

Altered Phospholipid Metabolism in a Sodium-Sensitive Mutant of *Escherichia coli*¹

JOAN E. LUSK² AND EUGENE P. KENNEDY

Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

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A mutant of *Escherichia coli* has been isolated, the growth of which is inhibited by low concentrations (1 mM) of NaCl. High levels of magnesium, calcium, or strontium in the medium permit growth in the presence of sodium. The metal content of the inhibited mutant is normal, but the strain is unable to tolerate levels of sodium to which the wild type is indifferent. Immediately after the addition of sodium to cultures of the mutant, rates of synthesis of protein, ribonucleic acid, deoxyribonucleic acid, and total lipid are unchanged, but more cardiolipin and less phosphatidylethanolamine are produced. The direct enzymatic cause of this change, which affects membrane function, is not known. Studies of the metabolism of phosphatidylglycerol in vivo after pulse-labeling with [2-³H]glycerol reveal that a major pathway both in wild-type and mutant strains involves the cleavage of labeled glycerol from phosphatidylglycerol.

In a previous communication (26), we reported the isolation of two mutants of *Escherichia coli* that depend on high concentrations of magnesium for growth. We expected such strains to be deficient in the transport of magnesium. The finding that relatively high concentrations of other divalent cations (Ca²⁺ or Sr²⁺) also supported growth of the mutants suggested that magnesium transport might not be directly affected. This is now known to be the case. Instead, one of these mutants (A324-1) is inhibited by sodium ions and the inhibition relieved by magnesium, calcium, or strontium. The second mutant (A324-2) requires high concentrations of divalent cations when grown on amino acids but not when the carbon source is glucose, galactose, lactose, or glycerol. Sodium or other constituents of the medium used during selection of strain A324-2 do not prevent growth in low magnesium on these latter carbon sources. The two strains thus differ markedly in their properties, although both were selected as requiring high levels of magnesium. No further work has been done with strain A324-2.

Salts such as NaCl and CaCl₂, which have

little or no effect on the wild type, can elicit dramatic responses in a mutant, suggesting that ion sensitivity may be a useful means of selecting conditional-lethal strains. In addition, the report by Silver and Kralovic (39) that calcium is not taken up by cells of *E. coli* suggests that calcium can affect only those proteins external to the permeability barrier. Thus, sensitivity to calcium may be used in selecting mutants having altered membrane-bound or periplasmic proteins.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain A324 (F⁻, thi⁻, pro⁻, i⁻z⁺y⁺a⁺, str^r) was obtained from S. E. Luria. Selection of the mutants A324-1 and A324-2 derived from A324 has been described (26). Cells were grown at 37 C with shaking, and growth was followed by measuring turbidity at 650 nm in a Coleman Junior spectrophotometer. A culture density of 4 × 10⁸ cells/ml corresponds to 100 arbitrary units.

Media. Medium I [tris(hydroxymethyl)amino-methane (Tris)-buffered Casamino Acids] has been previously described (26). Other media were derived from medium I by replacing the Casamino Acids with other sources of carbon and nitrogen. For growth on amino acids without the inorganic impurities of commercial casein hydrolysates, reagent grade L-alanine, L-aspartate, and L-glutamate (Tris-salts, each 0.2%, pH 7.4) and L-proline (25 μg/ml) replaced the Casamino Acids. In some experiments, a mixture of 13 additional amino acids (each at 10 to 60 μg/ml) was added to simulate medium I. The bac-

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² Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139.

teria were also grown on glycerol (20 mM) or glucose (10 mM), $(\text{NH}_4)_2\text{SO}_4$ (15 mM), and L-proline (25 $\mu\text{g}/\text{ml}$). Fatty acids were added as aqueous solutions of the potassium salts or by 1:100 dilution of 1% solutions of the free acids in 80% ethanol and solubilized by the addition of 0.1% Tween 20 or Tween 40.

Analytical methods. For measurement of sodium, potassium, and calcium, bacteria were harvested by centrifugation, washed with 0.4 M sucrose, and ashed at 550 C in platinum dishes. The ash was dissolved in 12 N HNO_3 and diluted for flame spectrometry of sodium and potassium. A sample of Casamino Acids was ashed and analyzed in the same manner. Calcium was measured by atomic absorption spectroscopy in samples of the ash dissolved in water plus an equal volume of a solution 1 N in HCl, 0.8 N in HClO_4 , and saturated with oxine. For measurement of magnesium by atomic absorption, bacteria were washed with 50 mM Tris-hydrochloride, pH 7.4, as previously described (26).

Protein was measured by the method of Lowry et al. (24) and ribonucleic acid (RNA) by the orcinol method as used previously (26).

Entry and efflux of ^{28}Mg were measured by membrane (Millipore Corp.) filtration as previously described (25). Efflux of ^{28}Mg , which occurs by exchange with ^{24}Mg added to the medium, was measured in cells that had been labeled with ^{28}Mg during several generations of growth in media containing the isotope. Influx was measured at 28 C, efflux at 37 C. Control curves were obtained both before and after those with sodium or calcium, a difference of 24 min (influx) or 8 min (efflux).

The assay system for *o*-nitrophenyl- β -galactoside (ONPG) hydrolysis (final volume 3.0 ml) contained 1 ml of a culture of strain A324-1 (or 0.2 ml of the same culture after 1 min of agitation with toluene), 3 mM ONPG, and, in control tubes, 10 mM 1-thio- β -D-galactosyl galactose (TDG), which blocks the permease-mediated entry of ONPG but not the enzymatic hydrolysis of ONPG by β -galactosidase. The reagents were warmed to 37 C before the cells were added. After 5 min of incubation at 37 C, the reaction was stopped by the addition of 1 M K_2CO_3 (5 ml). The absorbance of *o*-nitrophenol was determined at 420 nm in a Coleman Junior spectrophotometer.

Phosphatidylserine decarboxylase was assayed in membrane fractions sedimented at $40,000 \times g$ for 1 hr and dialyzed against 50 mM Tris-hydrochloride (pH 7.4), with 10 mM mercaptoethanol, by the method of Kanfer and Kennedy (19). The reaction mixture contained 0.2 M sodium phosphate buffer (pH 7.4), 0.2% Triton X-100, and 0.2 mM phosphatidyl [$1\text{-}^{14}\text{C}$]serine. After incubation for 1 hr at 37 C, the reaction was stopped by the addition of H_2SO_4 , and the $^{14}\text{CO}_2$ was collected on filter paper saturated with 1 M KOH.

The standard assay system for cytidine-5'-diphosphate (CDP)-diglyceride: L-serine phosphatidyl transferase contained: 0.1 M Tris-hydrochloride (pH 7.4), 0.1% Triton X-100, 0.5 mM CDP-dipalmitin, 1 mM L-[$3\text{-}^{14}\text{C}$]serine or 2 mM DL-[$3\text{-}^3\text{H}$]serine at a specific activity of 10^6 counts per min per μmole , and enzyme in a total volume of 0.3 ml (19). Many

variations were made in this assay, including the addition of NaCl or KCl (0.1 or 0.01 M), ethylenediaminetetraacetic acid (EDTA), cytidine monophosphate (CMP), phosphate buffer (pH 6.5), Tris buffer (pH 8.5), and lower concentrations of both substrates and detergent.

