ERGOSTEROL REPLACEMENT OF TETRAHYMANOL IN TETRAHYMENA MEMBRANES

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SUMMARY: The naturally occurring pentacyclic triterpene alcohol of Tetrahymena pyriformis, tetrahymanol, is replaced by ergosterol as a result of supplementation of the growth medium with that sterol. Since tetrahymanol is not metabolized by the ciliate, loss of tetrahymanol from sterol-supplemented cells appears to occur through dilution during growth. Ergosterol-grown cells are at least 20 times more sensitive to polyene antibiotics than normal cells. The substitution of ergosterol for tetrahymanol observed in whole cells is also reflected in isolated cilia and ciliary membrane preparations. In these membranes, the phospholipid/tetrahymanol ratio in normal cells is similar to the phospholipid/ergosterol ratio in ergosterol-grown cells.

The model proposed by Vandenheuvel (1) for phospholipid-sterol interactions within membranes can be tested in the ciliate Tetrahymena pyriformis W where the native triterpene alcohol, tetrahymanol, may be replaced by a variety of sterols supplied as nutritional supplements (2). Comparison of the phospholipid fatty acid compositions in both normal and ergosterol-grown cells showed that adjustments in fatty acid composition occurred in response to ergosterol substitution (3). Of central importance to the interpretation of these data was the determination of the sterol or triterpene alcohol content of isolated membranes from this ciliate, in order to show that ergosterol had indeed been substituted for tetrahymanol in the cellular membranes. Further, it was necessary to establish for each of these polycyclic alcohols the stoichiometric relationship between the alcohol and the phospholipids in the membranes, since in principle quantitative as well as qualitative differences in the polycyclic alcohol content could be involved in the alterations in fatty acid composition induced by ergosterol supplementation.

MATERIALS AND METHODS

Stock cultures of Tetrahymena pyriformis W were maintained and mass

cultures with and without sterol supplementation were obtained as previously described (4). Ergosterol was added to the culture fluid to give a concentration of 5 mg/500 ml medium. Cell counts were performed with a Coulter Counter.

For whole-cell analyses, cells were harvested, lyophilized and extracted with chloroform-methanol 2:1 (v/v) (4). The total lipid extract was purified from non-lipid contaminants by the method of Wuthier (5). Aliquots were removed from this purified total lipid for determination of phosphorus content by a modification of the method of Ames (6); the samples were ashed with ethanolic $Mg(NO_3)_2$, and then color development was achieved by a modification of the method of Bartlett (7).

The purified lipid extract was separated into a neutral and a polar lipid fraction by Unisil column chromatography (4). The neutral lipid fraction (chloroform eluate) was assayed directly for free ergosterol or tetrahymanol content by GLC on a 0.75% SE-52 column (4). The alcohols were quantitated by comparing peak areas to those of cholesterol added as an internal standard; fatty acyl esters of tetrahymanol or ergosterol do not occur in this strain (2).

Cilia were isolated by a modification of the method of Watson and Hopkins (8), employing glycerol rather than ethanol. Ciliary membranes were obtained by treating the isolated cilia with 0.6M KI to dissolve the axonemal components (9). The purity of cilia and ciliary membrane preparations was established by enzymatic, chemical and microscopic techniques (Kaneshiro and Ray, manuscript in preparation). Lipids were extracted from cilia and ciliary membranes with chloroform-methanol 2:1 (v/v), and analyses for phosphorus and tetrahymanol or ergosterol were performed as described above.

Radiolabelled tetrahymanol was prepared from cells grown with ^{14}C-mevalonolactone (New England Nuclear). The total lipid fraction from these cells was saponified and subjected to TLC on Silica Gel G plates

(benzene:ethyl acetate 10:1 (v/v)). The purified ^{14}C-tetrahymanol had an R_f of 0.31 (10), and autoradiograms of TLC plates showed a single component. Carrier tetrahymanol was added to give a specific activity of 2.7×10^4 cpm/mg.

One milligram of this material was added to each of two culture flasks containing 100 ml of culture fluid (4). The cells were harvested after 40 hours of growth at 25°, and the lipids were extracted and subjected to TLC and autoradiography.

RESULTS AND DISCUSSION

Tetrahymanol (2.1 μmoles/10^8 cells) is contained principally in the particulate fraction of these ciliates. Cells were disrupted by ultrasound, and the sonicate was centrifuged for 2 hours at 108,000 x g. In two experiments, all of the cellular tetrahymanol was recovered in the lipid extract of the high-speed pellet, and none was found in the supernate.

