

# Coordinate regulation of unsaturated phospholipid, RNA, and protein synthesis in *Mycoplasma capricolum* by cholesterol

(sterol synergism/membranes/lanosterol/epicoprostanol/phosphatidylcholine)

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**ABSTRACT** The effect of cholesterol, epicoprostanol, and phosphatidylcholine on phospholipid, RNA, and protein synthesis was investigated in the sterol auxotroph *Mycoplasma capricolum*. Cells growing poorly on lanosterol were stimulated to grow more rapidly by supplementing the medium with either 2  $\mu\text{g}$  of cholesterol or 2.2  $\mu\text{g}$  of egg phosphatidylcholine per ml. In such cells cholesterol caused a sequential stimulation of phospholipid, RNA, and protein synthesis. Enhanced oleate incorporation into phospholipid occurred early; the rates of RNA and protein synthesis increased later. In cells supplemented with phosphatidylcholine only RNA and protein syntheses were enhanced. The addition of 2  $\mu\text{g}$  of epicoprostanol per ml to cells growing on lanosterol promptly inhibited the rate of unsaturated phospholipid synthesis and subsequently the rate of growth. Inhibition of both processes was relieved by supplying 2  $\mu\text{g}$  of cholesterol or 2.2  $\mu\text{g}$  of phosphatidylcholine per ml along with the inhibitory sterol. The results suggest that cholesterol in small amounts exerts a positive regulatory effect and epicoprostanol exerts a negative one on unsaturated phospholipid synthesis and, in turn, that RNA and protein synthesis are coordinately controlled with phospholipid synthesis. The previously reported phenomenon of sterol synergism and the postulated novel role of sterols in membranes [Dahl, J. S., Dahl, C. E. & Bloch, K. (1981) *J. Biol. Chem.* 256, 87-91] are here defined in terms of specific metabolic events.

Recent studies in this laboratory have furnished evidence in support of the view that the structure of cholesterol is optimally designed for modulating the properties of both artificial and natural membranes (1-3). In order of importance, cholesterol appears to owe its unique properties to the following structural features: planarity of the  $\alpha$  face in the region of C-14, absence of methyl substituents at C-4, and insertion of a 5,6 double bond.

Further work, notably the discovery of sterol synergism, has led to the postulate that cholesterol may serve in more than one role as a constituent of biological membranes (4). It was suggested that, apart from its well-established ability to modify the bulk physical state of the lipid bilayer (5), cholesterol may function more specifically in localized membrane domains influencing certain metabolic processes. Cholesterol availability was suggested to be coupled to the synthesis of phospholipid in *Mycoplasma capricolum*, a sterol- and fatty acid-requiring prokaryote. In essence, a control mechanism distinct from that affecting the bulk physical state of the lipid bilayer was proposed (6), prompted by the following observations. A low level of cholesterol (0.5  $\mu\text{g}/\text{ml}$ ), insufficient to support significant growth, elicits a 2-fold stimulation of the *Mycoplasma* growth rate when combined with lanosterol (10  $\mu\text{g}/\text{ml}$ ). Alone the latter sterol supports growth only poorly. The response is synergistic, not

additive (4). A significant result was that the membranes of cells grown on lanosterol with low cholesterol show the same low microviscosity value as membranes isolated from cells cultured on lanosterol alone (4, 6). In experiments designed to elucidate the basis of the synergistic sterol effect, we have obtained evidence that cholesterol specifically stimulates the uptake of unsaturated fatty acids by *M. capricolum*. The presence of a small quantity of cholesterol in lanosterol-rich cells lowers the  $K_m$  value for the uptake of unsaturated but not saturated fatty acids (6).

Here we show that (i) the addition of a small amount of cholesterol to the medium of cells grown on lanosterol to midlogarithmic phase elicits a sequential stimulation, first of unsaturated phospholipid synthesis, followed by the synthesis of RNA and protein; (ii) substitution of cholesterol by epicoprostanol results in an inhibition of all three processes, which can be overcome by cholesterol; and (iii) phosphatidylcholine causes the same increase in RNA and protein synthesis as cholesterol causes but, unlike cholesterol, has no effect on fatty acid utilization for endogenous phospholipid synthesis.

