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Effect of Serine Hydroxamate on Phospholipid Synthesis in *Escherichia coli*

LEWIS I. PIZER AND JOHN P. MERLIE[1]

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174

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Serine hydroxamate, which inhibits the charging of seryl-transfer ribonucleic acid, reduced the synthesis of phospholipid and nucleic acids in *Escherichia coli*. This effect was analogous to depriving amino acid auxotrophs of their nutritional requirement and appears to be a manifestation of the stringent response shown by *rel*$^+$ strains of *E. coli*. Amino acid starvation (serine or methionine) alone or serine hydroxamate treatment alone results in 60 to 80% inhibition of lipid accumulation, 90% inhibition of ribonucleic acid accumulation, and an increase in guanosine tetraphosphate (ppGpp). These three effects were reversed by addition of chloramphenicol (CM). A combination of serine starvation and serine hydroxamate treatment resulted in inhibition of lipid and RNA accumulation as well as an increase in ppGpp, but the consequences of the double block were not reversed by CM. We conclude that a strong interrelationship exists among these processes and that CM acts to relax a stringent response by mechanisms other than interference with ppGpp formation. All species of phospholipid were affected by a stringent response evoked by amino acid starvation or addition of serine hydroxamate, but in all cases the synthesis of phosphatidylethanolamine was most severely inhibited. Serine hydroxamate was not incorporated into lipid but specifically caused phosphatidylserine accumulation. Serine starvation produced a dramatic alteration of the distribution of isotope incorporated into phospholipid, which resulted from the stringent response compounded with the limitation of a substrate for phosphatidylserine synthesis.

In a preliminary report (L. I. Pizer and L. Pylkas, Bacteriol. Proc., p. 134, 1967) we described that the rate of incorporation of phosphate into phospholipid was reduced when amino acid auxotrophs of *Escherichia coli* were depleted of their nutritional requirements. We observed that conditions allowing preferential accumulation of ribonucleic acid (RNA), eg., addition of chloramphenicol (CM) to a culture of starved stringent (*rel*$^+$) cells or starvation of a culture of relaxed (*rel*$^-$) cells, permitted lipid synthesis at a level intermediate between that found in a starved culture and a growing culture. Similar results were reported by Sokawa et al. (15), and recently information on phospholipid synthesis and turnover has been published which extends these initial observations to isogenic relaxed and stringent strains (10). A correlation between RNA accumulation and lipid synthesis was also found in cells shifted from a rich to a poor medium (2).

One aspect of our early work with the amino acid auxotrophs that was of special interest was the nature of the lipid labeled during serine starvation since this amino acid is directly involved in the synthesis of phosphatidylethanolamine. When starved of serine, $^{32}P_i$ was incorporated into phosphatidylglycerol and cardiolipin, exclusively. Availability of a serine analogue, serine hydroxamate, which allows serine synthesis but inhibits its attachment to transfer RNA (tRNA; 18) prompted us to reinvestigate the relationships between amino acid starvation, RNA synthesis, and the incorporation of radioactive precursors into phospholipid. The availability of strains resistant to the analogue and auxotrophs derived from the same parental strain permitted a number of experimental conditions to be tested without complications due to strain differences. We

[1] Present address: Department of Molecular Biology, The Pasteur Institute, Paris.

investigated the effect of inhibiting the charging of tRNA on lipid synthesis, the effect of the analogue (serine hydroxamate) on phosphatidylethanolamine synthesis, and the direct participation of the analogue in lipid synthesis. This report describes these investigations.