Fractionation of cells. For extraction of lipids, cells were concentrated by centrifugation at $10,000 \times g$ for 10 min in the cold or treated with cold 5% (w/v) trichloroacetic acid. The precipitate was collected by centrifugation at $2,000 \times g$ for 20 min in the cold, or, if small enough volumes were to be extracted, treated directly with 5 volumes of methanol and 10 volumes of chloroform. Cell pellets or trichloroacetic acid precipitates were suspended in 1 ml of water, and 5 ml of methanol was added, followed by 10 ml of chloroform. The lipid extracts were washed three times with 2 M MgCl_2 and once with water. A measured volume of the chloroform phase and 10 mg of Triton X-100, placed in a counting vial, were evaporated to dryness in a stream of air. The residue was then suspended in 1 ml of water plus 10 ml of Patterson-Greene scintillation fluid (30), and the radioactivity in the lipids was determined in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc.).

For determination of ^{32}P in lipids, deoxyribonucleic acid (DNA), and RNA (Fig. 4), cells labeled with $^{32}\text{P}_i$ were precipitated with 5% trichloroacetic acid in the cold, carrier *E. coli* sonic extract was added, and the samples were centrifuged at $6,000 \times g$ for 20 min. The acid precipitate was suspended in a mixture of 1 ml of water, 5 ml of methanol, and 5 ml of chloroform, and the samples were centrifuged at $10,000 \times g$ for 10 min. After removal of lipids in the chloroform phase, the residue was suspended in 1 ml of 0.5 M KOH and incubated at 37 C overnight to hydrolyze the RNA. The samples were chilled, 1 ml of 1 M HClO_4 was added, and the samples were centrifuged to sediment the alkali-resistant DNA. A portion of the supernatant solution containing hydrolyzed RNA was counted. The pellet was redissolved in 1 N KOH, neutralized with HCl, and reprecipitated with 12% trichloroacetic acid. The pellets were washed once more with 5% trichloroacetic acid, taken up in 1 ml of water, and counted in 10 ml of Patterson-Greene scintillation fluid. Samples of the lipid extract were evaporated and counted as above.

For fractionation into lipids, nonlipid residue, and soluble substances (Fig. 8-9), samples of a culture were centrifuged at $10,000 \times g$ for 10 min. The cells were resuspended in 1 ml of water and extracted with 5 ml of methanol plus 5 ml of chloroform. The lipid-free residue was removed by centrifugation at $10,000 \times g$ for 10 min. The lipid extract plus 5 ml of chloroform was washed once with 10 ml of water. The radioactivity in the insoluble residue (suspended in 1 ml of water) and in samples of the medium, the lipid extract, and the water wash was determined.

The phospholipids were analyzed after hydrolysis by a modification (5) of the method of Dawson (10). A portion of the lipid extract was evaporated to dryness and taken up in 2.5 ml of chloroform-methanol (1:1). NaOH (0.05 ml, 4 M) was added. After 10 to 15 min at room temperature, 1 ml of water and 250 mg

of Dowex 50 x-1 (H⁺ form) were added. The upper aqueous phase was removed, and the chloroform layer was washed with 1 ml of water. The combined water phases were neutralized to a phenol red end point with 0.15 M NH₄OH and dried under a stream of air. The deacylated lipids were then separated by descending chromatography on Whatman paper no. 43 in *i*-propanol-20 mM aqueous EDTA (sodium salt, pH 7)-concentrated NH₄OH (7:2:1, v/v/v). The deacylated cardiolipin had an *R_f* of 0.34, phosphatidylethanolamine, 0.46, and phosphatidylglycerol, 0.59 in this system. The chromatogram was cut into 1-cm fractions, and each fraction was counted in 1 ml of water and 10 ml of Patterson-Greene fluid.

Identification of cardiolipin. Lipids of strain A324-1 labeled from [2-³H]glycerol in the presence or absence of sodium were separated by column chromatography on DE32 (41). The major peak from the sodium-inhibited culture was chromatographed with beef-heart cardiolipin (Sylvania) on DE32 and upon thin-layer chromatography on silicic-acid plates (Eastman Kodak Co.). Its water-soluble hydrolysis product migrated with that from authentic cardiolipin upon paper chromatography in three solvent systems.

Gas-liquid chromatography of methyl esters of fatty acids. The lipids were extracted and hydrolyzed with 20% KOH in 80% methanol. After acidification with 6 N H₂SO₄, the fatty acids were extracted with three portions of ether, dried over Na₂SO₄, and esterified with diazomethane. Diazomethane was prepared from *N*-methyl-*N*-nitrosos-*p*-toluene sulfonamide by the method of Lipsky and Landowne (23) and stored in the freezer until use. The completeness of the hydrolysis and the esterification were checked by thin-layer chromatography on silicic acid plates developed in hexane-ether-glacial acetic acid (95:5:0.1, v/v/v).

The methyl esters were separated in a gas chromatograph (Research Specialties Co.) on a column of 11% polyethyleneglycol succinate on chromasorb W at 182 C at an argon pressure of 10 psi. The relative amounts of the fatty acid esters were assumed to be proportional to the areas of the peaks recorded by the argon ionization detector, and were measured by tracing the peaks and weighing the paper replicas. Radioactive methyl esters in the effluent were collected in glass U-tubes in a dry-ice-acetone bath. The fractions were washed into scintillation vials with three 1-ml portions of ether. After the ether had been evaporated, 10 ml of Patterson-Greene fluid was added, and the radioactivity in the samples were determined. Collection efficiency ranged from 20 to 60% of the injected radioactivity and was assumed to be equal for the various esters within each sample.

Radioactive materials. ²⁸Mg was purchased from Brookhaven National Laboratory, Upton, Long Island, N. Y. [2-³H]glycerol was purchased from New England Nuclear Corp., Boston, Mass., and purified by chromatography on silicic acid according to Chang and Kennedy (5). DL-[3-³H]serine was from International Chemical and Nuclear Corp. Waltham, Mass. Phosphatidyl [1-¹⁴C]serine was prepared enzymatically from DL-[1-¹⁴C]serine (New England Nuclear Corp.) in this laboratory (19). Sodium[1-

¹⁴C]acetate and ³²P_i were also purchased from New England Nuclear Corp. The sodium acetate was diluted with ammonium acetate for use so that the added sodium was reduced to 2 μM in the culture of strain A324-1, too low a concentration to inhibit growth.

Fatty acids. Palmitoleic acid was from Mann Fine Chemicals. All other fatty acids, products of the Hormel Institute, were gifts from C. Plate.

RESULTS

Inhibition of growth of strain A324-1 by sodium. During the selection of strain A324-1 for magnesium dependence, 3 mM sodium was always present, since the Casamino Acids (Difco) used in medium I contain 14% NaCl. Under these conditions, relatively high levels of magnesium are required to sustain growth. In media free from added sodium, the mutant requires no more magnesium than does wild type and can similarly deplete the medium of magnesium to levels below 1 μM (*data not shown*).