## MATERIALS AND METHODS

**Organism and Growth Conditions.** *M. capricolum* (California kid strain 14, ATCC 27342) was cultured statically at 37°C on lipid-depleted modified Edward media (7) supplemented with delipidated bovine serum albumin (8), sterols, and fatty acids as described under *Results*. Delipidated albumin was lyophilized prior to use in order to remove organic solvent. Sterols and fatty acids were premixed in ethanol, when added simultaneously, before addition to otherwise complete media. The ethanol content of the media never exceeded 0.6%.

**Phosphatidylcholine Liposome Preparation.** Egg phosphatidylcholine (3 mg) dried from hexane/ethanol, 9:1 (vol/vol), was sonicated under a stream of nitrogen in ice-cold 0.01 M Tris-HCl, pH 7.9/0.15 M NaCl for 10 min with a Branson sonifier (W-350) equipped with a microtip. Liposomes were sterilized by passage through a 0.45- $\mu\text{m}$  Millipore filter. Phosphate was determined by the method of Chen *et al.* (9), and the liposomes were diluted to a concentration of 2 mM.

**Fatty Acid Incorporation into Phospholipid by Growing Cells.** Carrier-free [ $^{14}\text{C}$ ]palmitate or [ $^{14}\text{C}$ ]oleate was added to growing cultures in midlogarithmic phase. At different times, 0.5-ml portions of the culture were mixed with 2 ml of chloroform/methanol, 2:1 (vol/vol). After reextraction with 1 ml of chloroform, the extracts were applied to channeled silica gel thin-layer plates (Si 250-PA, J. T. Baker) and developed in chloroform/methanol/acetone/acetic acid/water, 120:20:40:20:10 (vol/vol). Lipids were located by exposure to iodine. In this solvent system, the phospholipids of *M. capricolum* separated into three spots that cochromatograph with diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylcholine, respectively. The phospholipid cochromatographing with phosphati-

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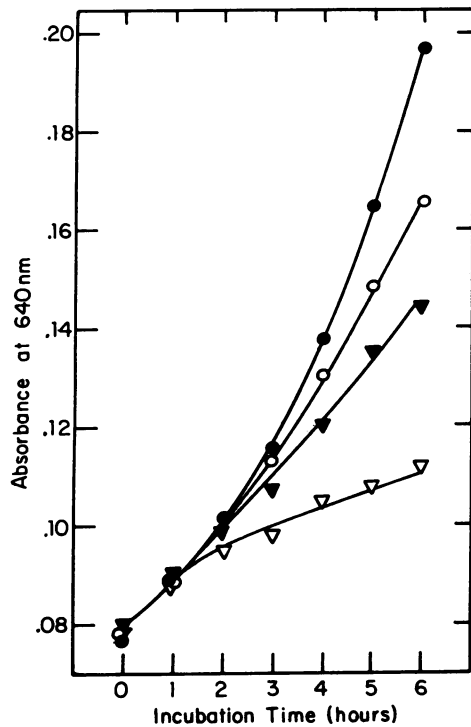


FIG. 1. Effect of supplemental sterol on growth of *M. capricolum*. A culture grown for 20 hr on a lipid-depleted medium containing 10  $\mu\text{g}$  of lanosterol, 5  $\mu\text{g}$  of palmitate, 6.5  $\mu\text{g}$  of oleate, and 0.05% delipidated bovine serum albumin per ml was divided and supplemented at 0 hr with one of the following sterols at 2  $\mu\text{g}/\text{ml}$ : cholesterol (●), lanosterol (control) (○), epicoprostanol (▽), or epicoprostanol and 2  $\mu\text{g}$  of cholesterol (▼). Growth was measured by increases in absorbance at 640 nm.

dylcholine is present in cells grown in the absence of added phosphatidylcholine and has not yet been identified. Silica gel fractions corresponding to the three phospholipids were transferred to scintillation vials containing Scint-A (Packard), and radioactivity was determined.

**Measurement of RNA and Protein Synthesis in Growing Cells.** Carrier-free [ $^3\text{H}$ ]uridine or [ $^{14}\text{C}$ ]labeled amino acids were added to growing cultures in midlogarithmic phase. At different times, 0.5-ml portions of the culture were mixed with 0.5 ml of 10% chilled trichloroacetic acid in an ice bath. After 15 min, the precipitates were pelleted in a clinical centrifuge and the supernatant fluids were discarded. The pellets were resuspended in 5 ml of 5% ice-cold trichloroacetic acid, collected on glass fiber filters (GF-B, Whatman), and washed once with 5 ml of 5% ice-cold trichloroacetic acid, followed by 1 ml of methanol. The filters were transferred to scintillation vials, 10 ml of scintillation fluid was added, and radioactivity was determined.

