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6-Fluorocholesterol as a Growth Factor for the Yeast Mutant GL7†

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ABSTRACT: 6-Fluorocholesterol supports the growth of the sterol-requiring yeast mutant GL7 albeit less efficiently than cholesterol or ergosterol. When the fluoro analogue is combined with very much smaller amounts of cholesterol, the growth response to the sterol pair is synergistic, i.e., greater than additive. On further addition of trace amounts of ergosterol to the 6-fluorocholesterol–cholesterol pair, an additional synergistic growth response is observed. On 6-fluorocholesterol alone, the growth rate of the yeast mutant is slow initially, but after several transfers of such cells to the same media containing the fluoro analogue, growth improves substantially. When incorporated into artificial membranes, cholesterol and its 6-fluoro analogue have essentially identical effects on membrane fluidity as judged from microviscosity measurements. The contrasting responses of artificial membranes and whole cells to the 6-fluoro analogue of cholesterol might be due to sterol–protein interactions in natural membranes.

There is growing evidence for multiple roles of sterols associated with eukaryotic plasma membranes. The first indication for such functional diversity came from the discovery of sterol synergism in the course of studies on the sterol requirement of pupating insect larvae (Clark & Bloch, 1959). At the time, already a distinction was made between structural and metabolic sterol functions. More recently, several laboratories have sought to define the phenomenon of sterol synergism in terms of two categories, with emphasis on the regulatory roles that sterols per se may play apart from modulating the bulk physical state of the membrane lipid bilayer (Dahl et al., 1980, 1981; Ramgopal & Bloch, 1983; Rodriguez et al., 1985).

Earlier this laboratory has reported extensively on the synergistic phenomenon displayed by the sterol auxotroph *Mycoplasma capricolum* on media supplemented with 20:1 mixtures of lanosterol and cholesterol (Dahl et al., 1980, 1981). Lanosterol can serve as the bulk sterol for this organism while the smaller cholesterol supplement controls the utilization of oleic acid for bacterial phospholipid synthesis. For studying sterol synergism in a eukaryotic cell, the yeast mutant GL7 has proven the organism of choice. Lacking squalene epoxide–lanosterol cyclase, this sterol auxotroph (Gollub et al., 1977) grows best on ergosterol, the principal sterol wild-type yeasts and fungi produce. Cholesterol supports growth of the mutant about half as effectively (Buttke & Bloch, 1981). In specified proportions, ergosterol–cholesterol combinations display the synergistic phenomenon as well (Ramgopal & Bloch, 1983). Here we compare the growth-promoting property of 6-fluorocholesterol with that of cholesterol, a choice motivated by the superiority of certain fluorosteroids as therapeutic agents. Cholesterol and its 6-fluoro analogue were

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also introduced into egg lecithin vesicles for comparison of their effects on membrane fluidity.

Materials and Methods

Saccharomyces cerevisiae strain GL7 (erg 12 heme 3), originally supplied by D. B. Sprinson (Columbia University) (Gollub et al., 1977), was stored at 4 °C on slants containing 2% dextrose, 0.67% yeast nitrogen base, 20 mg/L methionine, 50 μg/mL oleic acid, 1 μg/mL cholesterol, and 1.5% agar. Cultures were grown aerobically with shaking in the dark at 30 °C, and growth was monitored by measuring the absorbance at 540 nm. 6-Fluorocholesterol (mp 139.5–140 °C) was from DuPont Central Research, oleic acid, cholesterol, and ergosterol were from Sigma, 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Aldrich Chemical Co., and egg yolk lecithin was from Avanti. 6-Fluorocholesterol and cholesterol were recrystallized from methanol and ergosterol from ethanol/acetone.

Growth of GL7 on Various Sterols. GL7 from slants was inoculated into minimal medium containing 2% dextrose, 0.67% yeast nitrogen base, 20 mg/L methionine, 50 μg/mL oleic acid, and 1 μg/mL cholesterol. Oleic acid and sterols were added as solutions in Brij 58/ethanol, 1:4 (w/w), 100 μL/20 mL of medium. The cells were allowed to grow to an optical density of 0.5–1.0 and centrifuged, the supernatant was removed, and the pellet was washed twice with sterol-free minimal medium by resuspending and centrifugation. The cells were then dispersed in fresh medium. The number of viable cells per unit volume was determined in a hemocytometer after treating the cells with 0.1% methylene blue. The same number of cells (10^6) was inoculated into 20 mL of medium containing various sterols in desired concentrations and combinations. Cell growth was monitored by measuring the absorbance at 540 nm as a function of time.