When cells of strain A324-1 were grown in a medium containing 0.1 mM magnesium, the addition of NaCl to a final concentration of 10 mM prevented growth (Fig. 1). In the presence of higher levels of magnesium (10 mM), the addition of sodium had no effect (Fig. 1). Lithium did not inhibit growth as completely as did sodium, nor did magnesium completely prevent the inhibition by lithium. Potassium, rubidium, cesium, and ammonium ions (10 mM) did not affect growth.

Metal content of strains A324 and A324-1. The effects of sodium, calcium, and magnesium on strain A324-1 must result from an increased sensitivity to normal amounts of these ions rather than from an altered cellular metal content (Table 1). Although 3 mM sodium inhibits growth of strain A324-1 and not A324, there is little difference in metal content between the two strains. Calcium is bound to the same extent by both strains and reduces the sodium content of both to the same extent; it does not spare the requirement of either strain for magnesium for growth (*data not shown*). Thus, the fact that calcium allows strain A324-1 to grow in the presence of sodium is not a consequence of dramatic changes in the binding of sodium, calcium, or magnesium in the mutant as compared to the parental strain. The potassium content of strain A324-1 with or without calcium was 670 atoms/mg, comparable to the wild-type level (26).

Transport of magnesium. Transport of magnesium in strain A324, measured by using the radioactive isotope ²⁸Mg, has been described (25). The flux of ²⁸Mg either into or

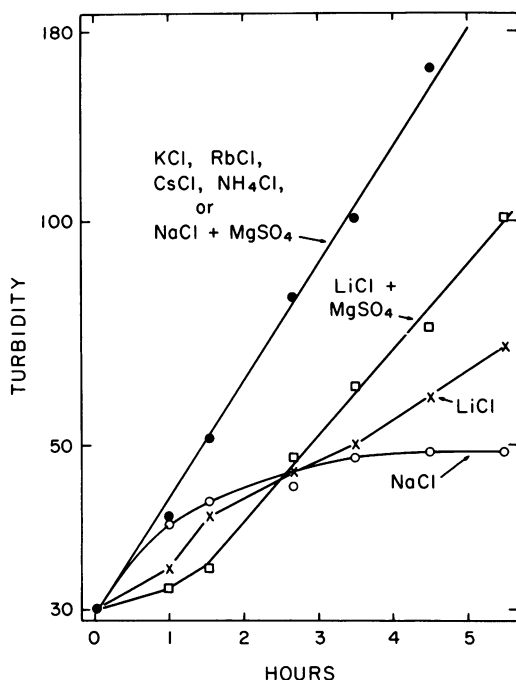


FIG. 1. Effect of monovalent cations on the growth of strain A324-1. To cultures growing on reagent grade amino acids in medium containing 0.1 mM magnesium, the following salts were added at a final concentration of 10 mM: O, NaCl; ×, LiCl; ●, KCl, RbCl, CsCl, NH₄Cl, or both NaCl and MgSO₄; and □, LiCl and MgSO₄. Turbidity was measured in a Coleman Junior spectrophotometer and is expressed in arbitrary units. A turbidity of 100 units corresponds to about 4×10^8 cells/ml.

out of strain A324-1 was as rapid as that of A324. Sodium did not inhibit the ²⁸Mg transport, although, at the same concentrations of sodium and magnesium, growth of A324-1 is inhibited. Calcium also had no effect on the flux of ²⁸Mg. These direct measurements (Fig. 2) confirm the suspicion aroused by the discovery that A324-1 does not truly require high concentrations of magnesium: the mutation does not affect magnesium transport.

Physiology during sodium inhibition. The physiological changes in cells of strain A324-1 after transfer from high to low magnesium in medium I (3 mM sodium) were examined in the hope of discovering why growth stops (Fig. 3). After transfer to low magnesium, growth as measured by increase in turbidity or protein continued at the same rate as in 10 mM magnesium for 30 min. Net RNA synthesis continued at the exponential rate nearly as long as protein synthesis. Neither protein nor RNA syntheses, therefore, seem likely to be directly affected by sodium in A324-1.

Net uptake of magnesium continued, but at a slower rate than was required to sustain steady-state exponential growth. The ratio of magnesium to protein or RNA, therefore, began to fall after transfer of the cells to low magnesium. After growth stopped, magnesium was lost from the cells and the amount of RNA decreased. The ratio of cellular magnesium to RNA was roughly constant during this period.

Similar increases in protein and total cellular magnesium occurred when NaCl (10 mM) was added to strain A324-1 growing in reagent-grade amino acids with 0.03 mM magnesium. Viable cell number increased and then remained constant for several hours of inhibition before declining. The inhibition of growth in either medium may be reversed by the addition of magnesium or calcium, exponential growth resuming at the normal rate after a lag time of 1 to 2 hr.

Incorporation of ³²P_i. The incorporation of ³²P_i into DNA, RNA, and phospholipids of strain A324-1 in the presence of inhibitory concentrations of sodium is shown in Fig. 4. Consistent with the results of Fig. 3, synthesis of all three components proceeded at the same rate for 30 min with or without sodium. Analysis of the phospholipid fractions, however, revealed gross differences between the cells given sodium and the controls (Fig. 5). The sodium-treated cells incorporated much more ³²P into cardiolipin and much less into phosphatidylethanolamine. Similar results were obtained by using [2-³H]glycerol as the radioactive precursor of phospholipids; the labeling of cardiolipin was up to five times greater in sodium-treated cells, and the labeling of phosphatidylethanolamine was reduced compared to that of control cells without sodium or with excess magnesium.

The increased incorporation of phosphate or glycerol into cardiolipin reflects a net increase in the amount of this lipid and not merely increased turnover. In contrast, more rapid incorporation of precursors into phosphatidylglycerol (Fig. 5) does not lead to an increase in the proportion of this lipid. The increased turnover of phosphatidylglycerol (Fig. 9) prevents its accumulation.

The relative amounts of the phospholipids were measured by growing the bacteria on ³²P_i for several generations, so that cellular phosphorus compounds attained equal specific activity, and the ³²P in each phospholipid served as a chemical measure of that lipid. During the exponential growth phase in the wild type, or in the mutant A324-1 without sodium, 70% of the total lipid phosphorus was in phosphatidylethanolamine, 20% in phosphatidylglycerol,

TABLE 1. Metal content of strains A324 and A324-1

Strain ^a	Phase of growth	Metal ions initially present in medium (mM)			Metals in cells (natoms/mg of protein)		
		Na	Ca	Mg	Na	Ca	Mg
A324	Exponential	3	—	0.1	130	4.5	ND
	Exponential	3	1	0.1	91	52	ND
	Exponential	3	—	0.02	ND ^b	ND	155 ± 20 ^c
A324-1	Stationary (24 hr)	3	—	0.02	ND	ND	79 ± 14 ^c
	Na-inhibited (2 hr)	3	—	0.1	130	4.6	100 ± 14
	Exponential	3	1	0.1	67	38	ND
	Exponential	—	—	0.03	ND	ND	140
	Stationary (24 hr)	—	—	0.03	ND	ND	100

^a The bacteria were harvested at about 5×10^8 cells/ml and analyzed as described in Materials and Methods.

^b Not determined.

^c Reference 25.

