MS I Accumulation Induced by Sodium Chloride*

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ABSTRACT: Both a stringent and a relaxed strain of *Escherichia coli* accumulated comparatively equal amounts of MS I (ppGpp) when placed in hypertonic NaCl solutions. This accumulation was shown to be dependent on nascent RNA synthesis. MS II, which is characteristically accumulated in this stringent strain upon amino acid starvation, was not accumulated in the NaCl solutions, and in fact the addition of NaCl to the arginine-starved stringent cells resulted in the rapid disappearance of MS II. These observations suggest that the mechanism governing the NaCl-induced MS I accumulation differs from the mechanism of MS accumulation during amino acid starvation.

Amino acid starvation or restricted aminoacylation of tRNA causes a severe reduction of RNA accumulation (termed the stringent response) and the rapid accumulation of two guanosine nucleotides (MS I and MS II) in stringent (*rel*$^+$), but not relaxed (*rel*) strains of *Escherichia coli* (Cashel and Gallant, 1969; Cashel, 1969). Cashel and Kalbacher (1970) identified MS I as guanosine 5′-diphosphate 3′- or 2′-diphosphate (ppGpp). MS II, whose structure is not yet determined, is accumulated in lesser amounts and is not characteristic of all stringent strains.

It is now clear that the accumulation of MS I is not specific to the stringent response but observable under various physiological conditions such as carbon, nitrogen, and sulfur starvation (Edlin and Donini, 1971). Furthermore, the fact that during carbon shift-down or glucose starvation both relaxed and stringent strains accumulate MS I concomitantly with the inhibition of RNA accumulation (Lazzarini *et al.*, 1971; Harshman and Yamazaki, 1971) indicates that its accumulation is not a specific property of stringent strains. However, it is not yet known whether the various physiological conditions which induce the accumulation of MS compounds do so by the same mechanism.

This paper reports that the addition of sodium chloride induced the accumulation of only MS I in both a stringent and a relaxed strain of *E. coli*. Also described are observations which suggest that the mechanism of this NaCl-induced MS I accumulation differs from that governing MS nucleotide accumulation during amino acid starvation.

Materials and Methods

Bacteria and Culture Conditions. Two strains of *E. coli* K-12 were used: CP 78 U$^-$ (F$^-$, *rel*$^+$, *arg*, *his*, *leu*, *thr*, *thi*, *ura*) and CP 79 U$^-$ (F$^-$, *rel*, *arg*, *his*, *leu*, *thr*, *thi*, *ura*). For bacterial growth, Tris–maleate glucose medium (Paranchych, 1966) supplemented with the required amino acids plus isoleucine (50 μg each/ml), uracil (20 μg/ml), and thiamine (10 μg/ml) was used throughout. Bacteria were grown at 37° on a gyrotory water bath shaker, and cell density was measured by an absorbance at 500 nm.

Chemicals. Uniformly labeled L-[^{14}C]arginine and [^{14}C]-uracil were obtained from International Chemical and Nuclear Corp.; carrier-free [^{32}P]orthophosphoric acid (in 0.1 N HCl) from Atomic Energy of Canada, Ltd.; rifampicin, from Calbiochem; chloramphenicol, from Sigma; D,L-β-2-thienylalanine and amino acids, from Nutritional Biochemical Corp.; polyethyleneimine-cellulose thin-layer plates (MN polygram cel 300 PEI), from Brinkmann Instruments.

Assay of RNA and Protein Accumulation. In order to assay accumulation of RNA, aliquots of the cultures were mixed with one-tenth the volume of the RNA-labeling mixture, which contained per milliliter: 5 μCi of [^{14}C]uracil, 100 μg of unlabeled uracil, and 200 μg of cytosine (to minimize the labeling of DNA). For the labeling of proteins, [^{14}C]arginine was added to the cultures to a final concentration of 0.1 μCi/ml, thus giving the final specific activity of 0.1 μCi/50 μg. At intervals, 0.1-ml portions of the cultures were withdrawn and assayed for the labeled RNA (cold trichloroacetic acid insoluble radioactivity) or labeled protein (hot trichloroacetic acid insoluble radioactivity) by the filter paper disk method (Bollum, 1968).

Assay of ppGpp. Carrier-free [^{32}P]orthophosphate was added to the cultures to final concentrations of 8.2×10^6 cpm/ml at the times indicated in the figure legends. The concentration of phosphate ions in the growth medium was 10^{-3} M. The intracellular phosphate pools were found to be equilibrated within 10 min of the addition of [^{32}P]orthophosphate as judged by the labeling of the GTP and ATP pools. At intervals indicated in the figures, samples were withdrawn and assayed for MS I and MS II by formic acid extraction and thin-layer chromatography as described previously (Cashel *et al.*, 1969).