**Protein Determination.** Protein was determined by the method of Lowry *et al.* (10) with bovine serum albumin as the standard.

**Chemicals.** Cholesterol was recrystallized from ethanol and dried *in vacuo* before use, and lanosterol was purified by the method of Bloch and Urech (11). Epicoprostanol (5 $\beta$ -cholestan-3 $\alpha$ -ol) was purchased from Steraloids (Wilton, N.H.), and egg phosphatidylcholine, from Avanti Biochemicals. [ $1\text{-}^{14}\text{C}$ ]Palmitic acid, [ $1\text{-}^{14}\text{C}$ ]oleic acid, [ $1,2\text{-}^3\text{H}$ ]cholesterol, [ $5,6\text{-}^3\text{H}$ ]uridine, and a uniformly [ $^{14}\text{C}$ ]labeled L-amino acid mixture was purchased from New England Nuclear. Bovine serum albumin (fraction V) was purchased from Sigma.

## RESULTS

A small amount of cholesterol along with nonlimiting lanosterol exerts a synergistic effect on the growth of *M. capricolum* (4). Under these conditions, cholesterol, the minor membrane sterol, appears to be regulating specifically the uptake of unsaturated fatty acids into phospholipid (6). For further study of the synergistic effect, we have chosen to interrupt the growth of cells raised on 10  $\mu\text{g}$  of lanosterol per ml at the midlogarithmic phase. At this stage (zero time), the culture medium was supplemented with either a small amount of cholesterol or icoprostanol, a sterol known to inhibit the growth of cholesterol-requiring mycoplasmas (12, 13). The addition of 2  $\mu\text{g}$  of cholesterol per ml to a lanosterol culture at midlogarithmic phase increased the growth rate of cells about 1.5-fold during a 6-hr period (Fig. 1). By contrast, the same amount of epicoprostanol retarded the rate of growth to less than half that of the control cells. The growth inhibition by epicoprostanol was relieved by supplying 2  $\mu\text{g}$  of cholesterol per ml along with the inhibitory sterol.

Changes in biosynthetic rates accompanying the stimulation of growth were examined next. As for palmitate incorporation into phospholipid, the rate was the same whether cholesterol or lanosterol was the supplemental sterol added to cells in midlogarithmic growth (Fig. 2). On the other hand, cholesterol significantly and promptly stimulated oleate incorporation into phospholipid compared to control cells that received at this point either additional lanosterol (Fig. 2) or no sterol (data not shown). In keeping with the increased growth rate measured by absorbance, the rates of both RNA and protein synthesis

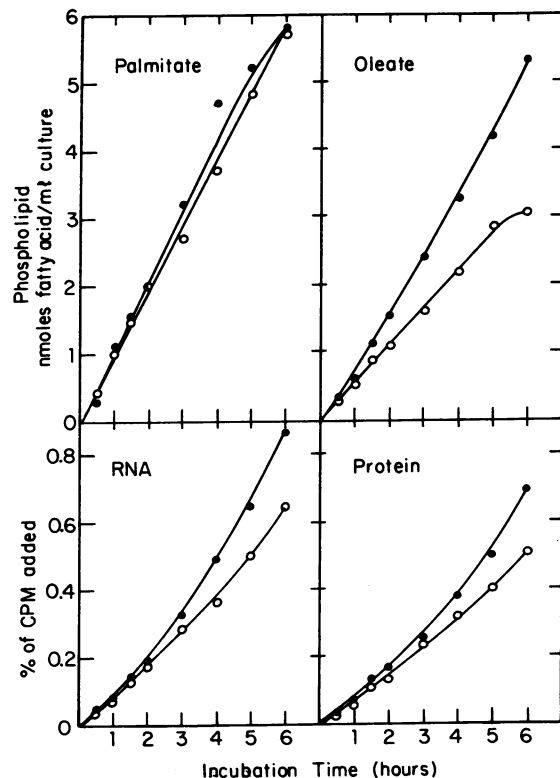


FIG. 2. Effect of cholesterol supplementation on phospholipid, RNA, and protein synthesis. A culture grown for 20 hr as described in Fig. 1 was divided and supplemented at 0 hr with either 2  $\mu\text{g}$  of cholesterol (●) or 2  $\mu\text{g}$  of lanosterol (control) (○) per ml along with 0.01  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]palmitate, 0.01  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]oleate, 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]uridine, or 1  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]labeled L-amino acids per ml. Samples were taken at the indicated times and assayed for radioactivity incorporated into total phospholipid, RNA, and protein.

were also increased by cholesterol supplementation but only after a 2- to 3-hr lag (Fig. 2).

