Sterol Synergism in *Paramecium tetraurelia*

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Paramecium tetraurelia is a naturally occurring sterol auxotroph with an absolute nutritional requirement for one of a small group of structurally related phytosterols. We report here a quantitative study demonstrating that a low, otherwise sub-supportive, concentration (≈ 0.020 – $0.050 \mu\text{g ml}^{-1}$) of an essential phytosterol (stigmasterol) is adequate for growth of this ciliate, provided that a second, relatively non-specific sterol is available at a higher concentration ($1.0 \mu\text{g ml}^{-1}$) to allow for membrane biosynthesis. This phenomenon, referred to as sterol synergism, has been observed in a broad taxonomic range of organisms, with the conclusion that small amounts of specific sterols are required to perform some previously unknown, vital metabolic or regulatory function. *Paramecium* promises to be an excellent model organism for the elucidation of essential sterol function.

INTRODUCTION

Ciliated protozoans of the genus *Paramecium* are auxotrophic for a phytosterol and octadecenoic acid (van Wagtenonk *et al.*, 1953; Soldo & van Wagtenonk, 1967). The nutritional requirement for sterol was described 30 years ago, when it was found that only a small group of structurally similar phytosterols support growth (Conner *et al.*, 1953; Conner & van Wagtenonk, 1955). One subsequent report has addressed the question of why specific phytosterols are required for growth of *Paramecium* (Conner *et al.*, 1971). An apparent link between growth support and fatty acyl esterification of sterols was demonstrated. Our recent study of the metabolism of phytosterols in *Paramecium tetraurelia* (Whitaker & Nelson, 1987) revealed that non-growth-supporting sterols are esterified and can constitute a high percentage of the cell's esterified sterols when growth support is conferred by a phytosterol other than stigmasterol. In addition, we have demonstrated the phenomenon of sterol synergism or sparing in this simple eukaryote, i.e. the adequacy of non-growth-supporting sterols for the cell's bulk membrane structural requirement when a much lower concentration of an essential phytosterol is provided to meet the nutritional requirement (Pinto *et al.*, 1983; Rampogal & Bloch, 1983; Whitaker & Nelson, 1987).

Over the past decade, sterol synergism has been described and characterized in a variety of organisms, including microbes, invertebrates and vertebrates, summarized in a review by Bloch (1983). Among the micro-organisms are the prokaryote *Mycoplasma*, several yeasts and *Tetrahymena*. The general conclusion from these studies is that, in addition to the relatively non-specific requirement for sterols as a structural component of cell membranes, there is often a requirement for small amounts of specific sterols which apparently perform some vital metabolic or regulatory function.

Paramecium offers several clear advantages for research directed toward the elucidation of essential sterol function: (1) it is a simple, single-celled eukaryotic micro-organism which is

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naturally auxotrophic for sterols; (2) it grows well in both a defined (but complex) axenic medium and a simple monoxenic medium consisting of a dense suspension of *Escherichia coli* plus added sterol in a buffered salts solution (Whitaker & Nelson, 1987); and (3) mutants are readily generated and selected, and the genetics is well understood. In this study we have quantified the synergistic effect of combinations of growth-supporting and non-growth-supporting sterols on the growth of *P. tetraurelia* in both axenic and monoxenic cultures.

Table 1. *Source and purity of sterols*

Sterol	Carbon no.	R group at C-24	Source	Purity* (%)
Stigmasterol (stigmasta-5,22-dien-3 β -ol)	29	α -C ₂ H ₅	Applied Science Labs	98
Cholesterol (cholest-5-en-3 β -ol)	27	-H	Sigma	99+
Campesterol ([24 <i>R</i>]-ergost-5-en-3 β -ol)	28	α -CH ₃	Sigma	99
Ergosterol (ergosta-5,7,22-trien-3 β -ol)	28	β -CH ₃	Aldrich	98
Tetrahymanol	30†	-	<i>Tetrahymena pyriformis</i>	99

* Determined by GLC (Whitaker & Nelson, 1987).