Adaptation Experiments. GL7 from slants was grown on cholesterol, centrifuged, washed twice with sterol-free medium, and then inoculated into fresh medium containing 6-fluorocholesterol. Several serial transfers were made. During the first transfer from cholesterol to the 6-fluoro analogue, the cells grew poorly. After the third transfer, cells grew more rapidly, showing that GL7 was adapting to 6-fluorocholesterol. To test whether this adaptation was permanent, the adapted cells were centrifuged, washed twice with sterol-free medium, and reinoculated into cholesterol medium. Five such transfers were made. These cells, after centrifugation and washing, were reinoculated into 6-fluorocholesterol medium. The growth rate was now similar to that of the adapted cells, indicating permanent adaptation.

Extraction of Sterol from Cells. The procedures were as described (Buttke & Bloch, 1981). GL7 adapted to growth on 6-fluorocholesterol was centrifuged; the pellet was washed twice with distilled water and then lyophilized. The dry cells were saponified by refluxing in methanolic KOH (20% KOH in 90% MeOH at 70 °C for 1 h), and the sterol was extracted into petroleum ether. After solvent evaporation, the residue was analyzed for sterol composition by gas–liquid chromatography (Buttke & Bloch, 1981). The extracted sterol was homogeneous and identical with 6-fluorocholesterol.

Microviscosity Measurements. Vesicles for microviscosity measurements were prepared according to published procedures (Szoka & Papahadjopoulos, 1979). The desired amounts of sterol and egg lecithin (1.29 μmol) in hexane were mixed in a round-bottom flask, evaporated to dryness under reduced pressure, and then kept under argon. To the deposited lipid film were added 1 mL of NaCl (10 mM) and 3 mL of peroxide-free diethyl ether. The biphasic mixture was sonicated for 5 min in a Branson sonicator at 4 °C to give a homogeneous opalescent dispersion. Solvent was evaporated under reduced pressure at 25 °C. The vesicle preparation was filtered through a Sepharose 4B column. To 1 mL of the vesicle preparation was added 1 mL of a 1 M solution of the fluorescent probe DPH (Shinitzky & Inbar, 1974; Shinitzky & Barenholz, 1974) in 10 mM NaCl. The mixture was incubated at 37 °C for 30 min. Fluorescence depolarization of DPH was measured at 25 °C with an Elscint Model MV-1A microviscosimeter, and microviscosities were calculated according to Shinitzky and Inbar (1976). We are aware of reports (Chen et al., 1977; Dale et al., 1977; Kawata et al., 1979) questioning the validity of absolute microviscosity obtained from fluorescence depolarization measurements. In our present work, we report relative microviscosity values of membranes differing only in the structures of the sterol component. We consider these comparisons to be valid.

Control analyses to ascertain whether the sterol:phospholipid ratios in the vesicles used for fluorescence depolarization measurements were unchanged from the relative amounts of sterol and phospholipid in the mixture before sonication were not carried out. In previous work from this laboratory [e.g., see Dahl et al. (1980)], isolated liposomes prepared by the same technique with eight different sterols contained in all instances sterol and phospholipid in the same ratios as added initially before sonication.

Results and Discussion

In order to study the various functional roles sterols play in eukaryotic cells, we investigated the effectiveness of 6-fluorocholesterol in supporting growth of the yeast mutant GL7. For the three sterols used here, the order of effectiveness was ergosterol > cholesterol > 6-fluorocholesterol. It should be noted that all three sterols remain structurally unchanged during growth. The effects seen are therefore attributable to the sterols per se and not to potential metabolites.

