitudinal microtubule bands is seen. Note that mitochondria do not tend to associate with the cortex, except in the close vicinity of a microtubular structure. D, cells transformed to the 'fast-swimmer' phenotype by starvation for 5 h. These cells express the strict single mitochondrial pattern characteristic of starved cells. All × 1550.

* By universal convention (e.g. Ng & Frankel, 1977), left and right in the ciliate cortex refer to the viewpoint of an observer standing inside the cell, parallel to the cell's anterior–posterior axis, and looking out. Hence left and right frequently seem to be reversed in many micrographs, since most are printed from a viewpoint outside the cell.

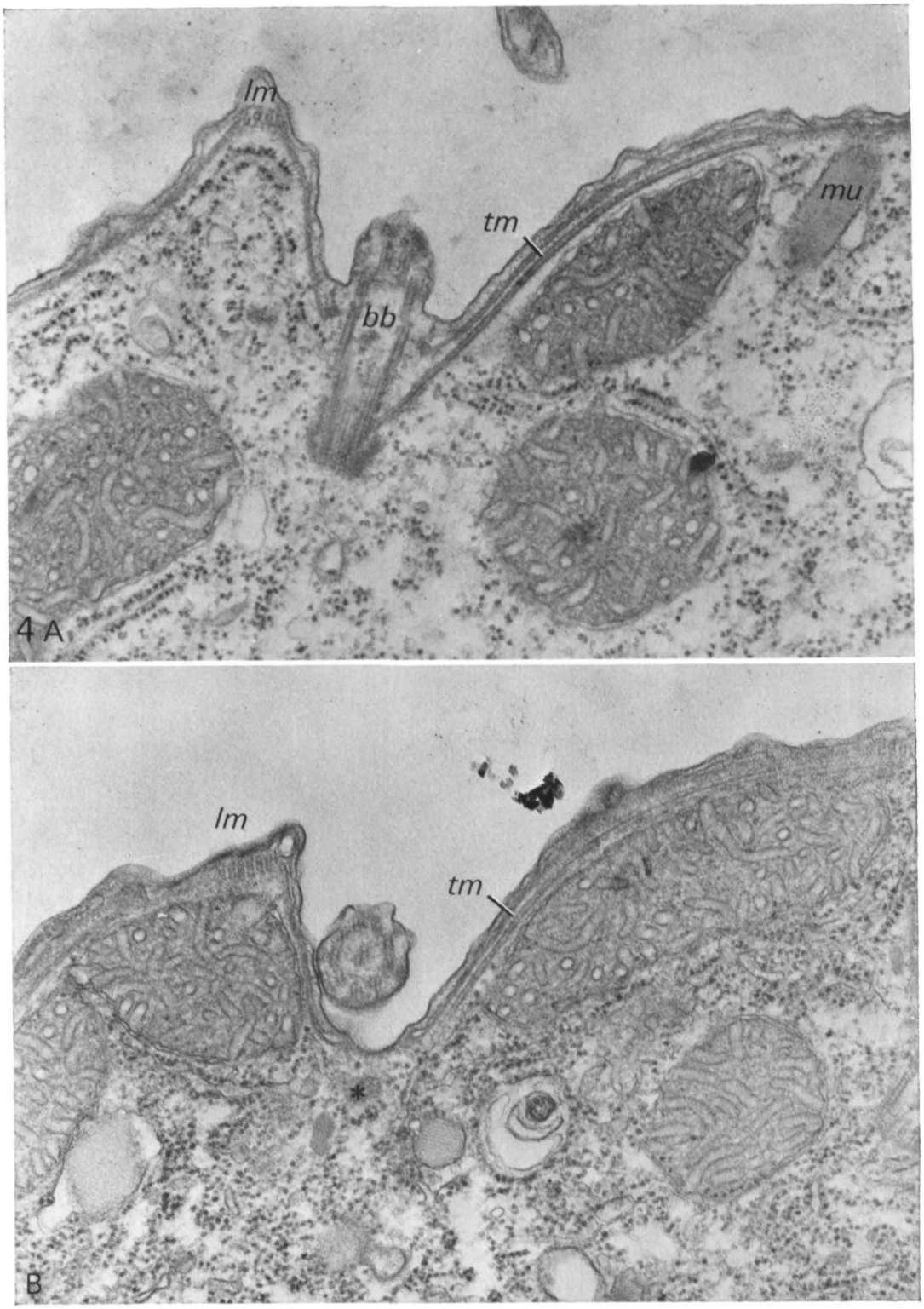

Fig. 4. For legend see facing page.

each kinety, is not seen. Grazing thin-section electron micrographs (Figs. 1 C, 2 C) of both pattern types confirm that the structures seen under phase-contrast and with protargol staining are indeed mitochondria and not some other cellular inclusion.

Structural correlates of cortical mitochondrial placement

Impregnation of *T. thermophila* with Merck protargol, followed by development with added Na_2CO_3, shows many of the microtubular elements of the cell cortex (Fig. 3 A). Basal bodies, with or without cilia, are organized into kineties. Emerging from the anterior of each basal body, the transverse microtubular band extends toward the cell's left. The band terminates before reaching the next kinety. The postciliary band starts immediately behind each basal body and extends a short distance to the posterior right. To the right of each kinety, the longitudinal microtubular band extends from nearly the anterior pole to the posterior pole of the cell. These observations are in agreement with descriptions published by various authors (e.g. Allen, 1967; Ng & Williams, 1977).

Cells, impregnated with Merck protargol and developed without added Na_2CO_3, may show both mitochondria and the microtubular components of the cortex (Figs. 3 B, C; 6). Careful study of these cells reveals a consistent association between certain microtubular elements of the cortex and the cortical mitochondria.