Epicoprostanol decreased the growth rate of cells raised on lanosterol, and this inhibition and its relief by cholesterol were reflected also in the rates of phospholipid and macromolecular synthesis (Fig. 3). Palmitate incorporation into phospholipid was insensitive to epicoprostanol or to epicoprostanol plus cholesterol. On the other hand, oleate utilization for phospholipid synthesis was severely inhibited in cells receiving epicoprostanol alone but was normal in cells receiving cholesterol along with epicoprostanol. Also in keeping with the growth data, RNA and protein synthesis were markedly retarded in cells supplemented with epicoprostanol alone. Again cholesterol relieved the inhibitory effect of epicoprostanol (Fig. 3).

In order to assess more accurately the time sequence of effects on the various biosynthetic processes after cholesterol stimulation or epicoprostanol inhibition, cells were pulse-labeled with either [ $^{14}$ C]oleate, [ $^3$ H]uridine, or  $^{14}$ C-labeled L-amino acids, 1 hr prior to sterol supplementation and again at various time intervals during the following 4 hr (Fig. 4). Clearly, unsaturated phospholipid synthesis was already enhanced at the earliest time point taken (i.e., within 15 min after cholesterol addition). In the same cells, increases in RNA and protein syntheses occurred much later. They became marked only 1–2 hr after cholesterol stimulation. In cells treated with epicoprostanol, the sequence of effects (in this case inhibition of synthesis) was again phospholipid, followed by RNA and protein; however, under these conditions, RNA and protein synthesis were inhibited already within 1 hr after epicoprostanol addition.

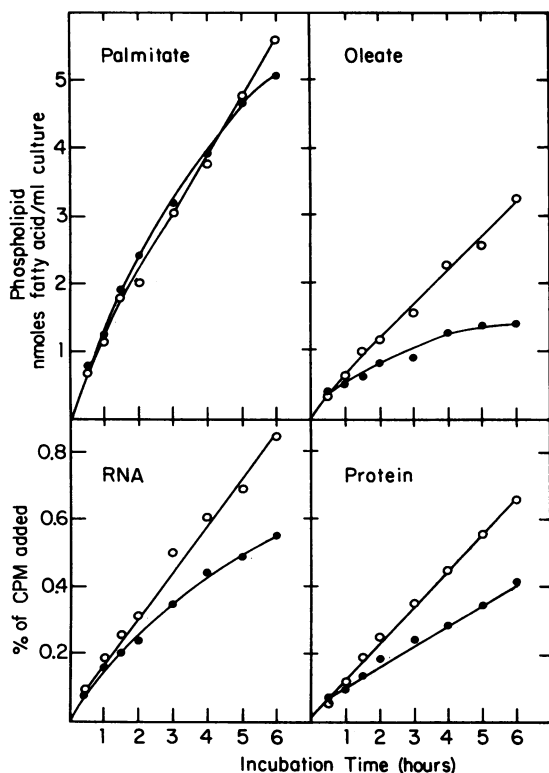


FIG. 3. Effect of epicoprostanol or epicoprostanol/cholesterol supplementation on phospholipid, RNA, and protein synthesis. A culture grown for 20 hr as described in Fig. 1 was divided and supplemented at 0 hr with either 2  $\mu$ g of epicoprostanol per ml (●) or 2  $\mu$ g of epicoprostanol and 2  $\mu$ g of cholesterol per ml (○) along with 0.01  $\mu$ Ci of [ $^{14}$ C]palmitate, 0.01  $\mu$ Ci of [ $^{14}$ C]oleate, 1  $\mu$ Ci of [ $^3$ H]uridine, or 1  $\mu$ Ci of  $^{14}$ C-labeled L-amino acids per ml. Samples were taken at the times indicated and assayed for radioactivity incorporated into total phospholipid, RNA, and protein.