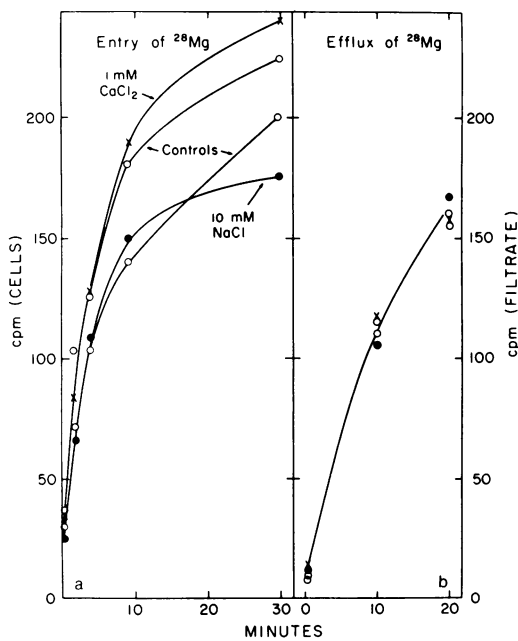


FIG. 2. Magnesium transport by strain A324-1. Strain A324-1 was grown on reagent grade amino acids and glycerol with $0.01 \text{ mM } ^{24}\text{Mg}$ (a) or ^{26}Mg (b). The flux of ^{26}Mg was measured in medium lacking amino acids. For measuring influx (a), $7.25 \text{ } \mu\text{M } ^{26}\text{Mg}$ was added at zero time, and samples of the cells were removed by membrane filtration. For measuring efflux (b), $0.1 \text{ mM } ^{24}\text{Mg}$ was added at zero time, samples were filtered, and the radioactivity in the filtrates was determined. Symbols: ●, 10 mM NaCl ; ×, 1 mM CaCl_2 ; ○, controls assayed before and after other curves.

and 5 to 10% in cardiolipin. In the sodium-inhibited mutant, the corresponding proportions became 43, 9 and 48%. If growth of either strain is limited by magnesium starvation, the pattern of phospholipids characteristic of ex-

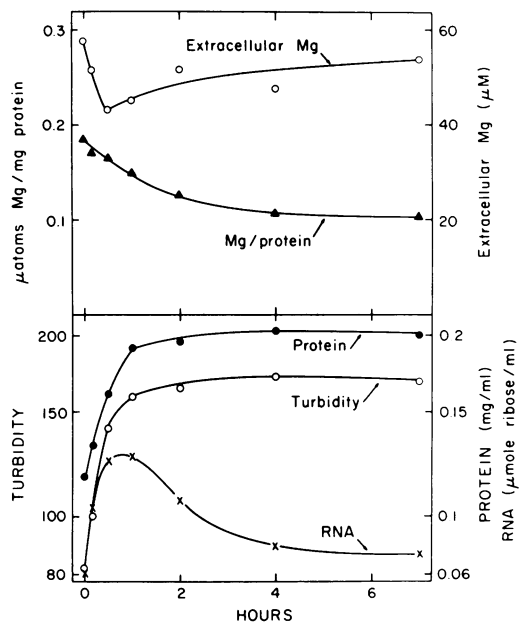


FIG. 3. Physiological changes in strain A324-1 during sodium inhibition. Exponentially growing cells in medium I containing 10 mM Mg and 3 mM Na were centrifuged and resuspended at zero time in medium I containing no added Mg and 3 mM Na .

ponential phase persists for at least 1 hr; thus the accumulation of cardiolipin in strain A324-1 during inhibition by sodium is not simply a manifestation of the conversion of phosphatidylglycerol to cardiolipin that normally occurs later in stationary phase (9). Calcium and magnesium, which allow A324-1 to grow at normal rates in the presence of sodium, also restore the wild-type pattern of phospholipids.

Protein synthesis is not required for the effect of sodium on lipid synthesis. The addition of chloramphenicol with inhibitory con-

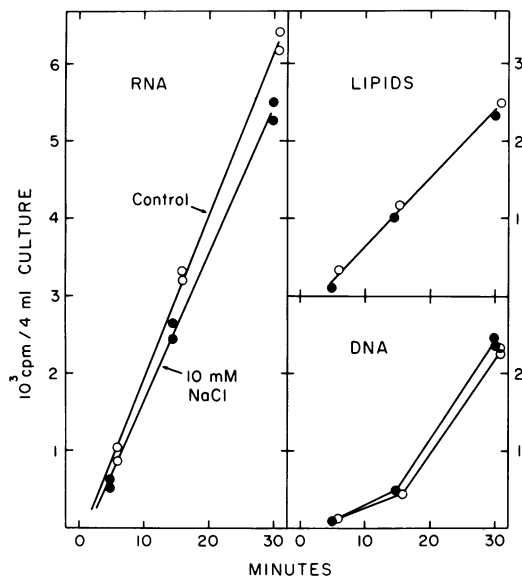


FIG. 4. Incorporation of $^{32}\text{P}_i$ into RNA, DNA, and lipids of strain A324-1. The medium contained reagent-grade amino acids and 0.1 mM Mg.

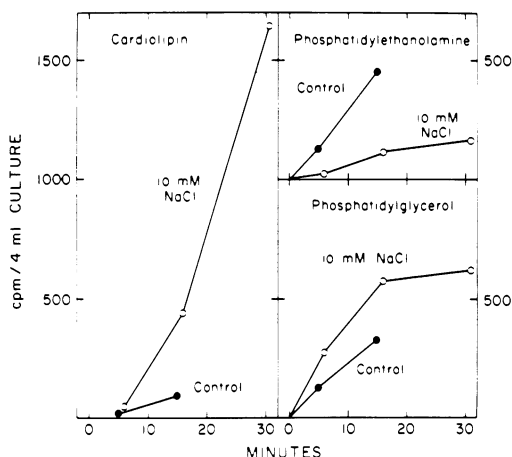


FIG. 5. Distribution of ^{32}P in the lipid fraction of strain A324-1, shown in Fig. 4. Symbols: O, 10 mM NaCl; ●, no NaCl.

centrations of NaCl did not prevent the typical increased synthesis of cardiolipin and decreased synthesis of phosphatidylethanolamine. Therefore, protein synthesis in the presence of sodium is not required for the effect of sodium on phospholipid synthesis in strain A324-1. A sodium-sensitive component must be present in cells of this mutant when grown without sodium.

Altered phospholipid metabolism in strain A324-1. If we assume the existence of a sodium-sensitive enzyme in strain A324-1, there

are several possibilities consistent with the observed changes in phospholipid composition during sodium inhibition. (See Fig. 6 for a summary of phospholipid metabolism in *E. coli*.) Synthesis of CDP-diglyceride cannot be inhibited because total incorporation of ^{32}P and glycerol into lipid is not inhibited. Rather, an inordinately large proportion of the CDP-diglyceride is converted to cardiolipin instead of phosphatidylethanolamine. Sodium might inhibit phosphatidylethanolamine synthesis directly, diverting CDP-diglyceride to an increased synthesis of phosphatidylglycerol and cardiolipin. Alternatively, synthesis of phosphatidylglycerol and its conversion to cardiolipin might be stimulated by sodium, leaving less CDP-diglyceride to form phosphatidylethanolamine. A third possibility is that conversion of cardiolipin to other products could be inhibited by sodium, causing excess cardiolipin to remain in the membrane. Inhibition of synthesis of phosphatidylethanolamine would then have to occur through some unknown control mechanisms.

