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# Functions of Trisporic Acid

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# Functions of trisporic acid

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[Plate 1]

Trisporic acid, the sex hormone of the Mucorales, elicits the formation of zygophores in (+) and (−) mycelia of *Mucor mucedo*. It can diffuse through the medium, from its site of synthesis, to affect mycelium a considerable distance away. There is no evidence for its translocation through hyphae. It causes an increase in content of β-carotene and ergosterol in recipient mycelium, and such increases are also observed during mating.

## Introduction

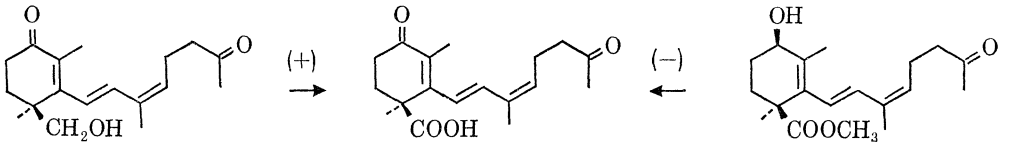
Fungi of the order Mucorales show two types of mating behaviour, heterothallism and homothallism. These two systems were elucidated by Blakeslee (1904), who arbitrarily designated the two mating types in heterothallic isolates as (+) and (−). He showed that isolates of all species do not interact with any other isolate of the same mating type, but that many (−) isolates undergo at least some sexual differentiation when grown with many (+) isolates. If the isolates are of the same species their interactions will culminate in karyogamy and the formation of zygospores; if of different species they will be confined to the early stages of mating – the formation, mutual attraction and growth together of zygophores – but plasmogamy will not occur. Thus the mating type of any isolate can be assigned by its interactions with strains already typed by the relation back to Blakeslee's original strains of *Rhizopus nigricans*.

The involvement of diffusible chemicals in the control of these sexual interactions was shown by Burgeff (1924), who observed zygophore formation in both mating types when (+) and (−) *Mucor mucedo* were grown on the same nutrient medium, but were separated by a collodion membrane.

The hormone responsible for the switch from asexual to sexual differentiation is trisporic acid, **1**. This compound, a C-18 terpenoid, is apparently biosynthesized via β-carotene and retinal, and is produced by both (+) and (−) cells in mated cultures (Austin, Bu'Lock & Drake 1970; Ende, Werkman & Briel 1972). It is scarcely, if at all, synthesized by the unmated strains. The initiation of its synthesis in mated cultures results from each mating type producing specific precursor molecules (e.g. **2**, **3**) that are converted to it by enzymes present in the opposite mating type; its synthesis is then accelerated, at least in part, by the action of trisporic acid itself in increasing the rate of synthesis of these precursors (Bu'Lock, Jones & Winskill 1976; Sutter, Harrison & Galasko 1974; Werkman & Ende 1973; Nieuwenhuis & Ende 1975). The details of this remarkable collaborative biosynthesis are reviewed by Bu'Lock (1976), Ende (1976, 1978), Gooday (1978) and Sutter (1977).

There is good reason to think that trisporic acid is the universal sex hormone of the Mucorales. Blakeslee's original detailed accounts of interspecific sexual reactions indicate a common mechanism. Trisporic acid has been fully characterized as a metabolite of mated cultures of

the heterothallic species, *M. mucedo* and *Phycomyces blakesleeanus*, where it is produced in small amounts, and *Blakeslea trispora*, where it is produced in large amounts (Austin, Bu'Lock & Gooday 1969; Sutter 1977). Its production and action in homothallic species has been studied and discussed by Werkman & Ende (1974) and Ende (1976).




trisporol B (**2**) trisporic acid B (**1**) methyl dihydrotrisporate B (**3**)

Trisporic acid is active in very small quantities in hormone bioassays with *M. mucedo*. Such bioassays are necessarily crude, both spatially and temporally, and so surely it can act *in vivo* in amounts much lower than our current limits of detection, especially at its site of synthesis. Perhaps only a few molecules produced *in situ* are sufficient to cause sexual differentiation in some cases.

As befits a regulator of sexual interactions among fungi found in a wide range of environments, reports of the phylogenetic specificity of production and action of trisporic acid remain exclusive to the Mucorales. For example, although Lynch & White (1977) describe shortening of shoots and lengthening of roots in barley plants treated with trisporic acid, they cannot ascribe this to the action of trisporic acid itself, as they found that it rapidly decomposed to several products in the plant culture solution. The specificity of the hormone ensures that the recipient fungus is only diverted from asexual reproduction, with the formation of cells for dispersal and survival such as sporangiospores, conidia, chlamydospores and arthrospores (Ingold 1971), when there is a good chance of it successfully mating with a compatible partner.

This account concerns the actions of trisporic acid as a hormone: eliciting zygophore formation, increasing the rate of accumulation of terpenoids, and increasing its own rate of synthesis.

## Trisporic acid and zygophore formation

Zygophores are the sexual cells that are the morphological consequence of the action of trisporic acid. They are not produced in unmated cultures of heterothallic species. The bioassay for trisporic acid involves counting the number of zygophores produced after the addition of a test solution (figures 1 and 2, plate 1). In *M. mucedo* they are distinctive aerial hyphae and are readily counted. Thus this species is used for bioassays sensitive down to about 10 ng of trisporic acid C. In *M. hiemalis* they are very similar in appearance to the sporangiophores, and although they are produced in younger mycelium than the sporangiophores this does not allow a reliable bioassay. In *P. blakesleeanus* (Sutter 1975) and in *B. trispora* they are stubby, branching structures, often developing embedded in the medium from vegetative hyphal tips.

Trisporic acids B and C are the only metabolites known to elicit zygophore formation in (+) and (−) mycelia. The mating type-specific precursors of trisporic acid such as trisporol B (**2**) from (−), and methyl dihydrotrisporate B (**3**) from (+), are active on (+) and (−) respectively (Bu'Lock *et al.* 1976), presumably via trisporic acid.