MATERIALS AND METHODS

Bacteria and growth conditions. *E. coli*, strain K-12 (λ), was utilized by Tosa and Pizer for their studies of the mode of action of serine hydroxamate (17, 18). Strains derived from this parent were: strain A111g, a mutant resistant to the analogue with an altered tRNA synthetase; strain C111d, a resistant mutant with reduced feedback inhibition of phosphoglycerate dehydrogenase and an elevated intracellular serine concentration; and sensitive nutritional auxotrophs produced by 2-amino purine mutagenesis which required serine or methionine. Bacteria were grown in a synthetic medium of the following composition: NaCl, 90 mM; KCl, 40 mM; NH_4Cl, 200 mM; $MgCl_2$, 10 μM; $CaCl_2$, 1 μM; K_2HPO_4, 0.48 μM; Na_2SO_4, 0.16 μM. This was buffered at pH 7.4 with 100 mM *N*,*N*-bis (2 hydroxyethyl) glycine. Glucose which had been sterilized separately by filtration was added to give a final concentration of 2 mg/ml. Serine, when present, was at 300 μg/ml, methionine at 50 μg/ml, chloromphenical at 100 μg/ml, and L-serine hydroxamate at 100 μg/ml. To avoid complications due to the effect of serine starvation on the supply of purines, 40 μg of adenine per ml was added to the medium for strain K-12 ser^-. Conditions for growing the bacterial cultures and the method for measuring growth were previously described (17, 18). In this investigation, cultures of bacteria were grown overnight, diluted 1/0 and allowed to grow to a Klett reading of 80 (no. 42 filter, 4×10^8 cells/ml). The cultures were then diluted to a Klett reading of 40 with prewarmed medium containing radioactive compounds and dispensed into growth tubes containing the compounds whose effect was to be tested. To deprive the serine and methionine auxotrophs of the amino acids they require, the cultures were filtered (HA filter, 0.6 μm pore size, Millipore Corp.), and the cells on the filter were washed with warm medium prior to resuspension in medium lacking the amino acid supplement.

Chemical and isotopic labeling procedures. L-Serine hydroxamate was synthesized as reported previously (17). CM was obtained from Parke-Davis, and other compounds were purchased from Calbiochem, Los Angeles, California. $^{32}PO_4$ and ^{3}H-uracil were purchased from New England Nuclear, Boston, Mass.

The conversion of radioactive precursors into acid-precipitable cellular material was measured by the paper disk method previously described (14). Phospholipids were extracted from 0.8 ml of bacterial culture by the method of Bligh and Dyer (4), and the components were separated by thin-layer chromatography on Bakerflex silica gel 1B (J. T. Baker Chemical Co., Philipsburg, Pa.). Routinely, chromatograms were developed in a single dimension with chloroform-methanol-water (65:25:4) as the solvent, but on occasion chloroform-methanol-glacial acetic acid (65:25:8) was used to develop in a second dimension (1). Lipids eluted from chromatograms were deacylated by the procedure described by Ballou et al. (3), and the water-soluble derivatives were separated as described previously (9).

The intracellular concentrations of ATP, GTP, and guanosine tetraphosphate (ppGpp) were measured in formic acid extracts of bacteria after chromatographic separation of the nucleotides on Polygram PEI plates (Brinkman Instrument Inc., Westbury, N.Y.) by the procedure of Cashel et al. (5). ^{32}P incorporation into phospholipid was measured by plating samples in stainless-steel planchets and determining the radioactivity in a Nuclear-Chicago gas flow counter. The positions of radioactive phospholipids or nucleotides on chromatograms were located by radioautography. The quantity of isotope present was determined by cutting out the spots, placing them in toluene-based scintillation fluid, and measuring the radioactivity in a Packard scintillation spectrophotometer.

RESULTS

Effect of serine-hydroxamate on isotope incorporation. The addition of serine hydroxamate to growing wild-type *E. coli* K-12 inhibits $^{32}P_i$ incorporation into lipid and ^{3}H-uracil incorporation into RNA (Fig. 1A and B). CM stimulates both RNA and phospholipid synthesis in serine hydroxamate-treated cells to the level found in cells treated with CM alone. It appears that, by inhibiting the charging of seryl-tRNA, serine hydroxamate produced a stringent response which reduced the accumulation of both lipid and RNA, and that CM released the cells from the stringent response. From a comparison of the data shown in Fig. 1A and B, it appears that in the presence of CM relatively more uracil was incorporated into nucleic acid than $^{32}P_i$ into lipid. A correlation existed between the synthesis of these two types of compounds, but the quantity of lipid synthesized was not adjusted to the amount of RNA.