The cortical mitochondria producing a single pattern are apparently associated with the transverse microtubular bands. This type of cortical mitochondrion may therefore be termed a transverse mitochondrion. In cells showing double patterns, the sausage-shaped mitochondria located to each kinety's right are apparently associated with the longitudinal microtubular bands; they can be termed longitudinal mitochondria. Thus, single pattern cells have only transverse mitochondria, while double pattern cells have both transverse and longitudinal cortical mitochondria (Figs. 3, 6).

Electron-micrographic observations confirm and extend the impressions gained from observations at the light level. Transverse mitochondria are in intimate association with transverse microtubule bands, and similar associations between the longitudinal mitochondria and the longitudinal microtubular bands are seen (Figs. 4, 5). Occasionally, in areas of close apposition between the mitochondria and the microtubules, electron-dense regions in the mitochondrial membranes are apparent (Fig. 5). These electron-dense regions are seen only in regions of close contact, and may indicate the presence of materials functioning to anchor the mitochondrion in its cortical

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Fig. 4. Electron micrographs of cross-sections of cells from cultures expressing predominantly single (A) or double (B) mitochondrial patterns. In A, a mitochondrion can be seen in apposition to the transverse microtubule band, which ranges from the basal body to the cell's left. The position under the longitudinal microtubule band is unoccupied, however. The mitochondrion in the lower left of A is in association with the transverse microtubule band of the next kinety to the cell's right. In B, a transverse mitochondrion can be seen under the transverse microtubule band, and a longitudinal mitochondrion under the longitudinal band. The basal body in this case should be above the plane of the section, in the vicinity of the star (*). A, × 49000; B, × 51000.