Synthesis of phosphatidylethanolamine.

There are only two enzymes specific to the synthesis of phosphatidylethanolamine: CDP-diglyceride:L-serine phosphatidyl transferase (phosphatidylserine synthetase) and phosphatidylserine decarboxylase. The decarboxylase is unlikely to be the defective enzyme in A324-1 because phosphatidylserine is not observed to be a major lipid in the sodium-inhibited mutant. Strain A324-1, with or without sodium, did not incorporate radioactivity from [$1\text{-}^{14}\text{C}$]serine into lipids under conditions which gave good incorporation of [$3\text{-}^{14}\text{C}$]serine

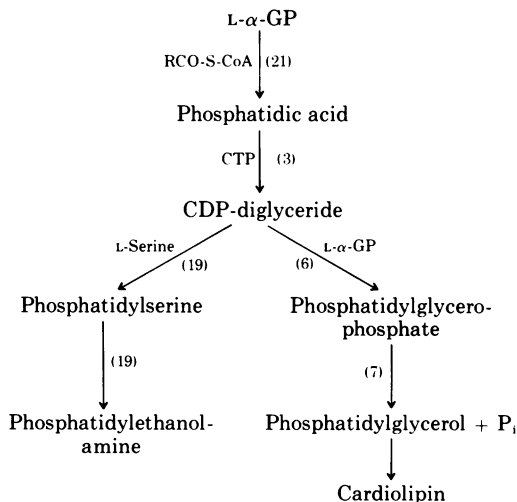


FIG. 6. Biosynthesis of phospholipids in *Escherichia coli*. L- α -GP, L- α -glycerophosphate. Numbers in parentheses indicate references.

into the ethanolamine moiety of phosphatidylethanolamine. Furthermore, the decarboxylase was fully active in the $40,000 \times g$ membrane fraction of a sonic extract of A324-1. The specific activity of the enzyme (in sodium phosphate buffer) was $0.58 \mu\text{moles}$ of $^{14}\text{CO}_2$ released per mg of protein per min by a dialyzed membrane preparation of strain A324, and 0.69 by a similar preparation from A324-1. Any direct inhibition of synthesis of phosphatidylethanolamine therefore must be mediated by an inhibition of the synthesis of phosphatidylserine.

Synthesis of phosphatidylserine. The synthesis of phosphatidylserine was measured in cell-free extracts of the mutant and wild type under a variety of conditions. Many attempts were made to demonstrate a difference between phosphatidylserine synthetase from strain A324-1 and the enzyme from A324. However, the enzyme from either strain had the same specific activity in both the $40,000 \times g$ supernatant fluid and the pellet prepared or assayed with or without sodium (0.01 or 0.1 M); at low serine (0.05 mM) or CDP-dipalmitin (0.1 mM) concentration; with or without CMP (1 mM), a product of the reaction, which might inhibit; at 10-fold lower detergent concentration; at various ionic strengths; at pH 6.5 or 8.5 ; and at temperatures up to 59 C . The synthetase was not displaced into the medium from intact cells of strain A324-1 in the presence of sodium. Kinetically and in intracellular localization, the mutant and parental strains have indistinguishable phosphatidylserine synthetases.

Synthesis of phosphatidylserine in intact cells could be impaired if sodium inhibits the accumulation or retention of serine. The activity of phosphatidylserine synthetase is likely to depend strongly on the intracellular serine concentration, which is normally 0.08 mM (32), well below the K_m of 0.8 mM of the synthetase (19). The lower K_m of the serine-activating enzyme, 0.03 to 0.07 mM (20), would insure that the supply of serine does not limit the overall rate of protein synthesis.

No evidence of starvation for serine during sodium inhibition was found. L-Serine (10 mM) in glucose minimal medium did not overcome the inhibition of growth of strain A324-1 by 10 mM NaCl. Incorporation of [$3\text{-}^3\text{H}$]serine into acid-precipitable, nonlipid material was undiminished in the same cells in which sodium severely inhibited its incorporation into lipid (*data not shown*); thus, serine must enter the cell. In a direct demonstration of the entry of serine, the uptake of [$1\text{-}^{14}\text{C}$]serine ($8 \mu\text{M}$) during a 2-min incubation period at 37 C in

the presence of chloramphenicol (32) was normal in the mutant and was stimulated by sodium in both the mutant and wild type. Retention of endogenous serine therefore is unlikely to be inhibited.

The second substrate of phosphatidylserine synthetase, CDP-diglyceride, must be available to the cells during sodium inhibition because the rate of synthesis of total phospholipid is unaltered. In the absence of detectable changes in enzymes or supply of substrates, it appears unlikely that sodium directly inhibits synthesis of phosphatidylethanolamine. Direct effects of sodium on the synthesis or degradation of cardiolipin were therefore examined.

Metabolism of phosphatidylglycerol and cardiolipin. The substrates of the enzymes catalyzing either the synthesis or degradation of cardiolipin are phospholipids. Unfortunately, the low solubility of phospholipids in water and their action as detergents preclude adding them to intact bacteria in order to investigate their metabolism. As an alternate way of adding labeled substrate, a pulse of [$2\text{-}^3\text{H}$]glycerol was used to label the bacterial lipids *in situ*, followed by unlabeled glycerol. Tritium from [$2\text{-}^3\text{H}$]glycerol, because it is removed in the oxidation of α -glycerophosphate, does not appear to any large extent in cellular material other than the glycerol moieties of lipids. Thus, it provides a much more specific label than ^{32}P for following the turnover of phospholipids. Because turnover of the glycerol moiety has not been studied, the process in the wild type was examined first.

The radioactivity in strain A324 after a pulse of [$2\text{-}^3\text{H}$]glycerol was almost entirely in the lipid fraction (Fig. 7). During the chase with unlabeled glycerol, one-third of the tritium disappeared from the lipid fraction and appeared in the medium. No radioactivity entered the lipid-free residue or the water-soluble cellular fraction. The lower half of Fig. 7 shows the distribution of tritium in the lipids during the chase. Radioactivity disappeared from phosphatidylglycerol, whereas the radioactivity in phosphatidylethanolamine and cardiolipin remained relatively constant.