Strain A111g, which has a seryl-tRNA synthetase resistant to serine hydroxamate inhibition was not affected in either lipid or RNA synthesis (Fig. 1 insert). The analogue acts in the wild-type strain K-12 primarily to inhibit the charging of seryl tRNA and it appears that the reduction in RNA and lipid synthesis are secondary consequences of this effect rather than independent effects of the analogue. In a similar experiment performed with another resistant strain (C111d), serine hydroxamate also failed to inhibit isotope incorporation into phospholipid or RNA.

Isotope incorporation in auxotrophic strains. Because serine hydroxamate appeared to evoke the stringent response, we compared

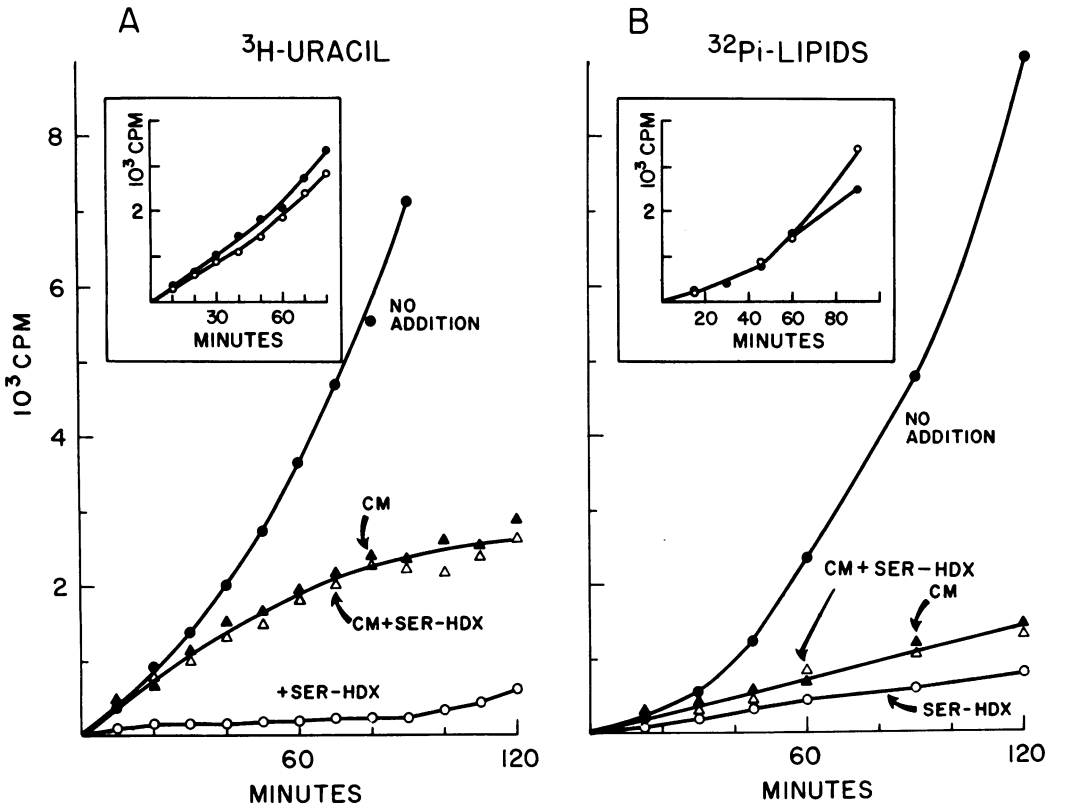

FIG. 1. *Effect of serine hydroxamate on isotope incorporation into nucleic acids and phospholipid.* $^{32}P_i$ *and* ^{3}*H-uracil were present in the medium at specific activities of 5 and 10 μCi/μmol. (A) Incorporation of* ^{3}*H-uracil per 100 μliters of culture. (B) Incorporation of* $^{32}P_i$ *into a lipid sample equivalent to 40 μliters of culture. Main figures show results with E. coli K-12, whereas inserts give results with strain A111g. Symbols used for both strains to describe culture conditions: (●) no additions, (○) plus serine hydroxamate (SER-HDX), (▲) plus chloramphenicol (CM), (△) plus SER-HDX and CM.*