† 4,4-Dimethyl pentacyclic triterpenoid alcohol.

METHODS

Cultures. Wild-type strain 51S of *Paramecium tetraurelia* (Sonneborn, 1975) was used throughout this study. In the first series of experiments, cells were grown in monoxenic culture in a medium devised to eliminate the problem of sterol contaminants. This medium consisted of a dense suspension of *Escherichia coli* B in a solution containing 15 mM-MOPS buffer, 3 mM-KCl and 1 mM-CaCl₂. The pH of the MOPS basal medium was raised to 6.2 with KOH and finally to 6.5 with NaOH. Sterols were added in an ethanolic solution to the MOPS basal medium prior to autoclaving. *E. coli* was grown in minimal glucose medium to stationary phase, harvested by centrifugation, washed once and resuspended in a small volume of sterile MOPS basal medium. Equal volumes of this suspension were then pipetted into each culture flask prior to inoculation with *P. tetraurelia*. This method of culturing *Paramecium* has been described in greater detail elsewhere (Whitaker & Nelson, 1987).

In the second series of experiments *P. tetraurelia* 51S was grown in Soldo's crude axenic medium (Soldo & van Wagtendonk, 1969). This medium is based on proteose and trypticase peptone plus yeast nucleic acid; it is rich in vitamins and unesterified fatty acids ($\approx 45 \mu\text{g ml}^{-1}$), and includes 1- α -cephalin (phosphatidylethanolamine) with a high polyunsaturated fatty acid content. The latter component of the medium included a substantial amount of cholesterol as a contaminant, and therefore was purified by silicic acid column chromatography (Whitaker & Nelson, 1987) prior to use. The desired combinations of bulk sterols plus stigmasterol were added in ethanolic solution prior to autoclaving culture flasks containing the axenic medium.

Commercial and natural sources of sterols. Table 1 presents the source and purity of sterols used in this study. With the exception of the pentacyclic triterpenoid alcohol tetrahymanol, all sterols were acquired from commercial sources and were recrystallized once or twice from hot 100% ethanol prior to use. Tetrahymanol was isolated from the ciliated protozoan *Tetrahymena pyriformis* and purified as reported previously (Whitaker & Nelson, 1987).

RESULTS

Requirement for exogenous sterol

Fig. 1 depicts the influence of exogenous stigmasterol concentration on the growth of *P. tetraurelia* in monoxenic culture. In these experiments, stigmasterol and its metabolite, 7-dehydrostigmasterol (Conner *et al.*, 1971; Whitaker & Nelson, 1987), served as both the bulk membrane sterol and the nutritionally required phytosterol. Under this condition, near maximal growth was realized at an exogenous stigmasterol concentration of $5.0 \mu\text{g ml}^{-1}$ (Pitt, 1980). The maximum cell density attained in monoxenic cultures, and to a lesser extent the exponential rate of growth, decreased with decreasing sterol concentration. The minimum amount of

Fig. 1. Dependence of growth of *P. tetraurelia* at 28 °C in monoxenic cultures on the exogenous stigmasterol concentration. Erlenmeyer flasks (250 ml) containing 50 ml sterile MOPS basal medium plus the specified concentration of stigmasterol and a dense suspension of *E. coli* B were inoculated at ≈ 100 cells ml^{-1} at time zero. The inoculum was drawn from an early stationary-phase culture (96 h growth; $\approx 5 \times 10^3$ cells ml^{-1}) supplemented with $1.0 \mu\text{g}$ stigmasterol ml^{-1} . Vertical bars represent ± 1 SD ($n = 5$). Stigmasterol was added at 5.0 ($\circ$), 2.0 ($\triangle$), 1.0 ($\blacksquare$), 0.5 ($\blacktriangle$) and 0.2 ($\bullet$) μg ml^{-1} .

stigmasterol required for significant growth, roughly three cell divisions during the 70 h post-inoculation period, was $0.2 \mu\text{g}$ ml^{-1} . Below this level of stigmasterol supplementation $\leq$ one cell division occurred in ≥ 48 h (data not shown).