The tritium appearing in the medium was nonvolatile and dialyzable. Most (80 to 90%) of the radioactivity was not adsorbed to a column of Dowex-1 formate, suggesting that it was not in a negatively charged compound (44). Treatment of the nonadsorbed radioactive material with an excess of adenosine triphosphate (ATP), 10 mM MgSO_4 , and glycerokinase (Boehringer) quantitatively converted it to a form that was eluted from a second Dowex column with carrier α -glycerophosphate. These

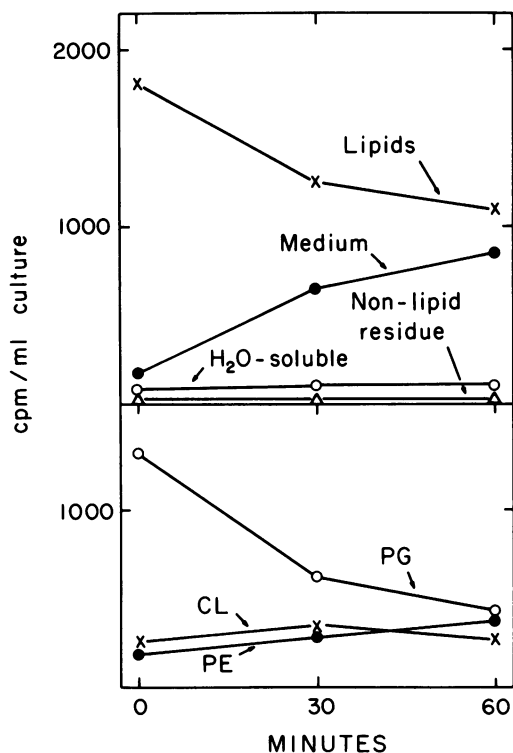


FIG. 7. Disappearance of tritium from cells of strain A324 pulse-labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ and distribution of tritium in the lipid fraction. A culture of strain A324 in medium I was labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ (0.04 mM), 3.8×10^8 counts per min (cpm) per μmole , for 1.5 min at 37 C. Unlabeled glycerol (20 mM) was then added, and the cells were centrifuged and suspended at 37 C in medium I containing 0.1 mM Mg with 20 mM unlabeled glycerol. Samples of the culture were fractionated as described. PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

properties suggest that the product of turnover of the tritiated lipids that is released into the medium is tritiated glycerol. Glycerol was also released from the cells when 20 mM α -glycerophosphate was present during the chase, suggesting that glycerol is the first water-soluble product derived from phosphatidylglycerol.

Lipid turnover in the mutant A324-1 was examined in a similar experiment (Fig. 8). During the chase with unlabeled glycerol, one-half of the culture received 10 mM NaCl and 0.1 mM MgSO_4 ; the control half of the culture received 10 mM MgSO_4 . The mutant differed from its parent in that cardiolipin accumulated as phosphatidylglycerol disappeared. In strain A324, all the radioactivity disappearing from phosphatidylglycerol was recovered in the

medium, whereas in strain A324-1 about half appeared in the medium and half in cardiolipin. Sodium increased the rate of accumulation of tritium in cardiolipin twofold and also slightly increased the rate of its appearance in the medium.

Figure 9 shows the loss of label from phosphatidylglycerol in one of several experiments in which cells were pulse-labeled with $[2\text{-}^3\text{H}]\text{glycerol}$. The rate of disappearance of the label from phosphatidylglycerol in the mutant was much faster when sodium was added. Although the direct hydrolysis of phosphatidylglycerol cannot be excluded, the results are consistent with the hypothesis that its conversion to cardiolipin is increased.

Fatty acids of strain A324-1. Although the amount of CDP-diglyceride synthesized by A324-1 must be unaffected by sodium, it is possible that the distribution of fatty acids is altered. De Siervo reported that the major phospholipids of *E. coli* B have markedly different fatty acids (11), in conflict with earlier results of Kanemasa et al. (17). If the fatty

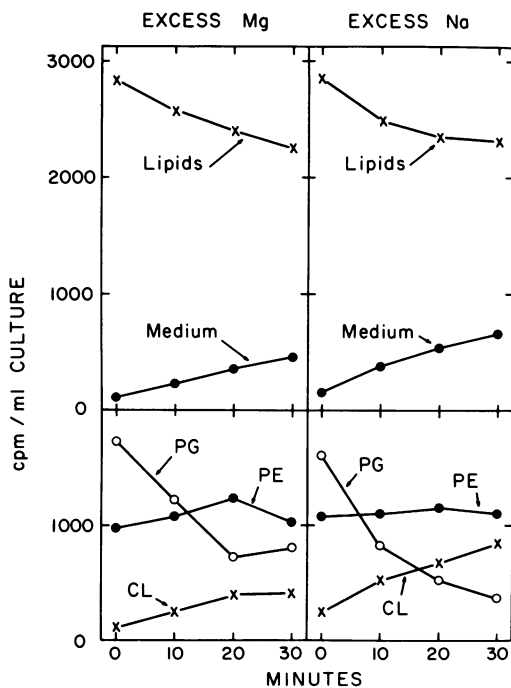


FIG. 8. Disappearance of tritium from cells of strain A324-1 pulse-labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ and distribution of tritium in the lipid fraction. The cells in medium I with 10 mM Mg were labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ for 2 min at 37 C, centrifuged, and suspended in two portions of medium containing 20 mM unlabeled glycerol and either 10 mM Mg or 0.1 mM Mg and 10 mM Na. Samples were treated as in Fig. 7.

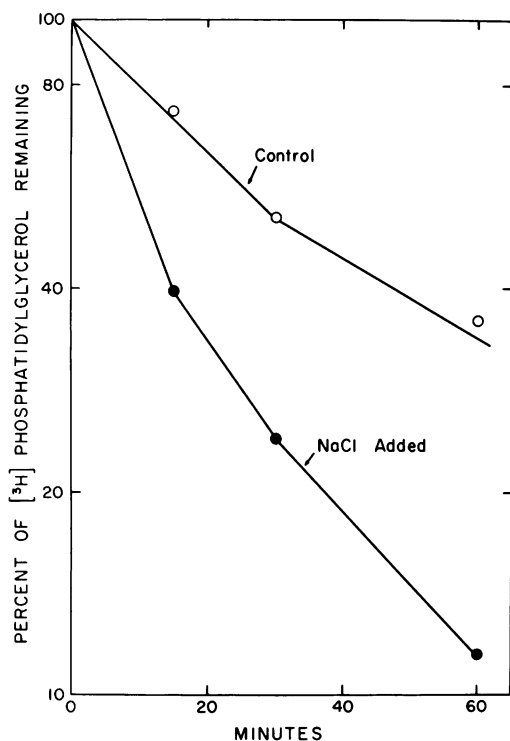


FIG. 9. Effect of sodium on loss of label from phosphatidylglycerol labeled with $[2\text{-}^3\text{H}]\text{glycerol}$ in strain A324-1.

acids of different phospholipids do differ, the enzymes synthesizing phosphatidylserine and phosphatidylglycerophosphate must discriminate among CDP-diglycerides having different fatty acids. By such discrimination or by more indirect control mechanisms, a change in fatty acid pattern might cause a change in the proportions of the phospholipids. Support for this contention may be found in the work of Henning et al. (16), who observed that, during starvation of an auxotroph of unsaturated fatty acids, synthesis of cardiolipin was stimulated and synthesis of phosphatidylethanolamine and phosphatidylglycerol was reduced.

Table 2 compares the principal fatty acids of strain A324-1 during exponential growth and after inhibition by sodium. Sodium consistently brought about a decrease in the proportion of *cis*-vaccenate ($\Delta 11$ 18:1). The amounts of palmitate (16:0), palmitoleate ($\Delta 9$ 16:1), and other minor fatty acid components were not greatly altered. A more sensitive method of examining the synthesis of fatty acids during sodium inhibition was to measure the incorporation of $[^{14}\text{C}]\text{acetate}$ into each fatty

acid. Acetate was converted to *cis*-vaccenate even in the presence sodium. Thus, although the rate of synthesis of *cis*-vaccenate was reduced relative to other fatty acids, sodium did not entirely block its formation.