the effects on lipid synthesis of methionine deprivation and serine hydroxamate addition in a methionine auxotroph. Methionine starvation or the addition of serine hydroxamate reduced the rate of $^{32}P_i$ incorporation to about 40% of the growing culture, and CM increased isotope incorporation in both inhibited cultures (Fig. 2). The rate of incorporation in the presence of CM was essentially the same as in the growing culture if the increase in mass of the growing culture is taken into account. These results indicate that blocking seryl-tRNA synthetase had quantitatively the same effect as amino acid starvation, and both states of inhibition were relieved by CM.

From amino acid analysis of acid-soluble pools (results not shown), we established that under the conditions of the previous experiments the intracellular concentration of serine was normal. We next examined the combined effects of serine hydroxamate with CM under conditions of serine deprivation by using a serine auxotroph. After the initial growth period, the culture was filtered, and the cells were suspended in medium lacking serine. The isotopically labeled compounds were added, and the culture was distributed. Samples were removed at 10-min intervals, and the curves of the type shown in Fig. 1 were constructed. The data obtained from the 60-min time point were selected as representative of each curve and have been presented in Table 1. The general correlation between incorporation of isotope into RNA and lipid demonstrated in Fig. 1A and B was again observed, but the flow of isotope into the two types of compounds was not directly proportional under all of the conditions tested. When serine was removed, relatively more $^{32}P_i$ was incorporated into lipid than uracil into RNA, but the stimulation produced by CM was more pronounced on RNA synthesis. The response to CM was quantitatively different in

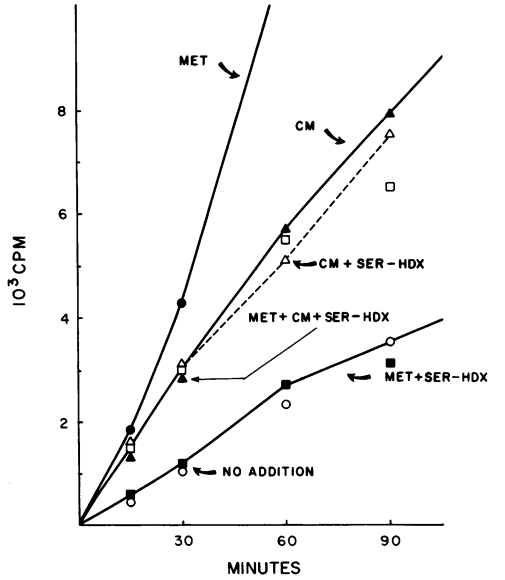

FIG. 2. *$^{32}P_i$ incorporation into phospholipids of E. coli K-12 met$^-$. $^{32}P_i$ was present at 40 μCi/μmol, and the curves show incorporation into a lipid sample equivalent to 40 μliters of culture. Symbols used to describe culture conditions: (●) plus methionine; (▲) plus CM; (△) plus CM and SER-HDX; (□) plus MET, CM, and SER-HDX; (○) plus MET and SER-HDX; (■) no addition.*

the serine-deficient cells than with the sufficient cells. In the absence of serine, neither lipid nor RNA synthesis was stimulated by CM to the level observed when serine and CM were present. In contrast, with *E. coli* K-12 *met*$^-$, incorporation of $^{32}P_i$ was the same in the presence of CM whether the culture was provided with methionine or not (data not presented). Moreover, CM failed to have any stimulatory effect if the culture depleted of serine was, in addition, supplemented with serine hydroxamate. We interpret these results to mean that, whereas CM can partially overcome the consequences of inhibiting seryl-tRNA synthesis (SER-HDX effect; Fig. 1 and 2) or a lowered serine pool (serine starvation of K-12 *ser*$^-$), the combination cannot be partially relieved by the antibiotic.