After the first transfer of cholesterol-grown cells to a medium containing 6-fluorocholesterol, the yeast mutant GL7 grew poorly. On subsequent transfers to the fluorosterol medium, the growth rate improved, reaching a maximum after the third transfer (Figure 1). No further changes occurred after additional (up to eight) transfers. To test whether this adaptation is permanent, the adapted cells, after being washed with sterol-free medium, were reinoculated into cholesterol-containing medium and harvested, and these transfers were repeated 5 times. These cells, readapted to cholesterol medium, were washed with sterol-free minimal medium and returned to 6-fluorocholesterol. At this stage, the cells maintained the adapted growth rate (Figure 2). It is important to note that this apparently permanent adaptation does not involve structural modification of the sterol. After several transfers on 6-fluorocholesterol medium, the cells contain only 6-fluorocholesterol; no defluorination occurs (Figure 3). A similar adaptation has been reported for cholesterol (Taylor & Parks, 1980).

That growth of the yeast mutant GL7 is affected synergistically by adding a small amount of ergosterol to cholesterol medium (ergosterol/cholesterol = 1:3) has been reported previously (Ramgopal & Bloch, 1983). Likewise, when 6-fluorocholesterol (0.3 g/mL) is supplied as the bulk sterol to unadapted cells, and supplemented with as little as 0.025 μg/mL cholesterol, a synergistic (2–2.8-fold), i.e., more than additive, growth response is observed (Figure 4, bars 1 and 3). In quantities that small, cholesterol alone fails to support any growth of the mutant (Figure 4, bar 2). A similar but large (4.6–9.3-fold) synergistic response is seen when 6-

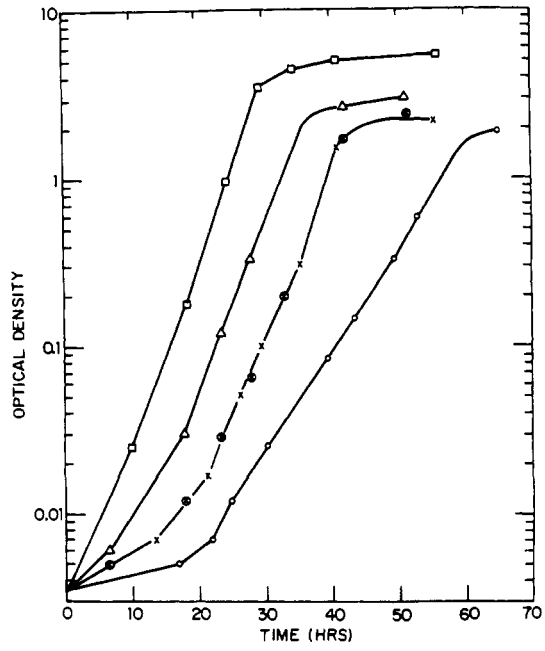

FIGURE 1: Growth rates of yeast mutant GL7 on media containing the following sterols (1 μg/mL): ergosterol (□); cholesterol (△); 6-fluorocholesterol, first transfer (○); 6-fluorocholesterol, third transfer (×); 6-fluorocholesterol, eighth transfer (⊗).

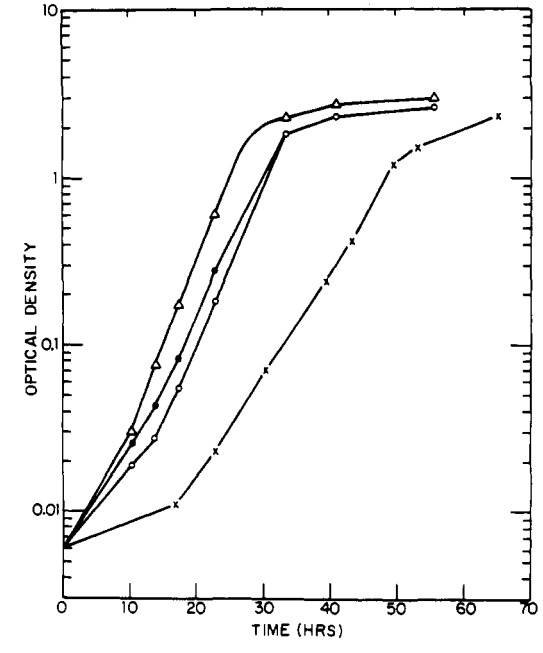

FIGURE 2: Adaptation of yeast mutant GL7 to growth on 6-fluorocholesterol (1 μg/mL); unadapted cells (×); adapted cells (three or more transfers) (○); adapted cells → cholesterol (five transfers) → 6-fluorocholesterol (●); cholesterol (1 μg/mL) (△).