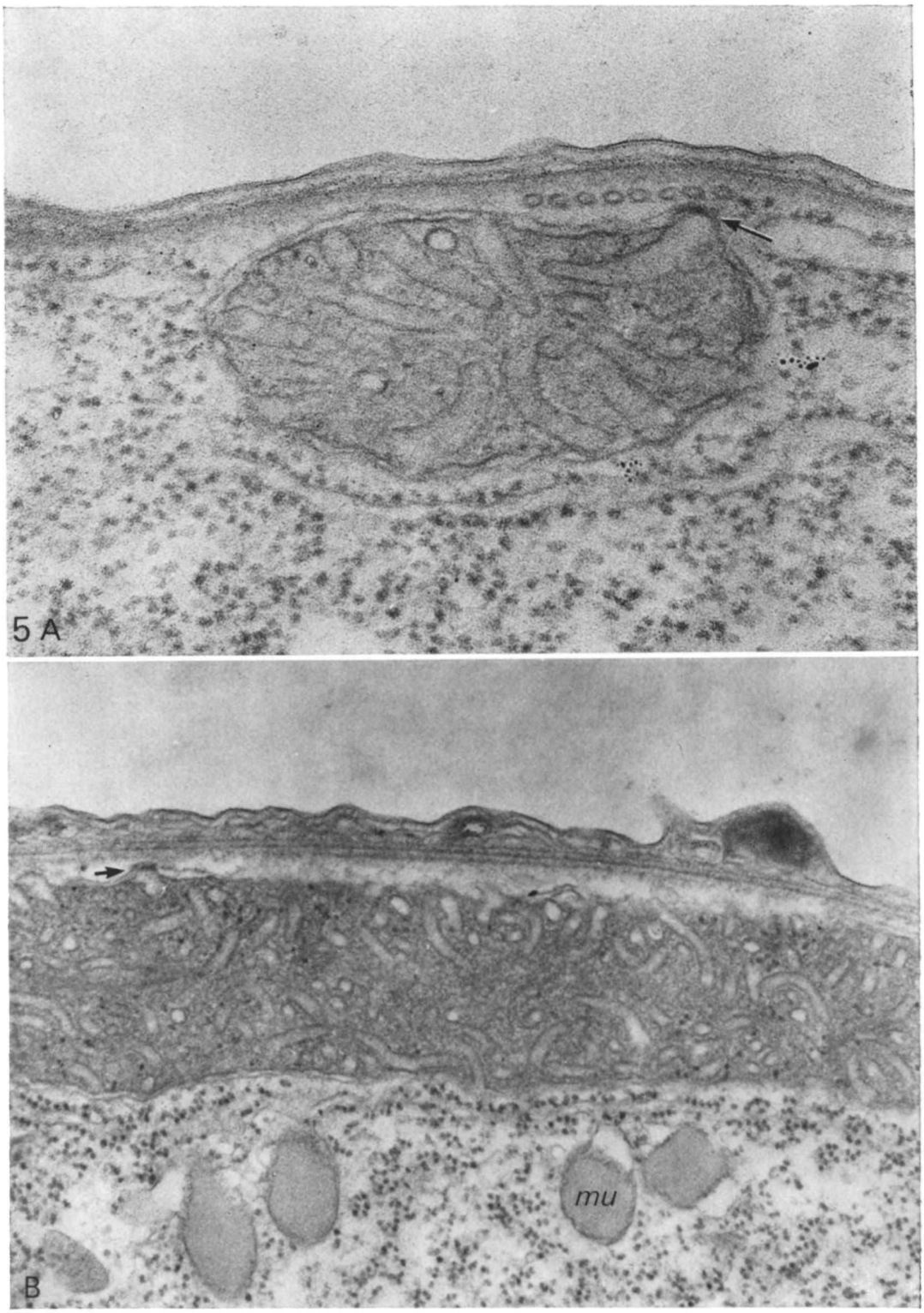

Fig. 5. For legend see facing.

position. Although differences in gross morphology between the transverse and longitudinal mitochondria are visible at the light-microscopic level (Figs. 1–3), the electron microscope reveals no obvious fine-structural differences between the 2 types.

Expression of double or single patterns

Most cultures contain a mixture of cells expressing either single or double mitochondrial patterns. Several hypotheses about the factors influencing expression were tested.