Distribution of $^{32}P_i$ among classes of phospholipid. The amount and nature of the phospholipids synthesized under the growth conditions described in the previous section were investigated by analyzing the distribution of $^{32}P_i$ in the individual lipid components. After the incorporation of isotope into total lipid was measured, samples were chromatographed. Chromatography in a single dimension was adequate to separate the major components phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL; reference 1; Fig. 3). The extent of movement of the lipids varied with the batch of plates, but their relative positions were constant.

The ^{32}P content of the phospholipids of *E. coli* K-12 *met*$^-$ grown under different conditions is shown in Table 2. The reduction in isotope incorporation into PE was the most pronounced consequence of removing the required amino acid, and the PG/PE ratio reflected this change. In the 15-min samples the PG/PE ratio went from 0.37 to 0.56, and in the 60-min sample it went from 0.21 to 0.40. Serine hydroxamate also reduced the incorporation of isotope into all major components, and CM released lipid synthesis from the restrictions imposed by either the addition of serine hydroxamate or amino acid starvation (Fig. 2).

Serine hydroxamate caused an increase in isotope incorporation into a minor component present in growing cells (Fig. 3) which we have identified as phosphatidylserine (PS) on the following grounds. It co-chromatographs with the standard compound (Serdary Research Labs, London, Ontario) in a two-dimensional chromatographic system, and after deacylation the water-soluble derivative co-chromatographs with the derivative produced from authentic PS added prior to the deacylation procedure (Fig. 4). If growing cells are sampled 15 min after adding $^{32}P_i$, PS constitutes about 1% of the total label. When 100 μg of serine hydroxamate per ml and CM were present, PS increased to about 10%, and if the L-serine hydroxamate concentration was increased to 500 μg/ml, 22% of the isotope in phospholipid was in PS. The

TABLE 1. *Isotope incorporation into nucleic acid and phospholipid by E. coli K-12 ser*$^-$[a]

Medium supplements	^{3}H-uracil into nucleic acid		$^{32}P_i$ into phospholipid	
	Counts/min/100 μliters of culture	%	Counts/min/40 μliters of culture	%
Serine	4,760	100[b]	6,460	100
None	160	4	730	11
Serine + CM	2,474	52	3,400	53
CM	1,379	29	1,200	19
CM + SER-HDX	199	4	740	11

[a] $^{32}P_i$ and ^{3}H-uracil (10 μg/ml) were present at 20 and 10 μCi/μmol, respectively. Samples were removed at 10-min intervals, and data obtained from the 60-min point are presented.

[b] Radioactivity in the growing culture supplemented with serine alone was set at 100%.

TABLE 2. *Distribution of $^{32}P_i$-labeled phospholipids of E. coli K-12 met⁻*[a]

Time (min)	Phospholipid	Medium supplements (counts/min)					
		Met	None	CM	Met + SER-HDX	SER-HDX + CM	Met + SER-HDX + CM
15							
	PE	28,200	11,400	33,900	13,600	26,600	28,500
	PG	10,400	6,350	9,440	5,100	9,150	8,700
	CL	2,360	1,900	2,160	1,060	2,180	1,900
	PS	550	145	555	1,200	5,310	5,150
	PG/PE	0.37	0.56	0.28	0.37	0.34	0.31
60							
	PE	225,000	49,600	116,000	36,600	83,000	108,700
	PG	47,000	19,600	26,800	17,900	18,950	20,000
	CL	14,000	7,920	8,920	4,620	9,800	9,120
	PS	1,800	180	640	2,520	8,250	9,450
	PG/PE	0.21	0.40	0.23	0.50	0.23	0.18

[a] Lipid samples corresponding to 400 μliters of culture were evaporated to dryness under a stream of N_2 redissolved in chloroform and chromatographed. From the radioautogram (Fig. 3) the locations of the phospholipids were established, and the areas of the chromatogram containing the lipids were cut out and counted. The abbreviations for the lipids are: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; and PS, phosphatidylserine.