fluorocholesterol (0.3 g/mL) is paired with $^1/_{12}$th the quantity of ergosterol (Figure 4, bars 1 and 5). Most notably, a double or superimposed synergistic growth response is achieved by supplementing 6-fluorocholesterol (0.3 g/mL) with $^1/_{12}$th the amount of *both* cholesterol and ergosterol (Figure 4, compare bar 7 with bars 3 and 5). It may be argued that the double-synergistic effect is simply due to the doubling of the amounts of synergistic sterols (0.025 μg/mL each of cholesterol and ergosterol) combined with 6-fluorocholesterol. The magnitude of the synergistic effect with a single sterol is in fact concentration dependent. However, calculations show that we are dealing with a second, superimposed synergism, not summation, of two individual synergistic effects. Thus, when

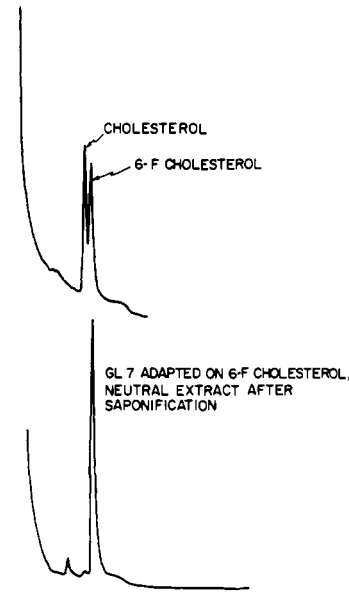

FIGURE 3: GLC trace of coinjected mixture of authentic sterols (cholesterol and 6-fluorocholesterol) (upper tracing) and of sterol isolated from the neutral extract after saponification of G17 cells adapted to growth on 6-fluorocholesterol (lower panel). Authentic 6-fluorocholesterol estimated to equal the amount of sterol in the unsaponifiable fraction of cell extract was added for coinjection. A peak corresponding to cholesterol was not seen in this chromatogram nor in GLC tracings when the unsaponifiable fraction for cells grown on 6-fluorocholesterol was injected as such (not shown).

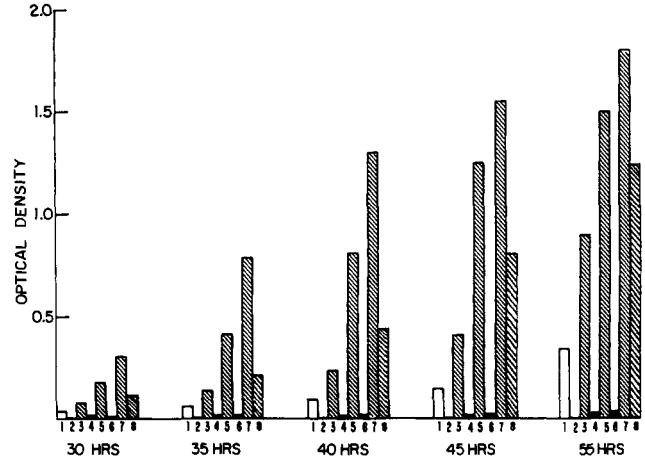

FIGURE 4: Growth of yeast mutant GL7 on single sterols and sterol mixtures. (Bar 1) 6-Fluorocholesterol, 0.3 μg/mL; (bar 2) cholesterol, 0.025 μg/mL; (bar 3) 6-fluorocholesterol, 0.3 μg/mL, + cholesterol, 0.025 μg/mL (1 + 2); (bar 4) ergosterol, 0.025 μg/mL; (bar 5) 6-fluorocholesterol, 0.3 μg/mL, + ergosterol, 0.025 μg/mL (1 + 4); (bar 6) cholesterol, 0.025 μg/mL, + ergosterol, 0.025 μg/mL (2 + 4); (bar 7) 6-fluorocholesterol, 0.3 μg/mL, + cholesterol, 0.025 μg/mL, + ergosterol, 0.025 μg/mL (1 + 6); (bar 8) 6-fluorocholesterol, 0.3 μg/mL, + cholesterol, 0.05 μg/mL, twice the amount of cholesterol used in the experiments shown in bars 2, 3, 6, and 7. Hatched bars show synergistic effects. Numbers in parentheses above refer to the numbered bars in the graph.