Results

Figure 1 shows that upon the addition of NaCl (0.35 M), there occurred an immediate accumulation of MS I with a concomitant cessation of the accumulation of labeled RNA and protein in both CP 78 U$^-$ (*rel*$^+$) and CP 79 U$^-$ (*rel*). Although CP 78 U$^-$ accumulates MS II during the stringent response (Figure 5), no detectable amount of MS II was accumulated here. The uracil incorporation curves indicate a transient degradation of labeled RNA shortly after the addition of NaCl–the orcinol RNA assay verified these results (unpublished data). This degradation period was followed by the reaccumulation of the label at a reduced rate. The fact that comparatively equal amounts of MS I were accumulated in both a stringent and a relaxed strain with similar kinetics of accumulation indicates that this NaCl-induced MS I

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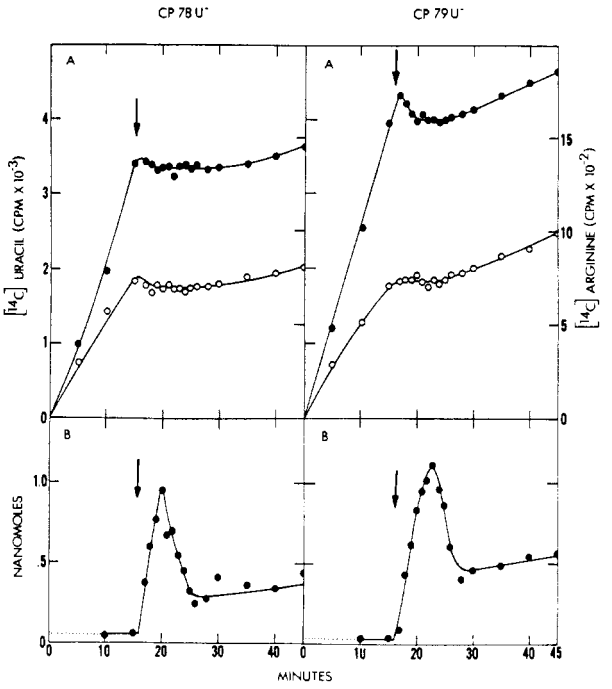

FIGURE 1: Accumulation of MS I by NaCl. CP 78 U⁻ (*rel*⁺) and CP 79 U⁻ (*rel*) were grown to a cell density of $A_{500\ nm}$ = 0.4. At 0 min, three equal portions of each culture were transferred to three culture tubes containing the RNA labeling mixture, [^{14}C]arginine, or [^{32}P]orthophosphate. NaCl was added at 16 min (as indicated by the arrows) to a final concentration of 0.35 *M*. The periodic assays of the accumulation of labeled RNA, protein and MS nucleotides were performed as described in the Materials and Methods. No detectable amount of MS II was accumulated. The left column indicates CP 78 U⁻ and right, CP 79 U⁻. Part A indicates the incorporation of precursors into RNA (●) or protein (○) in 0.1 ml portions of the culture. Part B indicates the amounts of phosphate incorporated into MS I expressed as nanomoles per ml of culture of $A_{500\ nm}$ = 1.0.

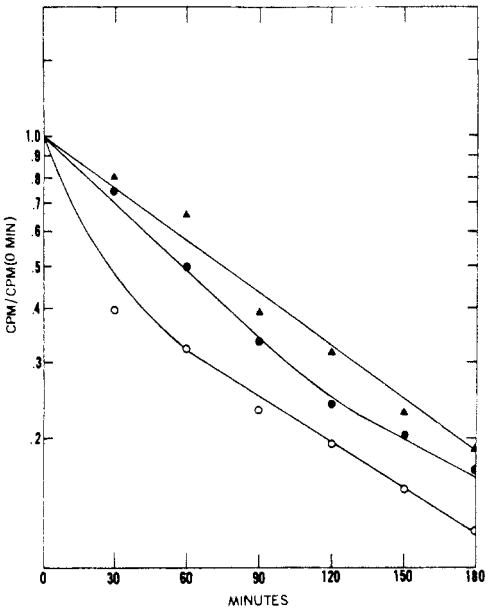

FIGURE 3: Alkali lability of MS nucleotides. A log-phase culture of CP 78 U⁻ was exposed to [^{32}P]orthophosphate for one generation time. The labeled culture was then divided into two portions. To one, D,L-β-2-thienylalanine was added to a final concentration of 500 μg/ml in order to cause phenylalanine starvation (Ezekiel, 1965). To the second portion, NaCl was added to a final concentration of 0.45 M. Both portions were incubated until the estimated maximal accumulation of MS nucleotides had occurred at which time acid extracts were prepared as described previously (Cashel *et al.*, 1969). The acid extracts were neutralized, and then exposed to 0.3 N KOH at 37°. At intervals indicated in the figure, samples were withdrawn, acidified with formic acid, and assayed for MS nucleotides as previously described (Cashel *et al.*, 1969). The radioactivity found in the MS spots are expressed as fractions of that found in the 0-min samples. MS I induced by β-thienylalanine (●); MS I induced by NaCl (○); MS II induced by β-thienylalanine (▲).