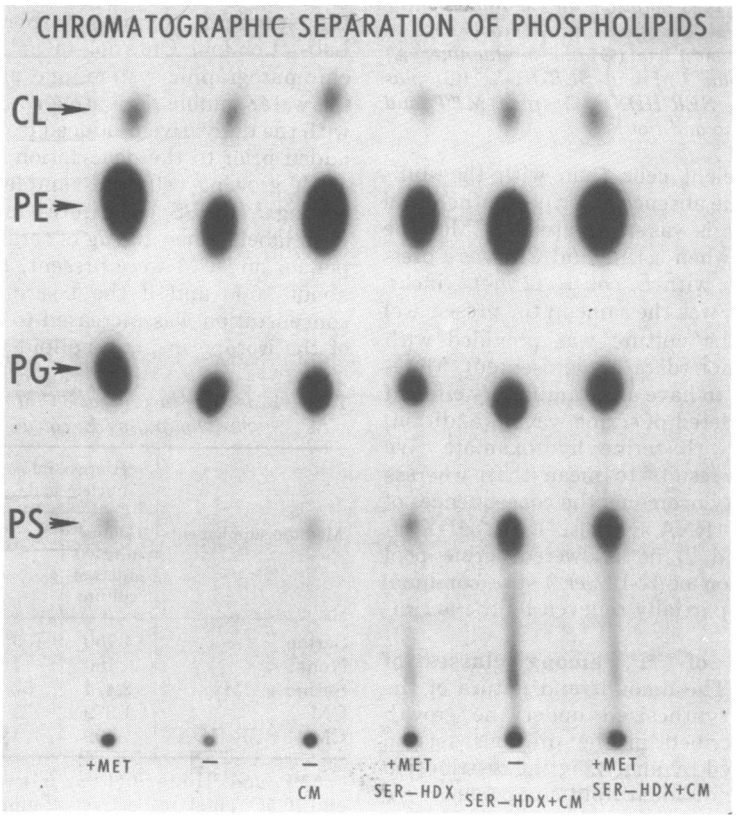

FIG. 3. *Chromatography of phospholipids. Lipid samples from the experiment shown in Fig. 2 were chromatographed. Supplements present in each culture are shown under the samples, all of which were taken 15 min after the start of the experiment.*

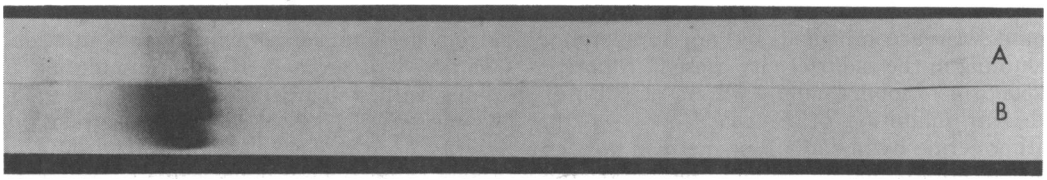

FIG. 4. *Chromatography of glycerolphosphorylserine. The phospholipid which accumulated in serine hydroxamate-treated cells was isolated from a thin-layer chromatography plate and mixed with 1 mg of authentic phosphatidylserine, and the sample was deacylated. Water-soluble compounds were chromatographed, and the radioactive regions were located by radioautography (strip B). Ninhydrin was used to locate the authentic glycerolphosphorylserine (strip A), and the strip which was developed in this way has been superimposed over half of the radioautograph to demonstrate the coincidence of the chemical and radioactive compounds.*

accumulation of PS appears to be due to serine hydroxamate specifically, since under the same conditions 100 μg of L-leucine hydroxamate per ml did not increase PS labeling. These results indicate that serine hydroxamate inhibits the conversion of PS to PE. Aside from the increase in PS and streaking from the PS region to the origin, chromatograms of lipids from cells treated with serine hydroxamate were the same as chromatograms of lipids from untreated cells and showed only normal lipid components (Fig. 3).