The zygophores have the unique property, among the different cell types produced in the Mucorales, of cell fusion. In *M. mucedo* they fuse together in mated pairs, often just behind their

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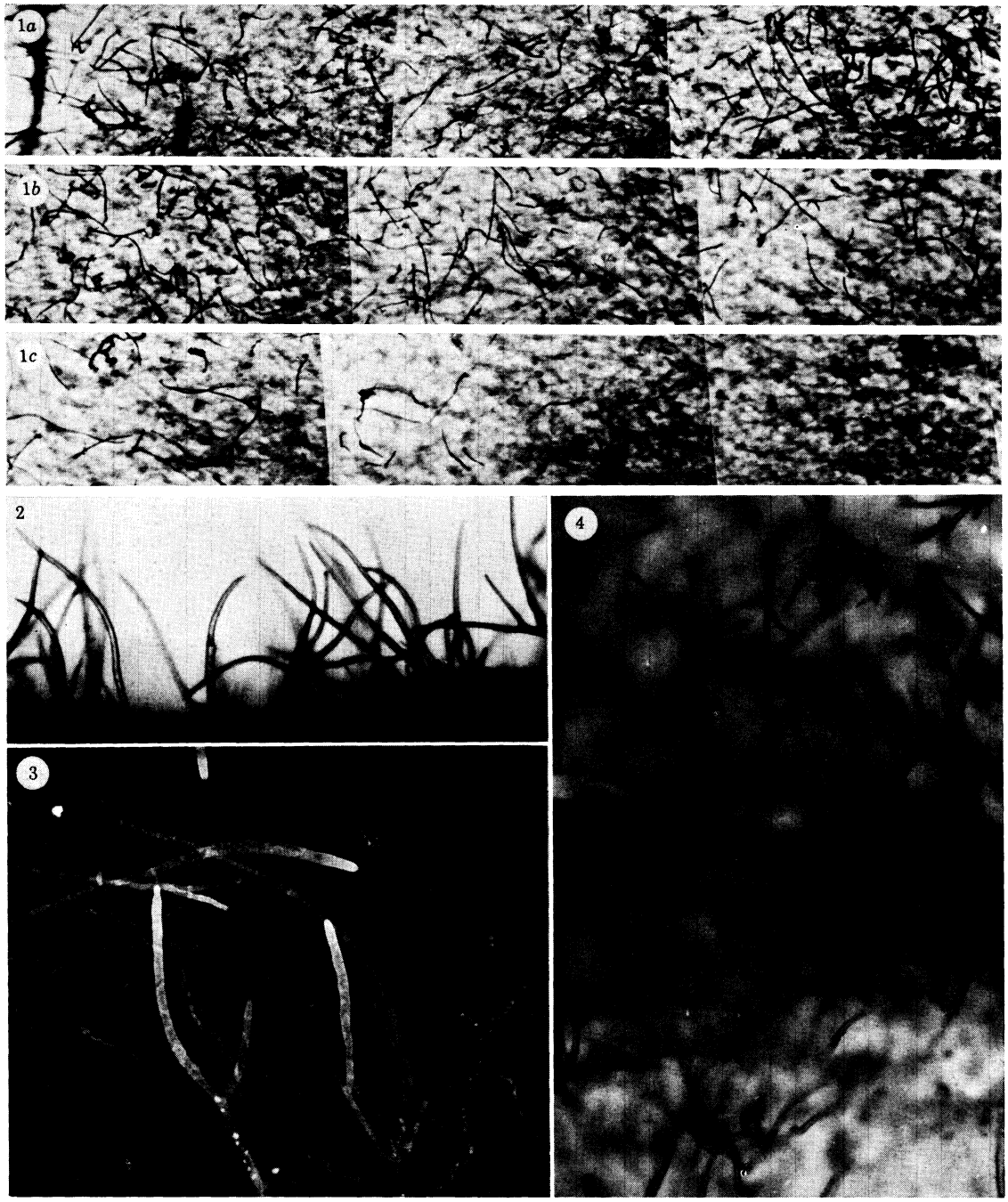


FIGURE 1. Appearance of bioassay plate of (−) *M. mucedo*, 15 h after addition of 100 ng trisporic acid C to a well just in front of hyphal tips. Edge of well is visible at left of 1*a*; photomontage of transect is, left to right, 1*a* to 1*b* to 1*c*; total distance is 14 mm. Note greatest density of zygophores around 5 mm from well.

FIGURE 2. Zygophores at edge of bioassay well of (−) *M. mucedo*, 15 h after addition of 1 μg methyl trisporate C. (Magn. × 64.)

FIGURE 3. Staining of (−) zygophores of *M. mucedo* by fluorescein-labelled wheat germ agglutinin. Conditions as described by Jones & Gooday (1977). Bright spots within hyphae represent autofluorescence of carotene-containing oil droplets. (Magn. × 150.)

FIGURE 4. Zygophores formed at zone of diffusion contact of (−) (top) and (+) (bottom) *M. mucedo*, grown separated by a Cellophane membrane cemented across a Petri dish. (Magn. × 64.)

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tips (cf. pl. 2 in Gooday 1973). A few minutes after touching they are firmly attached to each other, so that they cannot readily be pulled apart. Probably at initial contact they have, or quickly produce, complementary surface agglutinins such as have been described for some other mating fungal cells (Carlile & Gooday 1978).

A study of the surface properties of zygophores of *M. mucedo* has shown that they bind fluorescein-labelled wheat germ agglutinin very much more strongly than vegetative hyphae or sporangiophores (figure 3, plate 1; Jones & Gooday 1977). Zygophore-specific antibodies are produced in the sera of rabbits that have been injected with zygophore surface components (B. E. Jones and G. W. Gooday, unpublished). Thus the distinctive appearance of zygophores is mirrored in a distinctive wall chemistry, but it remains unclear how this chemistry relates to their functional properties. No differences have yet emerged in the surface components of (+) and (−) zygophores.

