Trisporic Acid Biosynthesis in *Blakeslea trispora* via Mating Type-specific Precursors*

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**SUMMARY**

Separate (+) and (−) mating type cultures of *Blakeslea trispora* synthesized: (a) labeled trisporic acid B and trisporic acid C when incubated with labeled, partially purified extracts isolated from opposite mating type cultures; (b) unlabeled trisporic acids when incubated with labeled glucose and unlabeled extracts isolated from opposite mating type cultures; and (c) over 100-fold less trisporic acids when incubated with labeled extracts isolated from the same mating type cultures. Thus, separate (+) and (−) cultures synthesize mating type-specific precursors of trisporic acids.

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Trisporic acids—oxidized, unsaturated derivatives of 1,1,3-trimethyl-2-(3'-methyl-2'-octyl)cyclohexane—stimulate zygophore (sex cell) development in *Mucor mucedo* and presumably in all other mucoraceous fungi including *Blakeslea trispora* (1–4). Extensive trisporic acid biosynthesis requires the cooperation of (+) and (−) mating type mycelia. Sutter and co-workers (4) demonstrated that separate (+) and (−) cultures of *B. trispora* synthesized small amounts of trisporic acid B and trisporic acid C when stimulated by a neutral fraction isolated from the culture medium of the opposite mating type culture of *B. trispora*. Each neutral fraction also stimulated zygophore development in the opposite mating type culture of *M. mucedo* (4, 5). The neutral fractions were presumed to contain mating type-specific precursors of trisporic acids (4–6). In this paper, we demonstrate that the neutral fractions do contain mating type-specific precursors of trisporic acid B and trisporic acid C.

Labeled PNF³ was prepared as follows. Ten 2-liter flasks, each containing 500 ml of PGT medium, were inoculated with (+) mycelia and incubated on a gyratory shaker at 25° (4). After 44 hours, 25 µCi of [U-¹⁴C]glucose (118 nmoles) were added to each flask and the incubation was continued for an additional 75 hours. The culture medium was collected and extracted in 620-ml portions with equal volumes of chloroform. (The culture medium was not adjusted to pH 2 prior to extraction, a step performed in previous work (4). This new procedure resulted in a 90% rather than a 40% recovery of the trisporic acid stimulating components from (+) culture medium.) The chloroform extract was evaporated to dryness in vacuo, and the residue dissolved in 6 ml of ethanol and then purified by Sephadex LH-20 chromatography (4). The active fractions from the column contained 900,000 cpm and 2,500 A₂₈₅ units of PNF. Unlabeled PNF containing 2,790 A₂₈₅ units was prepared at the same time. PNF absorbs ultraviolet radiation maximally at 285 nm. A₂₈₅ units are the A₂₈₅ reading of the sample times the dilution factor times the total milliliters of undiluted sample solution. Radioactivity measurements were made in a Beckman LS-230 liquid scintillation system after the sample solvent (ethanol) was evaporated from the counting vial by bubbling with nitrogen gas and after 5 ml of Bray’s (7) solution without 1,4-bis[2-(5-phenyl-oxazolyl)]benzene was added.

Labeled PNF was added to a 36-hour (−) culture, (+) culture, and a 0.5-liter flask containing 100 ml of uninoculated PGT medium and then incubated for 2.5 hours. After the incubation, the culture media were collected, and the acid fractions were isolated and partially purified by DEAE-Sephadex chromatography (4). Column fractions, in which trisporic acids were eluted if present, were pooled and analyzed for radioactivity and A₂₈₅. (Trisporic acids absorb ultraviolet radiation maximally at 325 nm.) The purified acid fraction from the (−) culture, but not the (+) culture, contained both radioactivity (29,280 cpm) and ultraviolet-absorbing material (116 A₂₈₅ units) above the levels found in the purified acid fraction from uninoculated PGT medium (Table I). In a control experiment in which uninoculated PNF and labeled glucose had been incubated for 2.5 hours with a (−) culture, (+) culture, and a flask containing uninoculated PGT medium, the purified acid fractions all exhibited the same low radioactivity (Table I). However, the purified acid fraction from the (−) culture, but not the (+) culture, contained ultraviolet-absorbing material (122 A₂₈₅ units) above the level found in the purified acid fraction from uninoculated PGT medium.

Trisporic acid B and trisporic acid C in purified acid fractions were resolved and purified by silica gel thin-layer chromatography (4). Trisporic acid B and trisporic acid C, isolated from the (−) culture incubated with labeled PNF, exhibited specific activities of 250 cpm per A₂₈₅ unit. When labeled trisporic acid C (13 A₂₈₅ units) and authentic unlabeled trisporic acid C (90
PCi of [U-\textsuperscript{14}C]glucose (118 nmoles) were added to each flask and were counted for 50 min or 50,009 counts and then back.

After 44 hours, 2 mg of partially purified trisporic acids and 25 medium, were inoculated with (-) mycelia and incubated.

Forty flasks, each containing 500 ml of PGT medium, were inoculated with (-) cultures incubated with labeled PNF. Each culture medium was extracted with chloroform, the chloroform extract evaporated to one fifth its volume and then extracted with 4% NaHCO\textsubscript{3} to remove trisporic acids. The labeled MNF contained 1,848,000 cpm and 5,300 A\textsubscript{325} units. Unlabeled MNF, prepared simultaneously, contained 5,800 A\textsubscript{325} units. MNF absorbs ultraviolet radiation maximally at 300 nm.