Sterol synergism in monoxenic cultures

We have previously reported that in monoxenic cultures supplemented with a high ratio of a non-growth-supporting sterol to stigmasterol ($\geq 4:1$), the non-growth-supporting sterol (and its dehydrogenated derivative, if any) generally constituted the majority of the unesterified (free) sterols in both whole cells and cell (ciliary) membranes (Whitaker & Nelson, 1987). This did not hold true, however, for cultures supplemented with the triterpenoid alcohol tetrahymanol plus stigmasterol in a ratio of 10:1. While tetrahymanol did constitute the majority of the free sterols in whole cells from these cultures, it accounted for only $\approx 40\%$ of the ciliary sterols. These and other data led us to conclude that tetrahymanol serves poorly as a structural component of the cell membrane of *Paramecium*, while $\Delta^{5,7}$ -diene sterols are optimal in this role.

The results from this present, quantitative study of stigmasterol sparing by cholesterol and tetrahymanol in monoxenic cultures of *P. tetraurelia* (Figs 2 and 3) support our previous conclusion. In these experiments, the concentration of stigmasterol was varied over the range 0.0 – $0.25 \mu\text{g}$ ml^{-1} , and the concentration of the sparing sterol (i.e. cholesterol or tetrahymanol) was held constant at $1.0 \mu\text{g}$ ml^{-1} . At the maximum ratio of stigmasterol to sparing sterol ($1:4$, or $0.25:1.0 \mu\text{g}$ ml^{-1}), both the exponential growth rate and maximum titre of cultures supplemented with cholesterol (Fig. 2) were substantially higher than those of comparable cultures supplemented with tetrahymanol (Fig. 3). In addition, the threshold ratio of

Fig. 2

Fig. 3

Fig. 2. Sparing of the requirement for stigmasterol by cholesterol in monoxenic cultures of *P. tetraurelia* at 28 °C. Erlenmeyer flasks (250 ml) containing 50 ml sterile MOPS basal medium, the specified concentrations of a dense suspension of *E. coli* B, cholesterol and stigmasterol were inoculated at ≈ 100 cells ml^{-1} at time zero. The inoculum (≈ 0.7 ml) was drawn from an early stationary-phase culture (96 h growth; $\approx 7 \times 10^3$ cells ml^{-1}) supplemented with $1.0 \mu\text{g}$ cholesterol ml^{-1} plus $0.2 \mu\text{g}$ stigmasterol ml^{-1} . Vertical bars represent ± 1 SD ($n = 3$, with replicate cultures in each experiment). SD was generally quite small at sub-supportive concentrations of stigmasterol, and is therefore not shown for these curves. Cholesterol at $1.0 \mu\text{g}$ ml^{-1} (●); plus stigmasterol at 0.010 (▼), 0.025 (▲), 0.050 (▽), 0.100 (■) and 0.250 (△) μg ml^{-1} . (See Fig. 1 for $1.0 \mu\text{g}$ stigmasterol ml^{-1} control.)

Fig. 3. Sparing of the requirement for stigmasterol by tetrahymanol in monoxenic cultures of *P. tetraurelia* at 28 °C. Erlenmeyer flasks (250 ml) containing 50 ml sterile MOPS basal medium plus a dense suspension of *E. coli* B and the specified concentrations of tetrahymanol and stigmasterol were inoculated at ≈ 100 cells ml^{-1} at time zero. The inoculum (≈ 3.3 ml) was drawn from an early stationary-phase culture (96 h growth; $\approx 1.5 \times 10^3$ cells ml^{-1}) supplemented with $1.0 \mu\text{g}$ tetrahymanol ml^{-1} plus $0.2 \mu\text{g}$ stigmasterol ml^{-1} . (Thus, the actual concentration of stigmasterol in sparing experiments was $\approx 0.013 \mu\text{g}$ ml^{-1} higher than the specified concentration.) Vertical bars represent ± 1 SD ($n = 3$, with replicate cultures in each experiment). Tetrahymanol at $1.0 \mu\text{g}$ ml^{-1} (●); plus stigmasterol at 0.010 (▼), 0.025 (▲), 0.050 (▽), 0.100 (■) and 0.250 (△) μg ml^{-1} . (See Fig. 1 for $1.0 \mu\text{g}$ stigmasterol ml^{-1} control.)