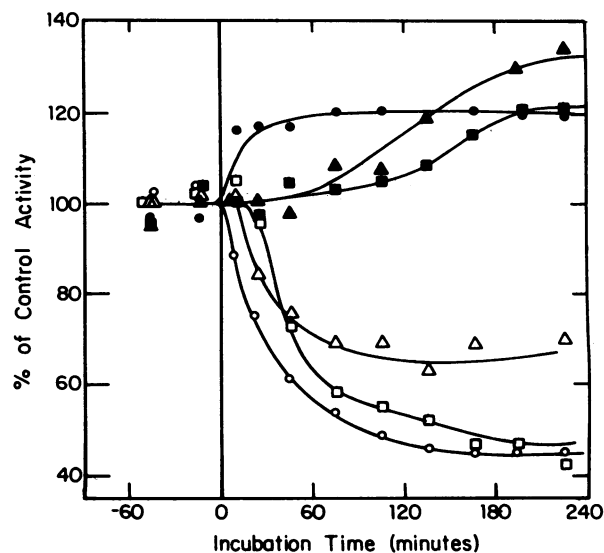


FIG. 4. Specific rates of oleate incorporation into phospholipids and of RNA and protein synthesis after cholesterol stimulation or epicoprostanol inhibition. A culture grown for 20 hr as described in Fig. 1 was divided and pulse-labeled by adding 0.1  $\mu$ Ci of [ $^{14}$ C]oleate, 1  $\mu$ Ci of [ $^3$ H]uridine, or 1  $\mu$ Ci of  $^{14}$ C-labeled L-amino acids per ml for two 30-min periods. At 0 min, cultures were supplemented with either 2  $\mu$ g of cholesterol (●, ■, ▲) or 2  $\mu$ g of epicoprostanol (○, □, △) per ml and were further pulsed at 0–15 min, 15–30 min, and at 30-min periods thereafter up to 4 hr. [ $^{14}$ C]oleate incorporation into total phospholipid (●, ○), [ $^3$ H]uridine incorporation into RNA (▲, △), and  $^{14}$ C-labeled L-amino acid incorporation into protein (■, □) were determined. Specific rates were calculated as [ $^{14}$ C]oleate (nmol),  $^{14}$ C-labeled amino acids (cpm), or [ $^3$ H]uridine (cpm) incorporated per min per  $A_{640}$ .

Prompted by our finding that an early manifestation of cholesterol supplementation is the stimulation of unsaturated phospholipid synthesis and by the report of Razin *et al.* (14) that *M. capricolum* readily takes up exogenous phospholipid, we chose to investigate the effect of supplementing midlogarithmic-phase cells growing on lanosterol with a small amount of egg phosphatidylcholine (Fig. 5). Indeed, phosphatidylcholine had a stimulatory effect on growth comparable to that shown by cholesterol. Furthermore, egg phosphatidylcholine like cholesterol counteracted the adverse effect of epicoprostanol on growth (see Fig. 1). Similarly, phosphatidylcholine had no effect on [ $^{14}$ C]palmitate incorporation into phospholipid (Fig. 6). However, the responses of cells to cholesterol and phosphatidylcholine differed crucially in one respect. Unlike cholesterol, phospholipid did not stimulate [ $^{14}$ C]oleate incorporation (Fig. 6). RNA and protein synthesis, the other processes activated by cholesterol in cells growing on lanosterol, were stimulated also by phosphatidylcholine.

## DISCUSSION

As previously observed and defined, the phenomenon of sterol synergism refers to the ability of cholesterol, in small amounts, to raise the growth rate of *Mycoplasma* growing poorly on lanosterol by a factor that is greater than additive (4). From the observations that disclosed the synergistic effect, it seemed likely that cholesterol controls some rate-limiting metabolic event, tentatively identified as the biosynthesis of unsaturated membrane phospholipids (6). Thus, our earlier work led to the postulate of a dual control function for sterols in membranes, one involving modulation of a physical parameter and the other involving a metabolic, presumably enzyme-catalyzed process. For further definition of the metabolic, cholesterol-specific membrane function, we designed experiments suitable for