the amount of synergistic cholesterol is doubled from 0.025 to 0.050 μg/mL (bar 8), the increment is significantly smaller than that given by a supplement consisting of 0.025 μg/mL each of cholesterol and ergosterol (bar 7). It should also be noted that both the single- and double-synergistic effects are largest during the logarithmic phase of cell growth (Figure 4, 35–45 h). At any rate, we interpret these superimposed responses as reflections of at least three distinguishable roles for sterols in yeast. Rodriguez et al. have also observed the phenomenon of multiple sterol synergism in yeast mutants

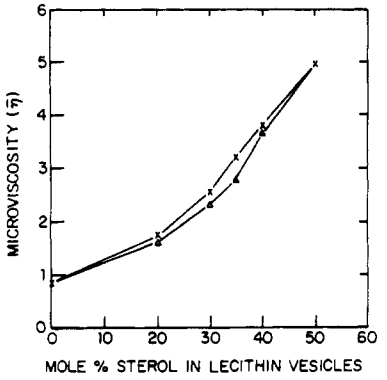

FIGURE 5: Microviscosity of egg lecithin vesicles containing increasing concentrations of cholesterol (△) or 6-fluorocholesterol (×).

(Rodriguez et al., 1985). In their studies, cholestanol served as the bulk sterol, a molecule that fails to support any growth of yeast sterol auxotrophs. By contrast, all the sterols tested in the present work satisfy this requirement by themselves albeit with varying efficiencies.

A likely candidate for one of the three sterol functions is a highly sterol-sensitive GL7 protein kinase which is activated by as little as 10^{-8} M ergosterol (Dahl et al., 1987). This laboratory has also reported stimulatory sterol effects on partial reactions of phospholipid biosynthesis, among them the methylation of phosphatidylethanolamine to phosphatidylcholine (Kawasaki et al., 1985), and third, Dahl and Dahl have shown sterol stimulation of phosphatidylinositol kinase (Dahl & Dahl, 1985). Stimulation of these phospholipid transformations seems to require higher sterol concentrations than the protein kinase but significantly lower ones than the sterol levels needed for the "bulk" function. On the assumption that the latter is physical, i.e., has to do with membrane stabilization, we estimate that of the total sterol the yeast auxotroph requires for optimal growth, at most 10–20% are involved in metabolic control processes and the remainder in maintaining the membrane physical state.

We do not imply or claim that the various sterols we have tested and which display the synergistic effect perform different physiological functions for the yeast cell *as long as they support some yeast growth* no matter how poorly. The differences are quantitative not qualitative. The very fact that the yeast mutant grows on single sterols and does so without metabolic alterations demonstrates that the yeast needs no more than one sterol structure for performing all of its essential functions.

When introduced into unilamellar egg lecithin liposomes, cholesterol and its 6-fluoro analogue exert essentially identical effects on acyl chain ordering in the phospholipid bilayer (Figure 5). Thus, the small fluorine atom, while highly electronegative, does not interfere with the bulk sterol function. The differences in biological effectiveness may therefore be attributed to direct sterol–protein interactions in physiological environments such as plasma membranes. Whether sterol–protein contacts occur only at selected positions of the ring system such as C_6 could be explored by placing the fluorine atom elsewhere into the sterol molecule.

Fluorosteroids are widely used as therapeutic agents. These are often superior to their natural counterparts but not necessarily because they are intrinsically more effective, i.e., at the molecular level. Some fluoro compounds are more slowly metabolized than the parent hormones (Friend & Chang, 1985; Hatano et al., 1980) and may therefore owe their superiority to different pharmacokinetics. According to other reports, fluorine introduction into steroids interferes with their binding to receptor proteins (Westpahl, 1971; Florini & Buyske, 1961). At any rate, the highly electronegative fluorine substituent can be envisioned to share hydrogen bonds with the various hydrogen donor groups at enzyme active sites or ligand binding sites or receptors.

Registry No. 6-Fluorocholesterol, 86361-68-4; cholesterol, 57-88-5; ergosterol, 57-87-4.

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