The zygophores of *M. mucedo* are not geotropic or phototrophic, as are the sporangiophores. Instead, they grow as aerial hyphae away from their parent mycelium, i.e. they are negatively autotropic. Most importantly, they show the remarkable property of zygotropism, as described by Blakeslee (1904): '. . . a mutual attraction . . . is exercised between the zygophoric hyphae belonging to opposite mycelia, and they may be seen gradually to approach each other . . .' A zygophore of *M. mucedo* under the influence of zygotropic effectors grows very much longer than one not exposed to an attractant (Plempel 1962; Mesland, Huisman & Ende 1974), but when it meets a zygophore of opposite mating type it stops growing immediately on contact and starts to form a progametangium (Gooday 1975). From a preliminary investigation of their nature, Mesland *et al.* suggest that the zygotropic attractants are, or are very closely related to, the two sets of mating type-specific precursors of trisporic acid. This is a very appealing idea, as we do not have to invoke new biosynthetic pathways for the biosynthesis and reception of the zygotropic hormones. It explains observations such as (*a*) (−) zygophores being attracted to a (+) sporangiophore (fig. 2 in Mesland *et al.* 1974), as in this experiment the (+) mycelium will be increasingly producing its trisporate precursors in response to the nearness of the (−) mycelium, and (*b*) zygophores of both mating types ceasing to be attractive on fusing with a compatible mate (fig. 7 in Gooday 1975), as after fusion the precursors that are the attractants are now very efficiently converted to trisporic acid by the partner, and no longer can diffuse into the air.

The zygophores are formed as a result of the action of trisporic acid, but they also are themselves sites of localization of its further synthesis and effects. Thus Werkman (1976) has shown by histochemical staining that the zygophores of (−) *M. mucedo* are rich in enzyme activity oxidizing the 4-OH of 4-dihydrotrisporin and methyl 4-dihydrotrisporate (**3**) to the ketone of trisporin and methyl trisporate. The presence of such transformations in the zygophores is consistent with the suggestion that zygotropism is mediated by trisporate precursors. Presumably after plasmogamy the gametangia and then the young zygospores continue to synthesize trisporic acid. In *M. mucedo* all stages of development, from the zygophores to the young zygospores (until they become too opaque for microscopic observation) are very rich in carotene, often in the form of crystals as well as in oil droplets. Also, trisporic acid can still be extracted from mated cultures three days after inoculation of a mixture of equal numbers of spores, when the mycelium chiefly consists of zygospores and moribund zygophores that have ceased to grow (Gooday 1968*a*).

Very little is known of the biochemical mechanism of the induction of zygophore formation.




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Bu'Lock *et al.* (1976) report increases in RNA turnover rate and cyclic AMP content following addition of trisporic acid to *B. trispora* and *M. mucedo* respectively. With the ready availability of trisporic acid, this seems a worthwhile field of investigation.

## Trisporic acid as a hormone: its intercellular diffusion

Hormones are transportable substances, coordinating activities of cells away from their sites of synthesis. As trisporic acid is produced by both (+) and (−) mating types of *M. mucedo* and *B. trispora*, and is active on both mating types, there appears to be no need for its action at a distance. It has been suggested that the true sex hormones of the Mucorales are the mating type-specific precursors of trisporic acid (Sutter, Capage, Harrison & Keen 1973). Thus, when β-carotene-deficient mutants of *P. blakesleeanus* are mated with wild-type strains, zygophores are only produced by the mutant mycelium (Sutter 1975). This can be interpreted as being the result of the mating type-specific precursors diffusing only from the wildtype mycelium to be converted to trisporic acid only in the mutant mycelium. Certainly, any model for the mating interactions in the Mucorales must involve the cross-diffusion of the mating type-specific precursors, but in addition in *M. mucedo* there is evidence for the diffusion of trisporic acid itself.

### *Kinetics of zygophore formation in* Mucor mucedo

When grown on a nutrient agar medium (conditions as for table 2, with or without cellophane), about 3 h after mycelia of the two mating types have met, zygophores grow up from the vegetative mycelium of the (+) mating type, to be followed after about an hour by (−) zygophores. This appearance of (+) zygophores before (−) zygophores has been observed with all combinations of mating five strains from different sources of both mating types (cf. pl. 1 in Gooday 1973). This observation has been rationalized by Mesland *et al.* (1974) who, in their account of the volatile induction of zygophores in both mating types of *M. mucedo* by extracts of unmated mycelium of opposite mating type, found that the (+) mycelium had a more vigorous reaction to (−) extracts than vice versa.

At first, the zygophores are confined to the immediate area of contact between the two mating types, but later are formed in a wider area (up to 10 mm) on either side of this zone, to arch over the earlier ones that are already forming zygospores. However, if activated charcoal (1% by mass) is incorporated into the medium, zygophores and the resultant zygospores are only formed in a very narrow zone where the hyphae from the two mating types intermingle. Activated charcoal strongly adsorbs trisporic acid and its precursors (cf. Gooday 1968*b*).

As shown by Burgeff (1924), zygophores, but no zygospores, also form without physical contact of the two mating types, when they are grown separated by a permeable membrane (figure 4, plate 1). Activated charcoal in the medium completely prevents zygophore formation in such cultures.

Mesland *et al.* (1974) have demonstrated mutual volatile induction of zygophores when (+) and (−) mycelia of *M. mucedo* were placed next to each other separated by an air gap. They showed that the volatile effectors were probably the mating type-specific precursors of trisporic acid, diffusing from the producing strain to be converted to trisporic acid in the recipient strain.




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*Hormone diffusion during outgrowth experiments*

The rôle of the diffusion of trisporic acid through the medium in the eliciting of zygophore formation in *Mucor mucedo* is indicated in 'outgrowth experiments'. The strain Z43 (−) had a faster radial growth rate than Z46 (+), and thus outgrew it when they were inoculated together on fresh medium as a disk of mixed mycelium in the centre of a 14 cm diameter dish (figure 5). The mixed mycelium produced abundant zygospores and no sporangia. The (−) outgrowth also did not produce sporangia, but formed zygophores. Only zygophores were produced for a definite distance out from the mixed mycelium, and not until then were sporangia produced. Growth beyond this point was as for a normal unmated (−) culture. The outermost annulus of zygophores changed slowly to sporangia, but the inner ones stayed as zygophores indefinitely. Thus when the culture had reached the edge of the dish there were, from the centre out: zygospores to 8 mm, 'set' zygophores to 26 mm, zygophores 'reverting' to sporangia to 42 mm, and then sporangia outside these.