Genetic determination. It is possible that the pattern expressed in a particular cell

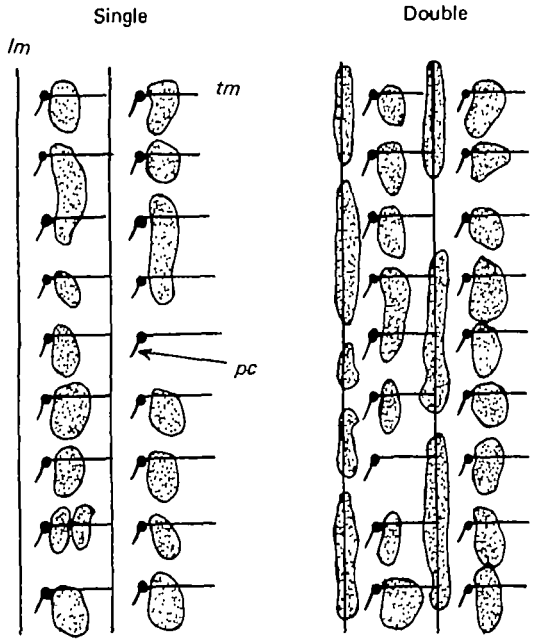

Fig. 6. Schematics of the appearance of the cortex of *T. thermophila* after protargol staining to show the microtubular and mitochondrial components of the cortex. A cell expressing a single mitochondrial pattern has only transverse mitochondria in its cortex, whereas a cell expressing a double pattern has both transverse and longitudinal mitochondria. *lm*, longitudinal microtubule band; *pc*, postciliary microtubule band; *tm*, transverse microtubule band.

Fig. 5. Electron micrographs of longitudinal sections of cells, selected to show microtubule-mitochondrion associations. A, a transverse mitochondrion located under a transverse microtubule band. A characteristic electron-dense area in the mitochondrial membranes (arrow) is noted in the region of close association, and is not seen elsewhere in cortical mitochondria. It is suggested that this density may reflect the presence of materials involved in 'anchoring' the mitochondrion to the microtubule band. × 94 500. B, longitudinal mitochondrion under the longitudinal microtubule band. A density in the mitochondrial membranes (arrow) structurally similar to that seen in A, is located in a region of particularly close contact between the mitochondrion and the epiplasm under the microtubule band. This density may be structurally and functionally homologous to the type seen in A. × 59 000.

is genetically determined and that the mixture of the 2 types seen in most cultures is the result of genetic or clonal heterogeneity. To test this, a 3-day-old tube culture of axenic *T. thermophila* was used as a source for 6 individual cell isolates. The parent culture was fixed and stained with protargol to show mitochondria. The 6 clones were grown to a similar age and density as the parent culture before they were fixed for protargol. The stained cells were scored for proportion of single versus double patterns. Results appear in Table 1. The results show that the clonal progeny of any cell will produce a mixture of cells expressing single or double patterns. Additionally, the proportions of single vs. double patterns in each of the 6 clones and the parent culture are statistically indistinguishable, suggesting that there are no inherited predelictions towards high or low expression of single or double patterns. The results allow one to reject the hypothesis as stated.

Relation to cell cycle. It is possible that the cell cycle could be a determining factor in the cell's decision to express a single or a double mitochondrial pattern. Thus, cells in an early phase of the cycle might express single patterns, while the same cells in a later phase might express only double patterns. This hypothesis was tested by comparing the proportion of single to double patterns expressed in cells in stomatogenesis or division against the proportion of single to double patterns in non-stomatogenetic cells from the same culture. Stomatogenesis and division are known to occupy a large fraction of the cell cycle (Frankel & Williams, 1973). If the single pattern was expressed exclusively, or mainly, in either the stomatogenetic or non-stomatogenetic phases of the cell cycle, one would expect to see a difference in the proportions of single patterns in the 2 sets of cells. The proportion of cells expressing single pattern mitochondria of those cells in stomatogenesis was not statistically distinguishable from the proportion of non-stomatogenetic cells expressing single pattern (Table 2). An absence of measurable difference between the 2 classes does not support the hypothesis that expression of mitochondrial pattern is influenced by certain stages of the cell cycle.