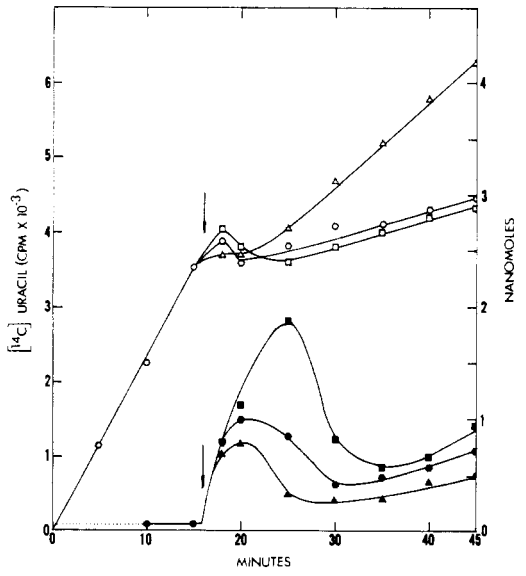

FIGURE 2: Effect of NaCl concentrations on the accumulation of MS I in CP 79 U⁻. The experimental plan used was identical with that described in Figure 1. The open symbols indicate the incorporation of [^{14}C]uracil into RNA: control (○) prior to the addition of NaCl, after addition of NaCl (0.25 M final) (△), 0.35 M NaCl (○), and 0.45 M NaCl (□). The closed symbols indicate the amounts of phosphate incorporated into MS I expressed as nanomoles per milliliter of culture of $A_{500\ nm}$ = 1.0: control (●) prior to the addition of NaCl, 0.25 M NaCl (▲), 0.35 M NaCl (●), and 0.45 M NaCl (■).

accumulation is independent of the allelic state of the *rel* gene. Figure 2 shows the effect of different concentrations of NaCl on the accumulation of MS I and labeled RNA. Upon the addition of NaCl the accumulation of labeled RNA exhibited two phases at each concentration, a brief period of cessation and/or degradation of labeled RNA, followed by a resumed accumulation of RNA at a reduced rate. Increasing the NaCl concentration resulted in (1) an increase in the level of MS I accumulated, (2) an increase in the extent of degradation of labeled RNA, and (3) a decrease in the subsequent rate of accumulation of labeled RNA. The level of MS I seems to be independent of the subsequent rates of accumulation of labeled RNA since no difference in the rates was observed at 0.35 or 0.45 M NaCl. However, there is an interesting correlation between the level of MS I and the extent of degradation of RNA. It is therefore possible that a nucleotide, pppGp, which cannot be separated from MS I (ppGpp) in this chromatographic system could be degradatively derived from the 5′ end of RNA by the action of NaCl. However, since MS I is alkali labile, it is clearly distinguishable from pppGp (Cashel and Kalbacher, 1970). Figure 3 shows that MS I and MS II formed by phenylalanine starvation (by the addition of β-thienylalanine) and MS I formed by NaCl addition in CP 78 U⁻ were equally susceptible to alkali treatment (0.3 M KOH at 37°).

Recently Erlich *et al.* (1971) and Watson and Yamazaki (1972) have demonstrated that the mechanism of MS I