The amount of ergosterol found in sterol-supplemented cells is greater than the amount of tetrahymanol produced and accumulated in normal cells, as evidenced by the ratio of μmoles of total chloroform-methanol extractable phosphorus (P) to μmoles of sterol or triterpene alcohol (S) (Table I). The ratio of P/S of 12.2 in normal cells is comparable to the value of 10 found by Thompson (11). No significant change in the cellular chloroform-methanol extractable phosphorus content was observed when the cells were grown with ergosterol (Table I).

The low value for P/S in whole cells grown with added ergosterol would be consistent either with a high membrane sterol content or with the inclusion of the sterol in lipid granules similar to those observed by Allison and Ronkin (12). The latter explanation is indicated, since the P/S ratio in cilia and ciliary membranes (Table I) is nearly the same for both normal and ergosterol-supplemented cells. However, this result does not necessarily require that the excess ergosterol in the latter cells be vacuolar, since it is also possible that it be present in internal membranes.

The cilia and ciliary membranes are enriched in tetrahymanol or

TABLE I

Ratios of Chloroform-Methanol Extractable Phosphorus (P) to Sterol or Triterpene Alcohol (S) in *Tetrahymena* Grown With and Without Ergosterol[1]

Cell Type	μMoles P/μMoles S		
	Whole Cells	Cilia	Ciliary Membranes
Normal cells (S = tetrahymanol)	12.2 ± 0.8 (6)	2.4 ± 0.1 (6)	2.1 ± 0.1 (5)
Ergosterol-grown cells (S = ergosterol)	3.8 ± 0.2 (6)	1.7 ± 0.2 (5)	1.9 ± 0.2 (5)

[1]No sterols are found in normal ciliates. Ergosterol-supplemented cells contain less than 1% of the normal amount of tetrahymanol, and their cilia contain no detectable triterpene alcohol. Data are expressed as mean ± S. E. (number of determinations). Tetrahymanol values were multiplied by 1.1 to correct for the GLC detector response (15). Chloroform-methanol extractable phosphorus (P) for normal cells was 24 ± 7 μmoles/10^8 cells, and for ergosterol-grown cells, 19 ± 2 μmoles/10^8 cells (data from six separate experiments).

ergosterol (relative to the phosphorus content) when compared to whole cells (Table I). In membrane preparations from ergosterol-grown cultures, tetrahymanol was completely replaced by the sterol. The fact that the P/S ratios are similar for tetrahymanol and ergosterol in ciliary membranes (Table I) implies that ergosterol molecules may be inserted into the membranes at locations which would ordinarily be occupied by tetrahymanol, and it further suggests that there may be a limited number of sites available.

Cells containing ergosterol in the ciliary membrane were found to be sensitive to the polyene antibiotic filipin, while normal cells were not. In a series of three experiments, it was noted that ergosterol-grown cells (at a density of 2 x 10^5/ml) were lysed within 2-3 minutes when suspended in a solution containing 10 μg/ml of filipin, while normal ciliates were not disrupted even after one hour in a solution containing 20 μg/ml of the polyene. Similar results were obtained with nystatin. It is assumed (13) that the antibiotic acts on ergosterol-grown cells to disrupt the sterol-phospholipid interactions in the outer membrane, thus leading to lysis. Presumably the structure of tetrahymanol is sufficiently different from that

of sterols to preclude association with the antibiotic; alternatively, the packing of tetrahymanol in membranes may exclude the antibiotic from the hydrophobic region.

It was of interest to determine if ergosterol replacement of tetrahymanol is due to metabolic removal of the triterpene alcohol, or if the process is simply one of dilution. Earlier data suggested the latter alternative, since the amount of tetrahymanol recovered from a culture supplemented with cholesterol was equivalent to the amount of tetrahymanol found in the initial inoculum (4). Removal by dilution was also indicated by incubations in which ^{14}C-tetrahymanol was added to the culture fluid: 80% of the radioactivity was recovered in the cellular total lipid fraction, and the remaining 20% was found in the culture fluid. Autoradiograms of TLC plates developed as described above showed only one radioactive component at an R_f of 0.32. Thus, the ^{14}C-tetrahymanol was incorporated into the cells, but no metabolic products were detected. Since tetrahymanol biosynthesis is inhibited by ergosterol (2), it is reasonable to assume that the triterpene alcohol initially present is replaced by dilution during growth.

In conclusion, the evidence presented above indicates that: a) tetrahymanol is associated with the membranes of Tetrahymena pyriformis W; b) the pellicular (limiting) membrane covering the cilia (14) is especially enriched in tetrahymanol; and c) tetrahymanol is replaced in the ciliary membrane of ergosterol-grown cells by approximately the same amount of ergosterol. Therefore, it is assumed that the changes observed in phospholipid fatty acid compositions with ergosterol supplementation (3) are due to qualitative differences between tetrahymanol and ergosterol molecules, and are not due to a quantitative difference in the relative amounts of these two alcohols in the membranes.

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