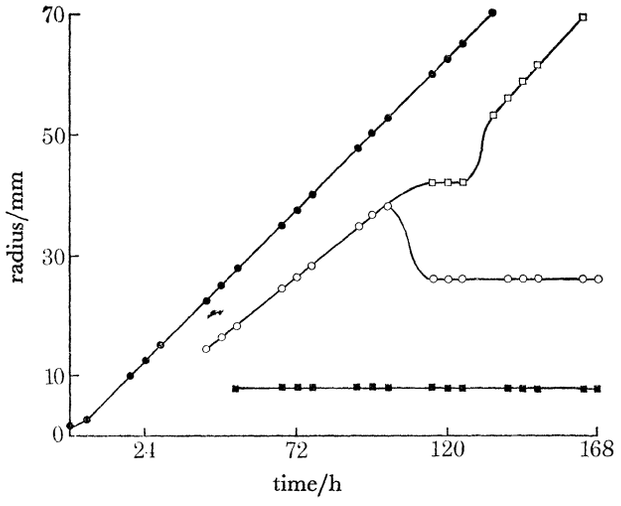


FIGURE 5. Characteristics of the 'outgrowth' of *Mucor mucedo* Z43 (−) from a mixed Z43 (−)/Z46 (+) inoculation. Culture grown on malt extract (2%), mycological peptone (0.5%), glucose (2%), agar (2%), in dark at 20 °C. Under these conditions the radial growth rates for Z43 and Z46 were 0.50 and 0.44 mm $h^{-1}$ respectively. ●, Outer limits of hyphal tips; ○, zygophores; □, sporangiophores; ■, zygospores.

These outgrowth experiments were repeated with different manipulations. Cultures grown on media containing 1% activated charcoal formed no zygophores at all outside the central area of zygospores, but sporangia were formed instead. Other cultures were grown for 60 h, when the outgrowth was established and zygophores were being formed. Untreated controls continued producing zygophores for a further 50 h (cf. figure 5), and so did cultures in which the central mating mycelium and the underlying agar was removed at 60 h. However, if the entire mycelium was lifted from the medium as a coherent mat and transferred to fresh medium, zygophore production immediately stopped, and sporangia formed from then on. The zygophores already produced stayed as such and did not 'revert' to sporangia. If the culture was transferred to water agar (2% by mass), not only were zygophores no longer produced, but those already formed reverted to sporangia.

When the zygophore-producing outgrowth mycelium, or its underlying agar, was extracted with acetone and purified by adsorption on charcoal and two successive partitions into ether from an acidic solution and back into aqueous sodium bicarbonate solution (Gooday 1968*b*; Bu'Lock & Winstanley 1971), it gave material active on both (−) and (+) in the zygophore bioassay (Gooday 1968*a*). These are characteristics of trisporic acids only. The outgrowth experiments thus suggest that in *M. mucedo* trisporic acids are produced where hyphae of the two mating types are in close contact, and then diffuse out through the medium to affect cells at a considerable distance (up to 34 mm in the conditions in figure 5). There is no indication of translocation through the hyphae. It could still be suggested that these outgrowth experiments represent the result of the diffusion of a precursor of trisporic acid from the mated mycelium, rather than of trisporic acid itself, but by using Occam's razor, trisporic acid as a diffusible hormone is the most likely explanation. The radial rate of initiation of zygophores was less than the radial vegetative growth rate (figure 5) and so presumably under these conditions the rate of diffusion of the trisporic acid was less than the hyphal growth rate.

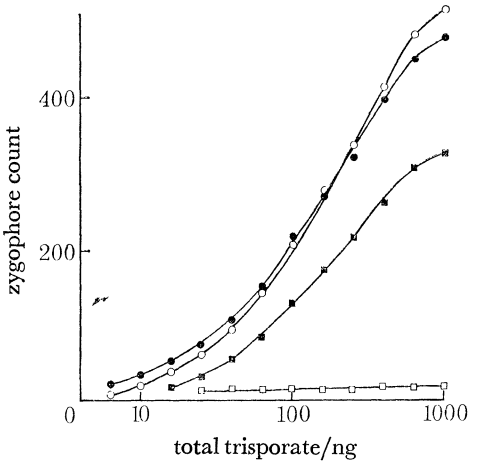


FIGURE 6. Bioassay response curves using *M. mucedo* Z43 (−) and Z46 (+); 15 h. Trisporic acid C, Z43 (●), Z46 (■). Methyl trisporate C, Z43 (○), Z46 (□).

### *Diffusion of trisporic acids in the bioassay*

The hormone bioassay involves counting the total number of zygophores in a transect from young to older mycelium after the test solution has been put into a well in the agar just in front of the hyphal tips of an advancing colony. Zygophores are formed at considerable distances from the assay hole (figure 1, plate 1). When activated charcoal was incorporated in the medium, no zygophores were formed when trisporic acid was added.

With the (−) culture, no difference is seen in the diffusion pattern, as detected by zygophore formation, between the trisporic acids and their synthesized methyl esters. Either the methylation of the carboxyl group makes no difference to the molecules' diffusion through the agar, or the methyl esters are hydrolysed by enzymes in the (−) strain, that may even be released extracellularly (cf. Werkman & Ende, quoted by Bu'Lock *et al.* 1976). The methyl esters have a very similar zygophore-inducing activity to that of the free acids for (−) *M. mucedo*, but not for (+) (figure 6), as the (−) alone has appreciable esterase activity on them (Werkman 1976).

*Uptake of trisporic acid*

The response to trisporic acid after its diffusion through the medium, as seen in bioassays and in the 'outgrowth' experiments, must require its uptake by the cell. Nothing is known of the nature or site of its putative receptor; it could be in the membrane or inside the cell. In either case the trisporic acid has to penetrate the wall. The walls of the Mucorales are rich in potentially charged components, the acidic polyphosphates and glucoran (poly-D-glucuronate) and the basic chitosan (poly-D-glucosamine, de-acetylated chitin) (Datema, Ende & Wessels 1977; Datema, Wessels & Ende 1977). The p*K* values of trisporic acids B and C are about 4.25 (as measured by titration; B. E. Jones, unpublished). Thus presumably they act as ionized weak acids in physiological conditions, and their passage through the wall would be facilitated or hampered by the state of charge of the wall components. Once through the wall their interaction with the cell membrane will again be influenced by their state of ionization. From its structure, trisporic acid might be expected readily to become incorporated into a biological membrane, but perhaps less readily to cross a membrane. The existence of a specific transport mechanism for trisporic acid has been suggested from the properties of some strains of (−) *M. mucedo* obtained after mutagenesis (Wurtz & Jockusch 1975). These do not respond to exogenous trisporic acid but do produce zygophores in crosses with wild type (+). One explanation is that they have lost a 'trisporate permease' but can still take up the (+) trisporate precursors and convert them to trisporic acid inside the cell. Sutter (1977) reports that *P. blakesleeanus* is $10^3$ to $10^4$ times less sensitive to exogenous trisporic acid than *M. mucedo*, and suggests that this is due to its being less permeable. As well as being able to take up and release trisporic acid, each mating type must be able to release and take up the appropriate mating type-specific precursors. If a specific transport mechanism exists for trisporic acid, it might be expected that it would also transport the related metabolites, but the results of Wurtz & Jockusch seem to argue against this.