Relation to nutrient conditions. Preliminary tests indicated that old, stationary phase cultures had a higher proportion of single pattern cells than young, exponential phase cultures. To analyse this phenomenon, axenic cultures of known concentration, growth rate, and time since inoculation were fixed and stained with protargol to demonstrate the mitochondrial pattern. The results are shown in Table 3. The general tendency appears to be an increase in proportion of single patterns expressed as a culture ages. Similar results are seen using bacterized medium. This result may suggest that a shift from double to single patterns is favoured as the nutrients available to the cells are depleted, or as other materials accumulate in older cultures.

The possibility that mitochondrial cortical patterns are influenced by nutrient conditions was tested by a shift-down experiment. A 2-day-old axenic culture was washed twice and resuspended in fresh Dryl's solution. Samples were taken before and at various times after the wash and resuspension, were stained with protargol, and were scored for mitochondrial pattern (Table 4). The results demonstrate that a culture with mostly double mitochondrial pattern cells can quickly shift to mostly single pattern cells when the culture is moved from nutrient to non-nutrient

Table 1. *Subcloning and mitochondrial pattern expression*

Culture	Mitochondrial pattern: No. of cells showing Single pattern	Double pattern	% single pattern
Parent	24	76	24
Subclones			
1	26	74	26
2	21	79	21
3	25	82	23
4	17	86	17
5	24	76	24
6	26	78	25

Table 2. *Cell cycle and mitochondrial pattern expression*

Cell type	Mitochondrial pattern: No. of cells showing Single pattern	Double pattern	% single pattern
Stomatogenetic	13	9	59
Non-stomatogenetic	44	34	56
Total	**57**	**43**	**57**

Table 3. *Culture age and mitochondrial pattern expression*

Culture age, days	Cell concentration, no./ml	Mitochondrial pattern: No. of cells showing Single pattern	Double pattern	% single pattern
1	2×10^4	41	146	22
1	1×10^5	81	145	36
2	$3{\cdot}5 \times 10^5$	99	180	35
3	$3{\cdot}3 \times 10^5$	146	213	41
4	$4{\cdot}1 \times 10^5$	191	268	42
12	*— —	125	81	61

Table 4. *Culture nutrient shiftdown and mitochondrial pattern expression*

Time after wash	Mitochondrial pattern: No. of cells expressing Single pattern	Double pattern	% single pattern
0 min	3	97	3
15 min	4	96	4
30 min	4	96	4
60 min	11	89	11
120 min	67	34	67
165 min	82	18	82
5 h (Fast swimmer cells)	100	0	100

* As many dead cells were present, actual viable conc. unknown.

conditions. Similarly, mating-reactive cells, conjugating pairs and cells transformed to the 'fast swimmer' phenotype (Fig. 3) (Nelsen & Debault, 1978; Nelsen, 1978), all have been starved for several hours, and all express virtually 100% single mitochondrial patterns.