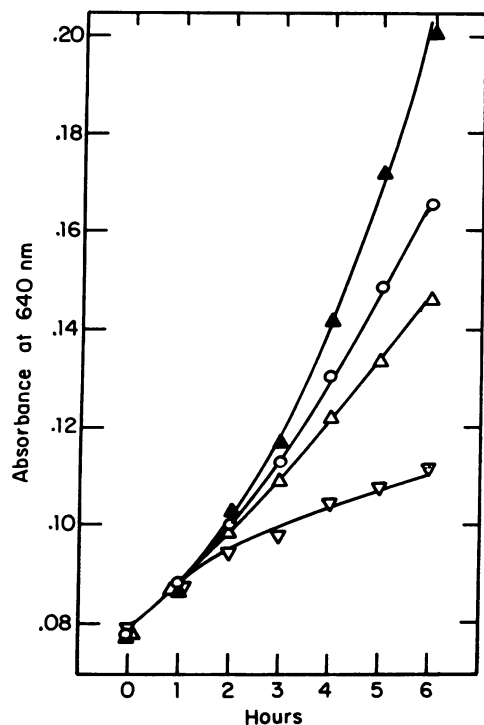


FIG. 5. Effect of phosphatidylcholine supplementation on growth of *M. capricolum*. A culture grown for 20 hr as described in Fig. 1 was divided and supplemented at 0 hr with 2.2  $\mu\text{g}$  of egg phosphatidylcholine per ml ( $\Delta$ ), no additional lipid (control) ( $\circ$ ), 2  $\mu\text{g}$  of epicoprostanol per ml ( $\nabla$ ), or 2  $\mu\text{g}$  of epicoprostanol and 2.2  $\mu\text{g}$  of egg phosphatidylcholine per ml ( $\Delta$ ). Growth was measured by increases in absorbance at 640 nm.

monitoring sterol-induced changes in rates of phospholipid, RNA, and protein synthesis *in vivo*. For this purpose cells were first cultured on medium containing lanosterol as the sterol source and then supplemented with cholesterol at the midlogarithmic phase of cell growth. Under these conditions there occurs stimulation of phospholipid, RNA, and protein synthesis. Enhancement of oleate incorporation into phospholipid is seen first, whereas the rates of RNA and protein synthesis increase subsequently. Most notably, under similar conditions but with phosphatidylcholine supplementation, phosphatidylcholine duplicates the stimulatory effects of cholesterol on growth and on the synthesis of RNA and protein, but it does not accelerate oleate uptake into phospholipids. This is a reasonable result because the external provision of phospholipid should obviate the need for the agent that stimulates endogenous phospholipid synthesis.

In similarly designed experiments, we examined the effect of epicoprostanol, a sterol with a nonplanar ring system (A/B *cis*). Epicoprostanol, which inhibits *Mycoplasma* growth (12, 13), was considered a likely antagonist of the various cholesterol-promoted processes. When cells cultured on a medium containing lanosterol were exposed to a small amount of epicoprostanol, a sequential effect on growth and the various biosynthetic processes was observed except that, in this case, all responses were negative. Oleate incorporation into phospholipid declined first, followed by a decreased rate of macromolecular synthesis (Figs. 3 and 4). Because the epicoprostanol-promoted inhibition of all processes was alleviated by cholesterol, the two sterols appear to compete for regulatory sites in the cell.

Several important questions arise from the data presented here. First, how does cholesterol stimulate the utilization of

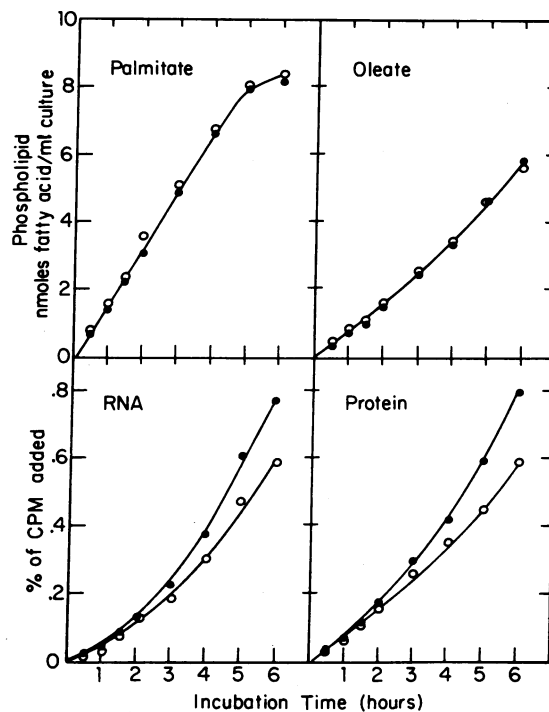


FIG. 6. Effect of phosphatidylcholine supplementation on phospholipid, RNA, and protein synthesis. A culture grown for 20 hr as described in Fig. 1 was divided and supplemented at 0 hr with either 2.2  $\mu\text{g}$  of egg phosphatidylcholine per ml ( $\bullet$ ) or no additional lipid (control) ( $\circ$ ) along with 0.01  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]palmitate, 0.01  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]oleate, 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]uridine, or 1  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]labeled L-amino acids per ml. Samples were taken at the times indicated and assayed for radioactivity incorporated into total phospholipid, RNA, and protein.