*Conclusions*

Thus it may be concluded that zygophore formation in a hypha of *M. mucedo* is initiated by the diffusion to it of either trisporic acid, or a mating type-specific precursor of trisporic acid. All the trisporic acid that is formed has apparently originated via diffusion from (+) to (−) or vice versa as the appropriate precursor. Once formed, it can then be released again to diffuse to other parts of the mycelium.

To decide whether the trisporic acid molecule itself, or perhaps a further metabolite of it, is actually the effector initiating the primary molecular event triggering sexual differentiation awaits the characterization of a specific receptor with appropriate characteristics.

## Trisporic acid and the control of terpenoid biosynthesis

*Effect on carotenogenesis*

Trisporic acid was characterized first, not as a hormone, but as a stimulator of carotene production in *Blakeslea trispora* (Caglioti *et al.* 1966). Its effect in increasing the β-carotene content of recipient mycelium of both mating types of *B. trispora* and of *M. mucedo* has been documented (Thomas & Goodwin 1967; Ende 1968; Gooday 1968*a*; Sutter & Rafelson 1968). Typical results are shown in table 1, which also records the large stimulation of carotene production in the (−) mycelium growing out from a mated inoculum (described above). This




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(−) outgrowth is continually under the influence of trisporic acid diffusing with it in the medium as it grows, and shows carotene contents almost double those of (−) mycelium responding to exogenous trisporic acid.

When cultures of (−) and (+) *M. mucedo* are grown to approach each other, their carotene contents increase rapidly at the line of their meeting. This increase in pigment then spreads back into the older mycelium at either side (table 2), and must represent the action of trisporic acid being produced in the mating zone and diffusing back from there into older mycelium.

An increase in carotenogenesis has been measured in artificial sexual heterokaryons and in rare sexually heterozygous diploids as well as in mated cultures (table 3), and presumably is a result of some trisporic acid synthesis in mycelium containing both mating-type genes. The sexual heterokaryons of *P. blakesleeanus* have the phenotype of producing 'pseudophores', zygophore-like structures (Murillo & Cerdá-Olmedo 1976), while the sexual diploids of *M. hiemalis* produce azygospores, structurally very similar to zygospores but held by only one suspensor (Gauger 1975).

TABLE 1. β-CAROTENE CONTENTS OF *MUCOR MUCEDO*

| age of mycelium/h | (−) control | (−) plus trisporate | (+) control | (+) plus trisporate | (−) 'outgrowth' |
|---|---|---|---|---|---|
| 18 | trace | 401 | trace | 169 | 913 |
| 54 | 174 | 833 | 162 | 603 | 1753 |
| 90 | 299 | 1378 | 201 | 1237 | 2375 |

Quoted as mean micrograms per gram dry mass for three 1 cm squares of mycelium. Cultures Z43 (−) and Z46 (+) grown on Cellophane film (PT300) on malt extract (2%), glucose (2%), agar (2%), 40 ml in 10 cm square Petri dishes, from inocula of spore suspensions on 9.5 × 0.4 cm strips of filter paper, at 20 °C in the dark. Fungus harvested, freeze dried, extracted exhaustively with acetone with sonic disruption, carotene estimated in hexane solution of extract after characterization by t.l.c. and spectrophotometry. All steps where appropriate in dark and under nitrogen to prevent decomposition of carotenoids. Trisporic acid solution (B and C, 435 μg $ml^{-1}$) was added where stated by pipetting it under the Cellophane at time zero and at each successive 24 h so that each square centimetre received 8.7 μg per day. Unpublished results of W. H. Leith and G. W. Gooday.

TABLE 2. β-CAROTENE CONTENTS OF MATING CULTURES OF *MUCOR MUCEDO*

(As for table 1, but (+) and (−) inoculated at opposite sides of Petri dish.)

| time after meeting/h† | distance from line of contact/mm ... | 32 (−) | 16 (−) | 0 | 16 (+) | 32 (+) |
|---|---|---|---|---|---|---|
| 0 | | 217 | trace | trace | 153 | 429 |
| 13 | | 427 | 467 | 2403 | 352 | 499 |
| 20 | | 336 | 1514 | 2519 | 1563 | 508 |

† At 13 h many zygophores had formed; at 20 h zygospores were forming.

### *Effect on other terpenoids*

As well as carotenoids, mated cultures of *B. trispora* also produce higher quantities of sterols, prenols and ubiquinones than unmated cultures (Thomas & Goodwin 1967; Bu'Lock & Osagie 1973). Ergosterol contents of both (+) and (−) mycelia of *M. mucedo* are increased by treatment with trisporic acids (table 4). The ergosterol content of the mycelium in the mating zone very quickly rises after cultures of the two mating types have met (table 4).

### *Rôles of increased terpenoid biosyntheses*

As trisporic acid is a metabolite of β-carotene, the increased carotene content resulting from the action of trisporic acid is part of the mechanism of positive feed-back whereby it greatly stimulates its own biosynthesis. In addition, some of the increased carotene content can be

incorporated into the outer warty layer of zygospores of *M. mucedo* and *P. blakesleeanus* as sporopollenin, an oxygenated polymer of carotenoids (Gooday, Fawcett, Green & Shaw 1973; Furch & Gooday 1978). Bu'Lock & Osagie (1973) suggest that increases in contents of steroids, polyprenols and ubiquinones may be necessary for the requirements of membranes, surface polymers and respiration of the zygophores and later stages in sexual differentiation.