The known unsaturated fatty acid auxotrophs differ from sodium-inhibited A324-1 in being unable to synthesize any unsaturated fatty acids (38). Strain A324-1 appears somewhat deficient in synthesizing *cis*-vaccenate in the presence of sodium, but synthesis of palmitoleate is hardly affected. Since the auxotrophs grow on palmitoleate without any *cis*-vaccenate in their lipids, neither a 50% decrease in synthesis of *cis*-vaccenate nor the change in overall proportions of unsaturated fatty acids can account for stasis in strain A324-1. These comprise at least 39% of the total fatty acids in sodium-inhibited cells, whereas, in fatty acid auxotrophs starved of unsaturated fatty acids, they make up only 18% of the total fatty acids in phosphatidylethanolamine (37). Furthermore, comparison of the fatty acid composition of A324-1 during sodium inhibition in two similar media (Table 2) reveals that, in medium I, sodium-inhibited cells have as much *cis*-vaccenate or total unsaturated fatty acid as do exponential cells grown on reagent-grade amino acids.

In spite of the differences between strain A324-1 and known fatty acid auxotrophs, the growth of A324-1 in the presence of sodium was affected by unsaturated fatty acids in the medium. Oleic acid (*cis* $\Delta 9$ 18:1) and *cis*-vaccenic acid at 100 $\mu\text{g}/\text{ml}$ allowed growth of the mutant at nearly normal rates in the presence of sodium. The mutant given oleic acid instead of high magnesium had a normal phospholipid pattern. Palmitoleic, linoleic, and linolenic acids, however, did not overcome inhibition by sodium, although they support the growth of fatty acid auxotrophs. It seems unlikely that the 18-carbon monounsaturated fatty acids could be specifically required by strain A324-1 and not by unsaturated fatty acid auxotrophs. Sodium inhibition of A324-1 was also overcome by oleate or *cis*-vaccenate in glucose minimal medium, which should have considerably repressed the uptake and incorporation of these fatty acids (22). Possibly they exert some physical effect on the membrane, unrelated to their incorporation into phospholipids (14).

Altered membrane function. The altered phospholipid and fatty acid composition of sodium-treated cells of strain A324-1 might be expected to alter membrane function. The lactose transport system was chosen for initial investigations of the membrane because of the

TABLE 2. Fatty acid composition of A324-1

Medium and phase of growth	Mg (mM)	Na (mM)	Total fatty acids (%)			Specific activity relative to 16:0	
			16:0	16:1	18:1	16:1	18:1
Reagent grade amino acids							
Exponential	0.1	—	43.4	34.4	14.2	1.2	1.46
Inhibited ^a	0.1	10	52.2	32.0	7.2	0.78	0.96
Medium I							
Exponential	10	3	36.0	30.0	23.5	1.18	2.1
Inhibited ^a	0.1	13	37.1	33.1	16.7	1.5	1.58

^a Half of each growing culture was treated with an inhibitory concentration of sodium. [¹⁴C]acetate, 2 mM, 2.2×10^5 counts per min per μ mole, was then added to label newly synthesized lipids. The sodium-inhibited cultures were harvested after growth had stopped; controls were allowed to grow to slightly higher density. The specific activities of the fatty acids are normalized to that of palmitic acid from the same culture.

wealth of information available about it and because strain A324-1 produces it constitutively. Hydrolysis of ONPG by whole cells was used to assay transport, because transport of ONPG into intact cells is rate-limiting for its hydrolysis by the intracellular β -galactosidase. Consequently, toluenized cells exhibit much higher rates of ONPG hydrolysis, which are a measure of the galactosidase itself. TDG inhibits mediated transport of ONPG by binding to the M protein (3); hence, subtracting the rate of hydrolysis in the presence of TDG controls for extracellular galactosidase, nonmediated diffusion, and nonenzymatic hydrolysis.

Samples of a culture of strain A324-1 growing on reagent-grade amino acids with 0.1 mM MgSO₄ were taken at various times after the addition of 10 mM NaCl. The rate of hydrolysis of ONPG by the intact cells with and without 10 mM TDG and by toluenized cells was measured at 37 C (Fig. 10). Mediated entry of ONPG at first increased in parallel with the growth of the culture. After 15 min, however, mediated transport slowed progressively. Addition of azide (10 mM) to the assay system gave similar results. Because azide-poisoned cells cannot accumulate galactosides, inhibition of ONPG hydrolysis in such cells must result from an inhibition of entry of ONPG rather than an inhibition of the energy supply for accumulation. The changes in membrane structure brought about by the addition of sodium to the mutant prevent proper functioning of the membrane.

DISCUSSION

To our knowledge, the mutant A324-1 is the first strain of *E. coli* known to be specifically inhibited by low concentrations of sodium. In theory, of course, any ion can interact deleteriously with any mutant protein, and mutations affecting the reaction of microorganisms to

specific metals do occur. In a mutant of *Neurospora crassa*, an increased affinity of tryptophan synthetase for zinc inactivates the enzyme at physiological zinc concentrations (43).

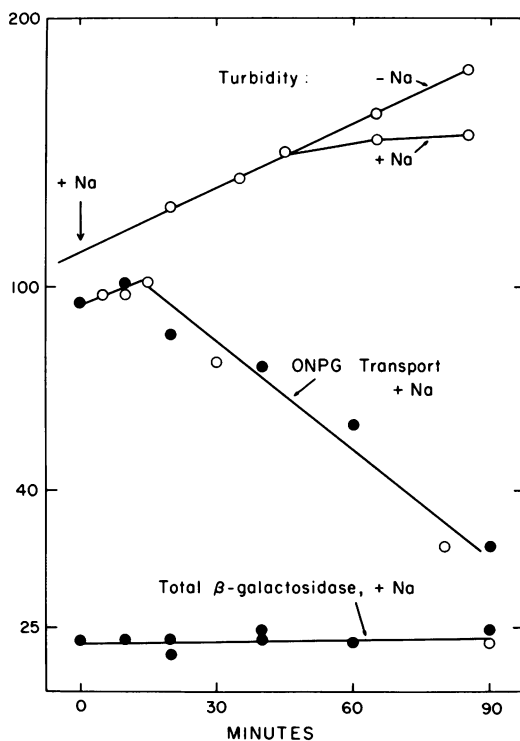


FIG. 10. Mediated *o*-nitrophenyl- β -galactosidase (ONPG) hydrolysis and total β -galactosidase in strain A324-1 inhibited by sodium. Samples of a growing culture were removed at the indicated times after addition of sodium. ONPG transport (hydrolysis by whole cells) and β -galactosidase in toluenized cells were measured. The ordinate represents arbitrary units of absorbance of *o*-nitrophenyl released by intact or toluenized cells from equal volumes of culture and turbidity of the culture.

Removing zinc from the mutant protein restores activity. Certain suppressor mutations which lower the amount of zinc in the organism allow the altered synthetase to function. A mutant of *Salmonella typhimurium* is inhibited by concentrations of Cr^{3+} less than one-fiftieth of those that affect the wild type (8); similar mutants are known in *E. coli* (45).