stigmasterol to sparing sterol (i.e. the lowest ratio allowing for measurable growth) was clearly lower in cultures supplemented with cholesterol ($\leq 1:50$) than in cultures supplemented with tetrahymanol ($\approx 1:20$).

Sterol synergism in axenic cultures

We have also done a quantitative study of stigmasterol sparing in axenic cultures by campesterol, ergosterol and cholesterol (Figs 4, 5 and 6, respectively). The data indicate a distinct order of effectiveness with respect to growth rate and maximum cell density for the three sterols tested: campesterol > ergosterol > cholesterol. The minimum concentration of stigmasterol required to elicit measurable growth also varied with the sparing sterol supplement as follows: $\leq 0.010 \mu\text{g}$ ml^{-1} in campesterol-supplemented cultures; $\leq 0.020 \mu\text{g}$ ml^{-1} in

Fig. 4

Fig. 5

Fig. 4. Sparing of the requirement for stigmasterol by campesterol in axenic cultures of *P. tetraurelia* at 28 °C. Erlenmeyer flasks (250 ml) containing 50 ml sterile, 'cholesterol-free' Soldo's crude axenic medium (see Methods), plus the specified concentrations of campesterol and stigmasterol, were inoculated at ≈ 150 cells ml⁻¹ at time zero. The inoculum (≈ 1.0 ml) was drawn from an early stationary-phase culture (96 h growth; $\approx 7.5 \times 10^3$ cells ml⁻¹) supplemented with 1.0 µg campesterol ml⁻¹ plus 0.2 µg stigmasterol ml⁻¹. (Thus, the actual concentration of stigmasterol in sparing experiments was ≈ 0.004 µg ml⁻¹ higher than the specified concentration.) SD values (not shown) were in the same range as indicated for sparing experiments with monoxenic cultures ($n = 3$, with replicate cultures in each experiment). Stigmasterol control (---○---) 1.0 µg ml⁻¹. Campesterol at 1.0 µg ml⁻¹ (●); plus stigmasterol at 0.010 (▼), 0.025 (▲), 0.050 (▽), 0.100 (■) and 0.250 (△) µg ml⁻¹.

Fig. 5. Sparing of the requirement for stigmasterol by ergosterol in axenic cultures of *P. tetraurelia* at 28 °C. Erlenmeyer flasks (250 ml) containing 50 ml sterile, 'cholesterol free' Soldo's crude axenic medium (see Methods), plus the specified concentrations of ergosterol and stigmasterol, were inoculated at ≈ 150 cells ml⁻¹ at time zero. The inoculum (≈ 2.5 ml) was drawn from an early stationary-phase culture (96 h growth; $\approx 3.0 \times 10^3$ cells ml⁻¹) supplemented with 1.0 µg ergosterol ml⁻¹ plus 0.2 µg stigmasterol ml⁻¹. (Thus, the actual concentration of stigmasterol in sparing experiments was ≈ 0.010 µg ml⁻¹ higher than the specified concentration.) SD values (not shown) were in the same range as indicated for sparing experiments with monoxenic cultures ($n = 3$, with replicate cultures in each experiment). Stigmasterol control (---○---) 1.0 µg ml⁻¹. Ergosterol at 1.0 µg ml⁻¹ (●); plus stigmasterol at 0.010 (▼), 0.025 (▲), 0.050 (▽), 0.100 (■) and 0.250 (△) µg ml⁻¹.

ergosterol-supplemented cultures; and ≈ 0.025 µg ml⁻¹ in cholesterol-supplemented cultures. Thus, growth support at both the maximum and threshold ratios of stigmasterol to sparing sterol was greatest with campesterol, intermediate with ergosterol, and poorest with cholesterol.