TABLE 3. EFFECT OF MATING, SEXUAL HETEROKARYOSIS AND SEXUAL HETEROZYGOSITY ON CONTENT OF β-CAROTENE (micrograms per gram dry mass)

| species and strains | (−) | (+) | mated | heterokaryon | diploid |
|---|---|---|---|---|---|
| *Phycomyces blakesleeanus* | | | | | |
| NRRL 1555 (−), 1554 (+) | 30 | 30 | 60 | — | — |
| C 131 (−), NRRL 1554 (+) | 46 | 56 | — | 450 | — |
| C 115 (−), M1 (+) | 2312 | 4163 | — | 15634 | — |
| *Mucor hiemalis* | | | | | |
| UNB6 (−), 5 (+), 792–3 | 116 ± 34 | 136 ± 32 | 334 ± 34 | — | 246 ± 9 |

Results for *P. blakesleeanus* are from Sutter (1977), Murillo & Cerdá-Olmedo (1976); C 115, M1 are *carS* mutants overproducing carotene. Results for *M. hiemalis* are calculated from results of Gooday & Gauger (1975); 792–3 is a diploid azygosporic strain derived from a cross of UNB6 and UNB5 (see Gauger 1975). Results are the means of three readings ± s.d.

TABLE 4. ERGOSTEROL CONTENTS OF *MUCOR MUCEDO*

| age of mycelium/h | (−) control | (−) plus trisporate | (+) control | (+) plus trisporate | mating zone |
|---|---|---|---|---|---|
| 13 | 3.9 | 6.1 | 1.7 | 2.6 | 14.0 |
| 26 | 9.4 | 12.2 | 4.8 | 7.8 | 16.5 |
| 52 | 14.3 | 18.2 | 13.0 | 16.1 | — |
| 104 | 15.6 | 26.5 | 14.3 | 23.0 | — |

Quoted as mean micrograms per gram dry mass for three 1 cm squares of mycelium. Conditions of growth, and extraction and characterization of ergosterol as for tables 1 and 2. Ergosterol estimated by absorbance at 282 nm of solutions in hexane.

### *Mechanisms of increased terpenoid biosynthesis*

From results obtained with carotene mutants of *P. blakesleeanus*, Murillo & Cerdá-Olmedo (1976) suggest that there are four separate accumulative mechanisms controlling β-carotene accumulation: feed-back inhibition by β-carotene; stimulation by vitamin A and β-ionone; stimulation by light; and sexual stimulation mediated by trisporic acid. As in this last case all classes of terpenoids increase, trisporic acid probably acts at an early stage of biosynthesis common to all. Thomas, Harris, Kirk & Goodwin (1967) have shown that cycloheximide inhibits the increase in carotene biosynthesis when trisporic acid is added to *B. trispora* (−), and suggest that the hormone is derepressing an enzyme in the biosynthetic sequence from acetyl CoA to carotene.

Preliminary experiments with the mevalonate kinase of *M. mucedo* indicate that this is probably not a limiting enzyme for terpenoid biosynthesis, in the presence or absence of trisporic acid (table 5). By analogy with other systems, the major regulatory enzyme for terpenoid biosynthesis is probably 3-hydroxy-3-methylglutaryl CoA reductase.

### *Effect on its own biosynthesis*

The finding of the two classes of mating type-specific precursors – the trisporols (**2**) produced by (−) and converted into trisporic acid (**1**) by (+); the methyl 4-dihydrotrisporates (**3**) produced by (+) and converted to trisporic acid by (−) – solved the qualitative problem of




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how trisporic acid can be made only by mated cultures (Bu'Lock, Jones, Quarrie & Winskill 1973; Ende *et al.* 1972; Sutter *et al.* 1973). However, these precursors are detected in only very small quantities in unmated cultures, and so the problem remained of how the very much larger amounts of trisporic acid can be made.

TABLE 5. CONTROL OF ENZYMES FOR TRISPORATE BIOSYNTHESIS FROM *MUCOR MUCEDO*

| enzyme | source | pH optimum | $K_m$/mM | comparative activity, mycelium trisporate-treated | reference |
|---|---|---|---|---|---|
| mevalonate kinase | (−), (+) | 7.5 | 1.0, ATP; 0.1, D,L-mevalonate | unchanged | M. Heyworth & G. W. Gooday (unpubl.) |
| esterase | (−), trace in (+) | 7.8 | 1.0, methyl trisporate | (−) doubled, (+) unchanged | Werkman (1976) |
| dehydrogenase | (−), none in (+) | 7.8 | (1.0), methyl dihydrotrisporate | (−) doubled | Werkman (1976) |

This was solved by the finding that trisporic acid itself stimulated the production of these precursors in unmated cultures of *B. trispora* and *M. mucedo* (Werkman & Ende 1973, 1974; Bu'Lock *et al.* 1976). This effect was inhibited by 5-fluorouracil and so presumably involves protein synthesis. Werkman (1976) has partly characterized two enzyme activities of the (−) strain of *M. mucedo*, one hydrolysing methyl trisporate and the other oxidizing the 4-OH of methyl 4-dihydrotrisporate. Specific activities of both were doubled by treatment of the mycelium with trisporic acid (table 5), and this effect was inhibited by cycloheximide.

## DISCUSSION

The gross outlines of the collaborative biosynthesis of trisporic acid via the mating type-specific precursors are now generally agreed, and the inability as yet to mimic this biosynthesis by addition of appropriate substrates to fungal cultures need not worry us. Such experiments cannot take into account factors such as diffusion rates, $K_m$ values for all of the enzymes involved, and mechanisms for the controls of activities of these enzymes. The observation that intermediates do not accumulate in mating cultures indicates that the rates of their conversions are well controlled. Perhaps they remain bound to enzyme complexes for the accomplishment of several metabolic steps.

The initiation and amplification of trisporic acid biosynthesis as the two mating types approach each other is a chemical display exactly analagous to the initiation and amplification of the visual displays of courtship that are well known in animals and birds: in each they serve functions of recognition of and attraction to a suitable partner, and preparation for mating. Thus the mechanism of feedback control of a biosynthetic pathway, so familar in other biochemical sequences, is here directly giving a primitive example of control of behaviour.