The results of a similar study in which the effects of nutrition on the incorporation of $^{32}P_i$ into the phospholipids of *E. coli* K-12 *ser*$^-$ are shown in Table 3. Serine deprivation preferentially reduced the incorporation of $^{32}P_i$ into PE, and CM stimulated incorporation into PG and CL, resulting in an appreciable difference in the PE/PG ratio from that found in growing or starved cells. CM did not increase total $^{32}P_i$ incorporation in serine-starved cells if serine hydroxamate was present (Table 1), but under these conditions the distribution of isotope was altered with CL containing 50% of the label. In this experiment where the cells were depleted of serine as in the previous experiment where the endogenous serine level was unaltered, no evidence was found for the incorporation of serine hydroxamate into lipids or the production of novel lipids when cells were grown with serine hydroxamate in the medium.

TABLE 3. *Distribution of ^{32}P labeled phospholipids of E. coli K-12 ser*$^-$ [a]

Phospholipids	Medium supplements				
	Ser	None	CM	CM + SER-HDX	Ser + CM
PE	70,280	740	510	500	11,770
PG	10,140	1,050	2,470	530	2,730
CL	3,740	380	2,410	1,190	770
PG/PE	0.14	1.40	4.80	1.06	0.23

[a] After incubation for 120 min under the conditions indicated, the lipids were extracted, and the samples were subjected to chromatography. The radioactive compounds were located by radioautography, cut out, and counted.

The effect of serine hydroxamate on nucleotide level. In view of the central role of ATP in both nucleic acid and lipid synthesis and the possibility that guanosine-tri and tetraphosphate are involved in regulating RNA accumulation, the levels of these nucleotides were measured in the serine auxotroph under the growth conditions described above. The results (Table 4) showed that when the auxotroph was starved of serine ATP and GTP levels dropped, whereas ppGpp levels increased. These changes did not occur in the presence of CM unless serine hydroxamate was added to the starved culture. From these data, a correlation cannot be made between changes in a single nucleotide level and the reduction in synthesis. However, the increase in ppGpp and the reduction in the biosynthetic processes observed in this experiment were also found under the conditions used to obtain the data presented in Fig. 1 and 2.

TABLE 4. *$^{32}P_i$ Content of purine tri and tetra phosphates*

Nucleotide	Medium supplements			
	Serine	None	CM	CM + SER-HDX
ATP	1870	580	4310	153
GTP	713	154	404	135
ppGpp	340	657	37	1373

[a] $^{32}P_i$ was present in the medium at 40 μCi/μmol, and 30 min after initiation of the experiment 2-ml samples of each culture were filtered. The cells on the filter were extracted with 1 ml of 2 N formic acid, and 50 μliters of the acid extract were chromatographed. After locating the nucleotides by radioautography, the spots were cut from the chromatogram, and the radioactivity present was determined.

Inhibition of *E. coli* K-12 by serine hydroxamate was accompanied by an approximate doubling in the radioactivity present in ppGpp (250 to 450 counts/min/50 μliters of culture), whereas inhibition of *E. coli* K-12 *met*⁻ by either serine hydroxamate or methionine starvation approximately tripled the radioactive ppGpp (1,480 counts/min increased to 3,930 and 4,743 counts/min/50 μliters of culture). In all cases CM lowered the ppGpp level to well below that found in growing cells, and no increase was produced by serine hydroxamate in the resistant strains.