DISCUSSION

The observed differences in growth support by the sparing sterols campesterol, ergosterol, cholesterol and tetrahymanol may reflect their relative suitability for the bulk membrane structural function, their relative level of (weak) growth support, or both. Solubility in the

Fig. 6. Sparing of the requirement for stigmasterol by cholesterol in axenic cultures of *P. tetraurelia* at 28 °C. Erlenmeyer flasks (250 ml) containing 50 ml sterile, 'cholesterol free' Soldo's crude axenic medium (see Methods) plus the specified concentrations of cholesterol and stigmasterol, were inoculated at ≈ 150 cells ml^{-1} at time zero. The inoculum (≈ 3.0 ml) was drawn from an early stationary-phase culture (96 h growth; $\approx 2.5 \times 10^3$ cells ml^{-1}) supplemented with $1.0 \mu\text{g}$ cholesterol ml^{-1} plus $0.2 \mu\text{g}$ stigmasterol ml^{-1} . (Thus, the actual concentration of stigmasterol in sparing experiments was $\approx 0.012 \mu\text{g}$ ml^{-1} higher than the specified concentration.) SD values (not shown) were in the same range as indicated for sparing experiments with monoxenic cultures ($n = 3$, with replicate cultures in each experiment). Stigmasterol control (---○---) $1.0 \mu\text{g}$ ml^{-1} . Cholesterol at $1.0 \mu\text{g}$ ml^{-1} (●); plus stigmasterol at 0.010 (▼), 0.025 (▲), 0.050 (▽), 0.100 (■) and 0.250 (△) μg ml^{-1} .

aqueous medium may also be involved in the apparent effectiveness of these four sterols, cholesterol being far more soluble than either campesterol or ergosterol, and these two in turn being more soluble than the triterpenoid alcohol tetrahymanol. It is noteworthy that the threshold concentration of stigmasterol required to elicit measurable growth in the presence of $1.0 \mu\text{g}$ cholesterol ml^{-1} was significantly lower in monoxenic cultures (≈ 0.015 – $0.020 \mu\text{g}$ ml^{-1}) than in axenic cultures ($\approx 0.025 \mu\text{g}$ ml^{-1}). This could be related to the observed initial lag phase following inoculation of axenic cultures and may reflect a difference in the relative 'availability' of exogenous sterols in the different media.

The demonstration of sterol sparing or synergism in *P. tetraurelia* indicates that the nutritional requirement for specific phytosterols is not related to the structural role of bulk sterol in the cell membrane. There is a growing body of evidence that specific sterols are required in relatively small amounts by micro-organisms, invertebrates and vertebrates to fulfil previously unknown metabolic or regulatory functions (Bloch, 1983).

To summarize what is known about the metabolic or regulatory roles of sterols in several organisms, it has been found in one or more instances that specific sterols have some critical function in fatty acid transport and/or phospholipid biosynthesis (Holz *et al.*, 1962; Dahl *et al.*, 1980, 1981; Guyer & Bloch, 1983), and in respiration/mitochondrial activity (van Wagtenonk

& Wulzen, 1950; Conner, 1957; Conner & Nakatani, 1958; Parks, 1978). For *Paramecium*, an apparent link between growth support and fatty acyl esterification of sterols has been reported (Conner *et al.*, 1971). This would clearly favour a hypothesis involving essential sterols in the transport, metabolism or transacylation of fatty acids. However, results from our study of sterol nutrition and metabolism in *P. tetraurelia* indicate that fatty acyl esterification is probably not related to the vital function of selected phytosterols in this ciliate (Whitaker & Nelson, 1987). An intriguing question awaiting further investigation is whether it is the growth-supporting phytosterol *per se* or instead a metabolite which is required for growth of *Paramecium*.

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