DISCUSSION

Both the single and double cortical mitochondrial patterns of *T. thermophila* are characterized by a right–left asymmetry in relation to the kineties: the single pattern in respect to the placement of the mitochondria, and the double pattern in respect to the placement and gross morphology of the cortical mitochondria. The asymmetry of cortical mitochondrial patterns reflects, and may be determined by, the asymmetrical organization of the cortical microtubule arrays. Evidence supporting this concept comes from several sources. In protargol-stained cells, a close association is seen between the cortical microtubular arrays and the cortical mitochondria in cells expressing either pattern. Electron-microscopic observations reveal ultrastructural differentiations of the mitochondrion, with a possible function in microtubule–mitochondrion interactions. The converse hypothesis, that the cortical mitochondrial pattern determines the pattern of microtubular arrays in the cortex, has little to support it. One can manipulate the expression of mitochondrial patterning, while leaving the microtubular pattern apparently untouched, by shifting nutrient conditions of the culture. Also, all mitochondria can be reversibly removed from the cortex by distortion of a cell in the rotocompressor. One might expect that cortical mitochondria would have to be more permanently positioned than they appear to be if they served as information sources for the assembly of the complex and precise cortical microtubular arrays. Certainly, longitudinal microtubular bands are present without the benefit of longitudinal mitochondria in dividing single-pattern cells. Independent studies of somatic morphogenesis in *Paramecium* and *T. thermophila* have shown that the nucleating site for assembly of a new basal body is a pre-existing basal body (Dippell, 1968; Allen, 1969). Once initiated, the new basal body then apparently serves as a nucleating site for its own structures, such as the transverse microtubule bands. Preliminary results (Aufderheide, unpublished data) indicate that a new basal body is not initially associated with a mitochondrion. The basal body appears to 'grow' a transverse microtubular band and only later is a mitochondrion seen to be associated with it. Thus, all the evidence points in favour of the former hypothesis: that the microtubular arrays of the cortex serve as a source of information for the localization and patterning of the cortical mitochondria.

Whether cortical mitochondria have any special function related to their intracellular placement is currently unknown. The fact that the cell can execute a wide variety of activities, such as development of the 'fast swimmer' phenotype, without longitudinal mitochondria shows that this class of organelle is not essential for such activities. However, the consistent expression of the transverse mitochondria under a wide range of conditions may reflect an important function for these organelles. Although they can be removed from the cortex, the rapid recovery of the cell in

re-establishing its transverse mitochondria at least indicates a strong affinity of the transverse cortical sites for mitochondria. Of course, the transverse mitochondria are located close to the basal bodies of the somatic cilia. Other systems, such as spermatozoa (Woolley, 1970; Tokuyasu, 1974) and trypanosomes (Simpson, 1972; Anderson & Ellis, 1965), have mitochondria associated with the basal body or the axoneme of a cilium or flagellum. The observations of *T. thermophila* are consistent with those made on other systems. An obvious hypothesis to explain this association is that the mitochondrion can serve as a nearby source of ATP for ciliary function. Tests of this hypothesis are being considered.

Another related question is whether any mitochondrion can occupy any cortical site, or whether certain mitochondria are 'pre-adapted' for their eventual cortical sites. This asks whether or not there is any real difference between transverse, longitudinal and endoplasmic mitochondria. In addition to a lack of observable ultrastructural differences, the *in vivo* observations argue against any specializations within the chondriome, since cortical mitochondria are constantly exiting the cortex and mixing with endoplasmic mitochondria. Occupation of cortical sites from the endoplasmic population also seems to be a concurrent activity.

In general then, the asymmetrical patterns formed by the cortical mitochondria of *T. thermophila* appear to be sensitive reflexions of the asymmetrical organization of the microtubular elements (the 'cytoskeleton') of the cell cortex. Although mitochondrion-microtubule associations have been reported in several other systems (Smith *et al.* 1977; Heggeness *et al.* 1978), none seems to be so complex as to generate the striking patterns produced by the associations observed here. This system serves as an example of the concept (previously termed cytotaxis) that, in morphogenesis, the organization of one structural system of the cell may serve as an inherent source of information guiding the organization of another adjacent set of structures in the same cell (Sonneborn, 1970; Ng & Frankel, 1977). Detailed tests of this concept are being performed.

The research for this paper was supported by NIH grant HD 08485 to J. Frankel. The author thanks J. Frankel and E. M. Nelsen for critical assistance.

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(*Received* 12 *March* 1979)