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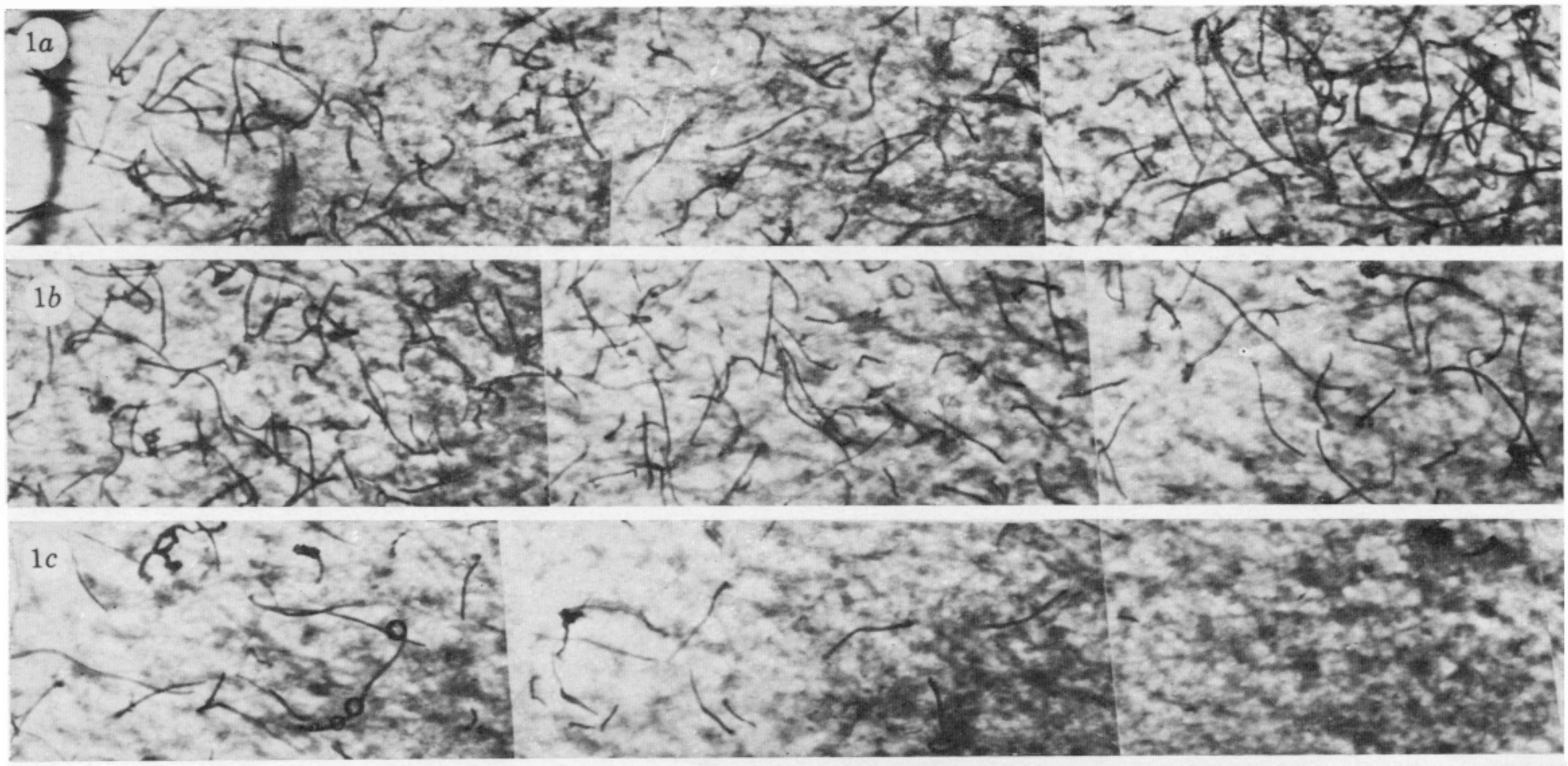