unsaturated fatty acids for phospholipid synthesis? Because *M. capricolum* is a natural fatty acid auxotroph, all of its fatty acid for phospholipid synthesis is of external origin. Thus, the biosynthetic process involves a set of reactions including entry from the external space, activation, and esterification of fatty acids. Together these steps constitute fatty acid "uptake." The specific enzymes in *Mycoplasma* have not yet been identified. Therefore, we cannot as yet identify the cholesterol-stimulated step in oleate utilization. However, certain modes of action can be ruled out. We have suggested previously that the synergistic growth response caused by a small amount of cholesterol supplied to lanosterol-rich cells is not due to changes in the bulk physical state of the membrane. This proposal stems from two observations. Membranes from cells raised on lanosterol alone and those from synergistically grown cells have the same low microviscosity value (4, 6). Moreover, 3 $\alpha$ -methylcholesterol, a sterol incompetent to order the acyl chains in phospholipid vesicles (3) or to raise the microviscosity of membranes from *M. capricolum* grown on this sterol (15), nevertheless supports good growth of *M. capricolum* (16). 3 $\alpha$ -methylcholesterol also promotes a synergistic growth response when supplied along with lanosterol (6). Presumably epicoprostanol, like 3 $\alpha$ -methylcholesterol, is unsuited structurally to order the acyl chains of phospholipids; both have bulky substituents at the 3 $\alpha$  (axial) position (17). Therefore, the opposite effect of these two sterols on growth and growth-related processes must be unrelated to the way they affect the bulk physical state of the membrane. Some other more-specific interactions between the sterol molecule and some membrane component, either lipid or protein, must come into play. This more-specific sterol interaction appears to be involved in some way in the synergistic phenomenon (e.g., the role of cholesterol in regulating membrane-associated

metabolic processes). Principal structural features common to cholesterol and 3 $\alpha$ -methylcholesterol but not shared by epicoprostanol are an equatorial hydroxyl group and *trans* A/B ring fusion, which renders the sterol nucleus planar.

Our results also raise the issue of whether unsaturated phospholipid synthesis is rate-limiting or mandatory for RNA and protein synthesis. The fact that egg phosphatidylcholine entering the cells from the growth medium fully replaced the low level of cholesterol otherwise needed for stimulating cultures growing on lanosterol suggests that the relationship between unsaturated phospholipid and enhanced macromolecular synthesis may be mandatory, not fortuitous. (It should be emphasized that phosphatidylcholine cannot replace sterol in the original medium; a bulk sterol requirement persists regardless of the phospholipid content of the medium.) In support of this contention is the related observation that changing from low to high external fatty acid concentrations increased unsaturated phospholipid synthesis and, in turn, the rate of cell growth (unpublished data).

Evidence for a coordinate regulation of lipid and macromolecular synthesis in other organisms (e.g., *Escherichia coli*) has existed for some time. The experiments of Goldberg *et al.* (18) with the fatty acid synthesis inhibitor cerulenin and of McIntyre *et al.* (19) with an *E. coli* glycerol auxotroph strongly suggest an obligatory coupling between phospholipid and macromolecular synthesis, the former preceding the latter. What distinguishes the mechanism controlling these processes in *E. coli* and *M. capricolum* respectively, is that in *Mycoplasma* the participation of cholesterol as an initiator is essential. Therefore, it follows that in cholesterol-requiring cells, cholesterol may play a fundamental role in membrane biogenesis and cell growth.

There is also evidence for a regulatory role of cholesterol in animal cells. Thus, a sequential onset of the synthesis of cholesterol and DNA after mitogen stimulation of lymphocytes has been observed (20); with the aid of 25-hydroxycholesterol, the sequential inhibition or onset of the synthesis of cholesterol,

phospholipid, RNA, DNA, and protein has been demonstrated in myoblasts (21).

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