DISCUSSION

The results of experiments with serine hydroxamate extend the observations made with amino acid auxotrophs that phospholipid accumulation is regulated in conjunction with RNA accumulation. Because the primary effect of serine hydroxamate was to inhibit the charging of Ser-tRNA (18) and mutants with altered Ser-tRNA synthetases accumulated $^{32}P_i$ into lipid at an uninhibited rate, it appears that it was the ability to charge tRNA rather than the availability of amino acids that regulated lipid synthesis as well as RNA accumulation. With the inadequate data we are unable to decide, at present, whether lipid and RNA accumulation were regulated by the same mechanism. However, several experiments support this possibility; both types of compounds were synthesized under conditions of amino acid starvation if the strain was of the *rel*⁻ genotype or if CM was added to the starved stringent strain (*rel*⁺), and the synthesis of both types of compounds was reduced when cells were shifted from rich to poor growth medium.

The nutritional conditions that reduce lipid synthesis caused an increase in ppGpp, and the possibility is attractive that this nucleotide which has been implicated in control of RNA accumulation (13) has a similar role in lipid synthesis. The correlation between the level of ppGpp and the inhibition of lipid synthesis was well demonstrated by the action of CM on strain K-12 *ser*⁻. Unlike the other situations tested, CM failed to block ppGpp production or allow lipid synthesis in this strain when the cells were both starved for serine and treated with serine hydroxamate. The combined effect of a block in endogenous synthesis of serine and inhibition of seryl-tRNA synthetase should lower the level of charged seryl-tRNA. Under the influence of the double block, ppGpp levels increased even when CM was present. It appears that the level of charged-tRNA regulates the concentration of ppGpp, and CM does not inhibit ppGpp synthesis. This conclusion is in agreement with earlier experiments in which CM failed to promote RNA accumulation in strains with a temperature-sensitive charging enzymes (8, 11, 12). Whereas the correlations described in this paper are consistent with the proposal that ppGpp plays a role in regulating lipid synthesis, we cannot conclude which effects are causally related until a mode of action for ppGpp is demonstrated.

Whatever the mechanism that serine hydroxamate and amino acid starvation evoked to reduce lipid synthesis, all classes of phospholipid were effected with PE showing the greatest changes. Sokawa et al. reported that the synthesis of PG is unaffected by amino acid starvation (16). The reason for the discrepancy between our data and those of Sokawa et al. is unknown, but we are in general agreement that lipid synthesis is under amino acid control. In addition, there were two conditions which arose from serine being involved directly in PE synthesis. Serine starvation not only reduced incorporation of $^{32}P_i$ into total lipid but drastically reduced PE synthesis, leaving PG and CL synthesis relatively high. These data show that a degree of flexibility exists in the production of the different lipid classes. The second condition was the accumulation of PS when serine hydroxamate was added to the culture. It was reported in the review by Cronan and Vagelos that hydroxylamine inhibits PS decarboxylation in vivo (7). Presumably, serine hydroxamate was having its effect because of the hydroxamate group, but the effect was specific because, at comparable concentrations, leucine hydroxamate failed to cause PS accumulation. The quantitative aspects of PS accumulation and serine hydroxamate concentration were only studied in strain K-12 *met*⁻, but PS accumulation was observed in the lipid of *E. coli* K-12 *ser*⁻ and the resistant strain A111g grown with serine hydroxamate. It is possible that the reduction in growth rate observed when the resistant strains A111g and D111g were treated with high levels of the analogue (17, 18), was caused by PS accumulation. Slow growth does not appear to be due to the synthesis of an aberrant serine hydroxamate containing phospholipid since only the normal lipids were present on chromatograms of lipids from treated cells, even when intracellular serine levels were reduced by starving K-12 *ser*⁻ for serine. A recently reported "serine suicide" procedure for isolating mutants blocked in PE synthesis makes use of serine hydroxamate to inhibit serine incorporation into protein (6). The accumulation of PS was not noted. How-

ever, had PS accumulated, the selective value of this technique would not have been effected.

The experiments described in this paper were undertaken to test whether the early observations on the correlation between lipid and RNA regulation could be extended and might provide an indication of which compounds should be investigated at the enzyme level. The results suggest that a study of the action of ppGpp on the enzymes of phospholipid synthesis might enable the cellular events to be explained at the chemical level, and we have started experiments to test the properties of this nucleotide in vitro.

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