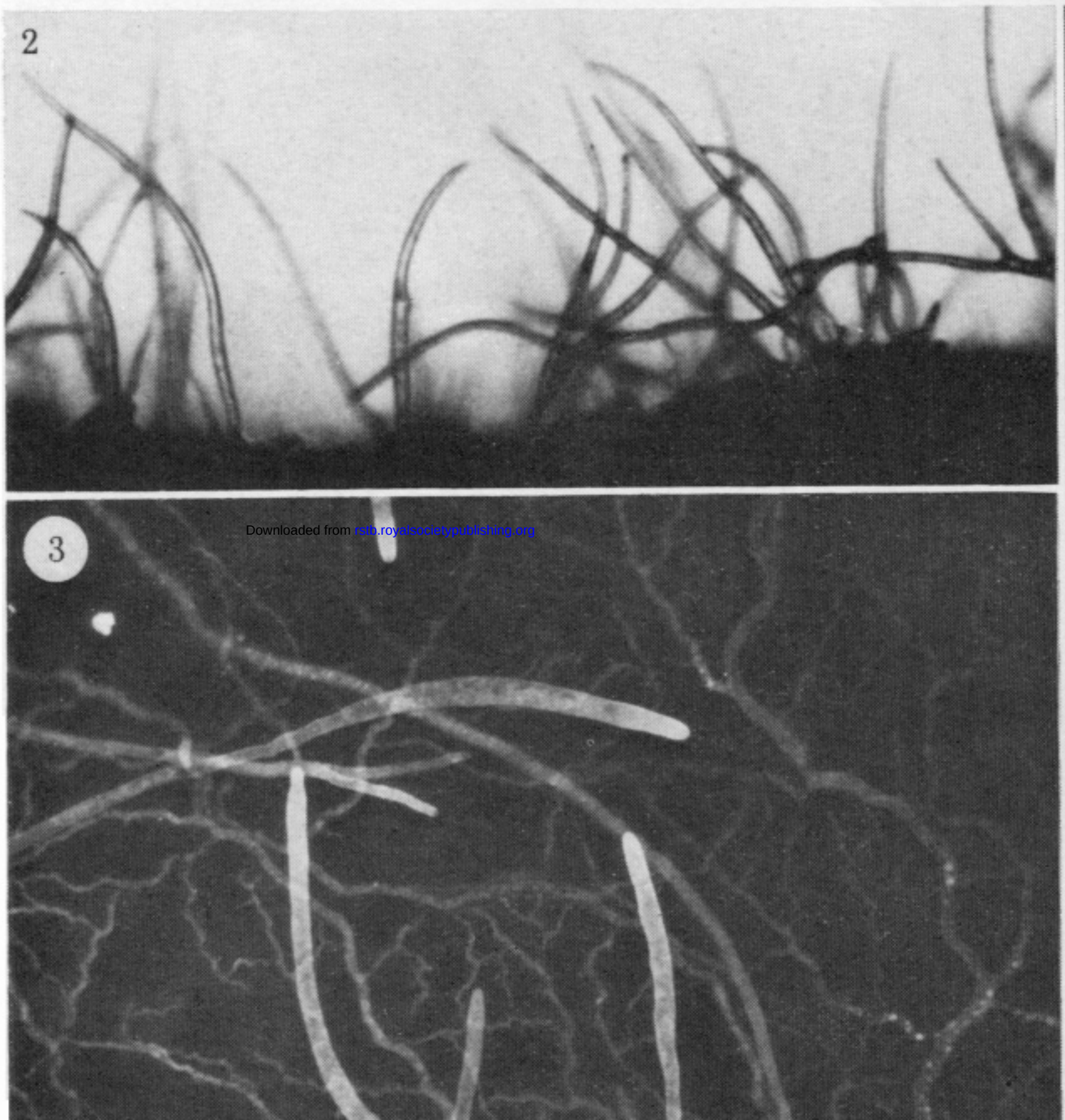







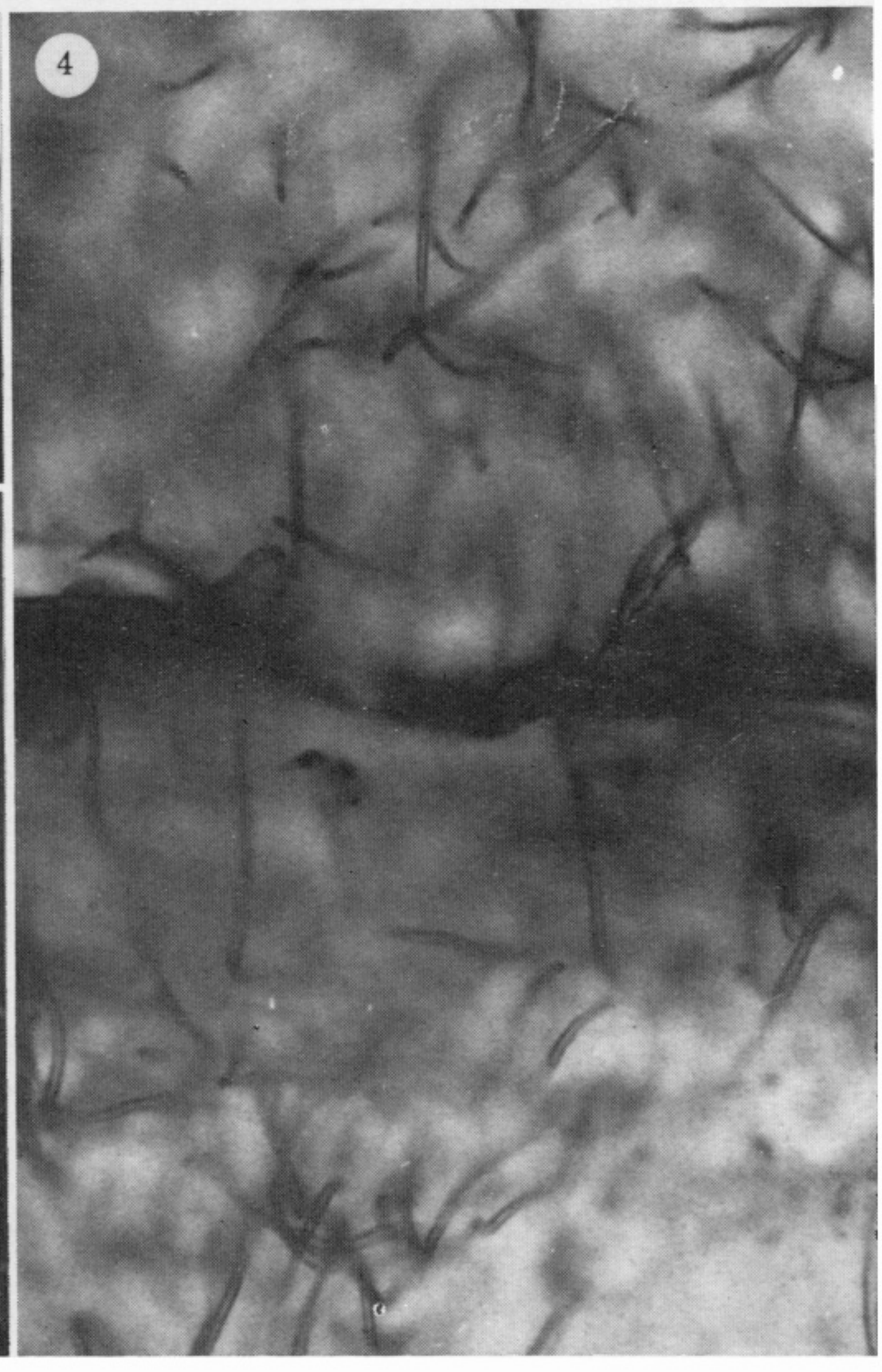


FIGURE 1. Appearance of bioassay plate of (−) *M. mucedo*, 15 h after addition of 100 ng trisporic acid C to a well just in front of hyphal tips. Edge of well is visible at left of 1*a*; photomontage of transect is, left to right, 1*a* to 1*b* to 1*c*; total distance is 14 mm. Note greatest density of zygophores around 5 mm from well.

FIGURE 2. Zygophores at edge of bioassay well of (−) *M. mucedo*, 15 h after addition of 1 μg methyl trisporate C. (Magn. × 64.)

FIGURE 3. Staining of (−) zygophores of *M. mucedo* by fluorescein-labelled wheat germ agglutinin. Conditions as described by Jones & Gooday (1977). Bright spots within hyphae represent autofluorescence of carotene-containing oil droplets. (Magn. × 150.)

FIGURE 4. Zygophores formed at zone of diffusion contact of (−) (top) and (+) (bottom) *M. mucedo*, grown separated by a Cellophane membrane cemented across a Petri dish. (Magn. × 64.)