The mutant A324-1 is specifically inhibited by sodium (and partially by lithium), demonstrating that it is not principally sensitive to osmotic pressure or ionic strength. A precedent for specific biochemical effects of low concentrations of sodium is provided by the requirement of *Aerobacter aerogenes* when growing anaerobically on citrate (27). Sodium activates oxaloacetic decarboxylase in this organism, 12 mM giving maximal activity (42). The 10-fold higher optimal concentration for growth may be a result of low permeability to sodium. At higher concentrations (2%), at which osmotic effects may be important, NaCl can phenotypically reverse a temperature-sensitive *dnaB* mutant of *E. coli*; the mutant protein functions at low temperature or in high salt concentration (34, 36).

The combination of sensitivity to sodium and distortion of the phospholipid pattern is reminiscent of the situation in *E. coli* K-12 (λ) infected with rII mutants of phage T4. Infection with these phage is abortive if the medium contains as little as 1 mM monovalent cation; any monovalent cation inhibits, and magnesium, or to a lesser extent calcium, overcomes the inhibition (15). Cardiolipin synthesis is increased in rII-infected cells compared to controls infected with r^+ (31). The increase in cardiolipin occurs too late to be considered the cause of abortive infection, however, and the similarity of ionic requirements and phospholipid synthesis between rII infection and sodium inhibition in strain A324-1 may be coincidental.

Increased cardiolipin is found in many other conditions that correlate with death or stasis of the cell. During stationary phase, phosphatidylglycerol decreases from about 20% of the total phospholipid to less than 10%, with a corresponding rise in cardiolipin (9, 18). Cardiolipin increases in cells infected with amber mutants of phage $\phi 1$ (H. Smilowitz, *personal communication*). The action of colicins A, K, Q, E1, and E3 (but not E2) on sensitive strains causes an increase in lysophosphatidylethanolamine and in cardiolipin, although not to such a large extent as in strain A324-1 with sodium (4). Similar increases in cardiolipin occurred in cells incubated with phen-

ethyl alcohol, chloramphenicol, penicillin, formaldehyde, dinitrophenol, toluene, or chloroform, without a carbon source or at temperatures above 55 C (1, 4, 33, 40). The effects of penicillin or chloramphenicol on phospholipid metabolism are undoubtedly indirect, secondary to the well-known action of these antibiotics on the synthesis of cell wall or protein. Since changes in lipid composition qualitatively so similar to that produced by sodium in strain A324-1 can be attributed to secondary effects, and since no direct effect of sodium upon an enzyme in strain A324-1 has been found, it would be premature to assert that altered phospholipids are the cause of inhibition by sodium. However, the change in phospholipids is the earliest known event following the addition of sodium to A324-1, occurring while the rates of synthesis of protein, nucleic acid, and total phospholipid are normal. The other agents that distort the pattern of phospholipids also simultaneously decrease the total amount of lipid synthesis and inhibit growth.

It is not clear that the altered pattern of phospholipids is responsible for sodium-induced stasis in strain A324-1, but it seems unlikely that the concomitant slight reduction in synthesis of *cis*-vaccenic acid can of itself stop growth. Fatty acid auxotrophs are able to grow without any *cis*-vaccenic acid and can grow on a wide variety of unnatural unsaturated fatty acids (38). This ability, however, depends somewhat on the growth medium, *trans*-18:1 or 18-ynoate supporting growth on glycerol but not on tryptone (35). Possibly some subtle difference in conditions of growth will account for the peculiarly limited range of fatty acids that are able to overcome sodium inhibition of strain A324-1.

It remains to be seen whether slight changes in the proportions of various fatty acids can cause more dramatic changes in the proportions of phospholipids synthesized from them. The work of Henning et al. (16) indicates that a reduction in total unsaturated fatty acid in CDP-diglyceride shifts the products of lipid synthesis from predominantly phosphatidylethanolamine to predominantly cardiolipin. Such a shift, if it is not simply the result of conditions unfavorable to growth, may be difficult to demonstrate *in vitro* where highly artificial conditions, including detergents, are necessary for assaying the enzymes involved. In particular, phosphatidylserine synthesis is routinely assayed using CDP-dipalmitin as substrate; it is not known if a more natural CDP-diglyceride would be a better substrate or

if the enzyme would have different properties when acting upon a natural substrate. Our failure to demonstrate an altered phosphatidylserine synthetase in strain A324-1 is subject to the limitations of the in vitro assay.

The experiments of Fig. 7 and 8 led to the unexpected finding that there is a very substantial release of free glycerol from phosphatidylglycerol in normal and mutant strains of *E. coli*. If glycerophosphate is released, it is dephosphorylated without equilibrating with an exogenous pool. Since intact glycerophosphate is transported by this organism, internal and external pools should be equilibrated. About 10% of the water-soluble products could be other glycerol derivatives. The pulse-chase experiments of Fig. 8 and 9 may not reveal the fate of the acylated glycerol moieties of phospholipids. In a short pulse, phosphatidylglycerol is probably labeled preferentially in the nonacylated glycerol moiety because the immediate precursor of this residue is α -glycerolphosphate and not CDP-diglyceride. Phosphatidic acid, CDP-diglyceride, and the phosphatidyl moieties of phosphatidylglycerol become labeled from α -glycerolphosphate more slowly, after several enzymatic reactions (21).

De Siervo and Salton (12) recently found that the final step in the synthesis of cardiolipin in *Micrococcus lysodeikticus* may occur in a reaction independent of CDP-diglyceride. Two molecules of phosphatidylglycerol may combine to yield one molecule of cardiolipin and one of free glycerol. Experiments in this laboratory by C. B. Hirschberg indicate that a similar pathway exists in *E. coli*. That the release of free glycerol is accompanied by conversion of phosphatidylglycerol to cardiolipin is especially evident in the studies of strain A324-1 in the presence of sodium, since a striking accumulation of cardiolipin is observed under these conditions. Whatever the mechanisms involved, however, these studies reveal for the first time that the metabolism of phosphatidylglycerol in vivo in both mutant and wild-type strains involves major pathways leading to the release of free glycerol.

Fox (13) observed a decrease in the ability of an oleate-starved fatty acid auxotroph to transport galactosides. Transport cannot be induced in the absence of oleate, and function of preexisting M protein decreases if oleate is withdrawn. Induction of transport in an inducible derivative of A324-1 has not been examined, but the decline in the rate of ONPG transport after sodium inhibition is very similar to that observed by Fox. The slight alteration in fatty acids of strain A324-1 in sodium-

inhibited cultures possibly could account for the inhibition of transport; the minimum change in fatty acid composition necessary to inhibit transport is not known. Alternatively, changes in phospholipid pattern, which are most evident in strain A324-1 with sodium but also occur during oleate starvation (16), could be the primary cause of diminished ONPG transport. Present information is insufficient to distinguish changes in fatty acids from changes in phospholipids and to decide which is more critical for function of the M protein. The magnitude of the respective changes favors attributing the effects on transport to fatty acids in the auxotroph and to phospholipids in strain A324-1. In demonstrations that the temperature dependence of transport depends on the type of unsaturated fatty acid in the membrane (29, 35, 46), possible changes in the phospholipids when different unsaturated fatty acids support growth have not been examined.

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