Labeled MNF (320 A\textsubscript{325} units) was added to each of five 34-hour (+) cultures, (-) cultures, and flasks containing 100 ml of uninoculated PGT medium and then incubated for 5 hours. The purified acid fraction isolated from (+) cultures, but not (-) cultures, contained significant radioactivity (18,700 cpm) and ultraviolet-absorbing material (84 A\textsubscript{325} units) above the levels found in the purified acid fraction from uninoculated PGT medium (Table II). In a control experiment in which unlabeled MNF and labeled glucose had been incubated with (+) cultures, (-) cultures, and flasks containing uninoculated PGT medium, the purified acid fractions all exhibited the same low radioactivity (Table II). However, the purified acid fraction from (+) cultures, but not (-) cultures, contained ultraviolet-absorbing material (91 A\textsubscript{325} units) above the level found in the purified acid fraction from uninoculated PGT medium.

Trisporic acid B and trisporic acid C, isolated from the purified acid fraction of (+) cultures incubated with labeled MNF, were co-chromatographed on a Sephadex LH-20 column (4), the radioactivity and A\textsubscript{325} units in each fraction collected were coincident (Fig. 1). A similar coincidence between radioactivity and A\textsubscript{325} was observed when labeled trisporic acid B (7 A\textsubscript{325} units) and authentic unlabeled trisporic acid B (80 A\textsubscript{325} units) were co-chromatographed. These observations demonstrate that both trisporic acid C and trisporic acid B were radioactive and therefore PNF contains mating type-specific precursors of trisporic acids. Radioactive trisporic acid C and trisporic acid B were also isolated from uninoculated PGT medium incubated with labeled PNF, indicating that the PNF preparations contained the trace amounts of trisporic acids which accumulate in the culture medium of 5-day (+) cultures (4).

Similar experiments were performed with MNF. The quantity of trisporic acid-stimulating components synthesized by (-) cultures, however, is less than \(\frac{1}{4}\) of the amount synthesized by (+) cultures (4). Werkman and van den Ende (6) reported that the tracer studies of Werkman and van den Ende (6) in three ways. (a) The final radioactivity measurements were made with pure trisporic acid C and trisporic acid B rather than with a partially purified extract of trisporic acids. (b) The radioactivity in the neutral fractions was shown to be incorporated into trisporic acids in a mating type-specific fashion. (c) The labeled compounds in a neutral fraction were shown not to be degraded to metabolites of glucose prior to incorporation into trisporic acids.

### Table I

| Incubation | PNF | [U-\textsuperscript{14}C] Glucose | Trisporic acid analysis: purified acid fraction | A\textsubscript{325} units | cpm\textsuperscript{a} |
|------------|-----|-------------------------------|-----------------------------------------------|-----------------|----------------|
| Culture    |     |                               |                                               |                 |               |
| (-)        | 2.35| 610                           | 0                                             | 119             | 30,160       |
| (+)        | 1.01| 320                           | 0                                             | 88              | 18,840       |
| None       | 2.35| 610                           | 0                                             | 3               | 830          |
| None       | 1.01| 320                           | 0                                             | 4               | 320          |
|            | 0   | 670                           | 2                                             | 125             | 30           |
|            | 0   | 670                           | 2                                             | 3               | 30           |
|            | 0   | 670                           | 2                                             | 3               | 30           |

\textsuperscript{a} Total counts per min per sample: 10% portions of each sample were counted for 50 min or 50,009 counts and then background (38 cpm) subtracted before calculating total counts per min.

### Table II

| Incubation | MNF | [U-\textsuperscript{14}C] Glucose | Trisporic acid analysis: purified acid fraction | A\textsubscript{325} units | cpm\textsuperscript{a} |
|------------|-----|-------------------------------|-----------------------------------------------|-----------------|----------------|
| Culture    |     |                               |                                               |                 |               |
| (-)        | 2.35| 610                           | 0                                             | 119             | 30,160       |
| (+)        | 1.01| 320                           | 0                                             | 88              | 18,840       |
| None       | 2.35| 610                           | 0                                             | 3               | 830          |
| None       | 1.01| 320                           | 0                                             | 4               | 320          |
| (-)        | 0   | 670                           | 2                                             | 125             | 30           |
| (+)        | 0   | 330                           | 1.1                                           | 85              | 20           |
| (-)        | 0   | 330                           | 1.1                                           | 4               | 30           |
| None       | 0   | 330                           | 1.1                                           | 4               | 30           |

\textsuperscript{a} As described in the legend to Table I.

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**TABLE I**

**Trisporic acid synthesis from precursors made by (+) cultures**

**TABLE II**

**Trisporic acid synthesis from precursors made by (-) cultures**

![Figure 1](http://www.jbc.org/)

**FIG. 1.** Sephadex LH-20 chromatography of unlabeled trisporic acid C isolated from (+/-) cultures and labeled trisporic acid C isolated from (-) cultures incubated with labeled PNF. Each fraction contained 2.1 ml; 1.5-ml portions of each fraction were counted for 10 min and the counts per min (× 10\textsuperscript{4}) uncorrected for background, were plotted versus A\textsubscript{325} units/1.5 ml. (● ... ○).
These experiments do not exclude the remote, ad hoc possibilities that labeled compounds in the neutral fractions were degraded to unknown, hypothetical metabolites which were then utilized in the de novo biosynthesis of trisporic acids. Experiments are now in progress to isolate pure mating type-specific precursors of trisporic acids and to determine their structures.

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