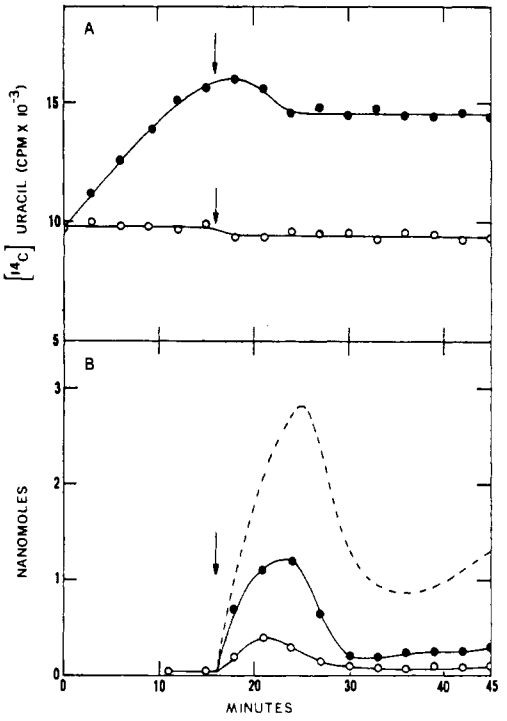

FIGURE 4: Accumulation of MS I by NaCl in the presence of rifampicin or chloramphenicol. A log-phase culture of CP 79 U⁻ was divided into two portions to which the RNA labeling mixture or [^{32}P]orthophosphate was added. After 30-min incubation, both the [^{14}C]uracil and ^{32}P-labeled cultures were further divided into two portions which were exposed to rifampicin (200 μg/ml) or chloramphenicol (100 μg/ml) at 0 min. NaCl was added (0.45 M final) at 16 min as indicated by the arrows. Samples were withdrawn and assayed for MS nucleotides and labeled RNA as described in Materials and Methods. Part A indicates the labeled RNA in the presence of rifampicin (○) or chloramphenicol (●). No detectable amount of MS II was accumulated. Part B indicates the amounts of phosphate incorporated into MS I expressed as nanomoles per ml of culture of $A_{500\ nm}$ = 1.0 in the presence of rifampicin (○) or chloramphenicol (●). The broken line indicates the amounts of MS I accumulated by NaCl (0.45 M final) in the absence of rifampicin and chloramphenicol (replotted from Figure 2).

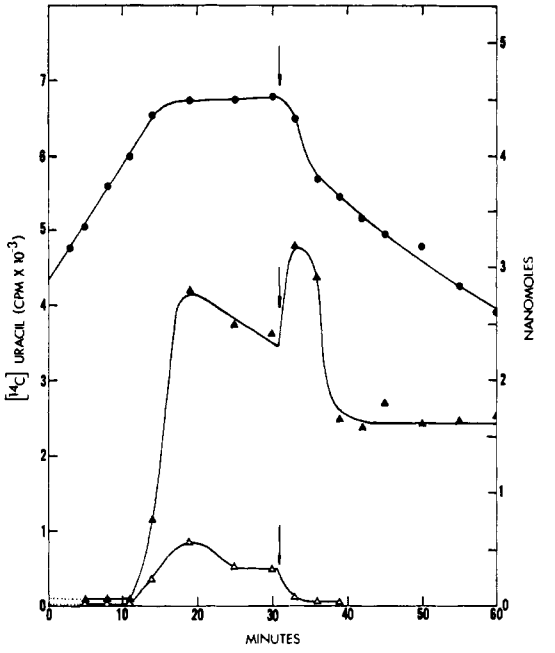

FIGURE 5: NaCl-induced MS I accumulation in an arginine-starved culture of CP 78 U⁻. A log-phase culture of CP 78 U⁻ was grown in the medium containing [^{32}P]orthophosphate or the RNA-labeling mixture for 30 min. The cells were collected on a membrane filter, and resuspended (0 min) in the medium lacking arginine but containing either [^{32}P]orthophosphate or the RNA-labeling mixture (each at the same concentration as used during the 30-min incubation). The suspension was shaken at 37°. NaCl was added (0.45 M final) at 31 min as indicated by the arrows. Samples were withdrawn and assayed for MS nucleotides and labeled RNA as described in Materials and Methods. Labeled RNA (●), MS I (▲), and MS II (△). The amounts of MS nucleotides are expressed as nanomoles of phosphate incorporated per milliliter of culture of $A_{500\ nm}$ = 1.0.

accumulation induced by amino acid starvation or restricted aminoacylation of tRNA can operate even when RNA synthesis is inhibited by rifampicin. However, Figure 4 shows that when RNA synthesis was inhibited by rifampicin prior to the addition of NaCl, the amount of MS I accumulated upon NaCl addition was greatly reduced, as compared to that in an untreated control culture. Figure 4 also shows that a substantial amount of MS I was accumulated upon NaCl addition in the culture pretreated with chloramphenicol. These results indicate that the NaCl-induced accumulation of MS I is markedly dependent on the continued RNA synthesis, but relatively less dependent on protein synthesis.

During amino acid starvation RNA synthesis in the stringent strain CP 78 continues at a reduced rate (approximately 30% during threonine starvation) (Lazzarini and Dahlberg, 1971). Figure 5 shows the effect of NaCl addition, during arginine starvation, on MS I accumulation in CP 78 U⁻. This stringent strain exhibited a typical stringent response when the intracellular arginine was exhausted; that is, the severe inhibition of [^{14}C]uracil incorporation accompanied by the accumulation of both MS I and MS II. When NaCl was added at a period when the levels of MS compounds were declining, significant reaccumulation of only MS I was observed. On the other hand, the amount of MS II immediately dropped to its basal level. The transient kinetics of the NaCl-induced MS I accumulation, which was observable in the previous experiments, is particularly noticeable here when it is compared with the kinetics obtained through arginine starvation.

Discussion

The NaCl-induced MS accumulation clearly differs from the accumulation of MS nucleotides induced by amino acid starvation or restricted aminoacylation of tRNA in the following ways: (1) in the presence of all required amino acids, comparatively equal amounts of MS I were accumulated in both the stringent and relaxed strains; (2) no detectable amount of MS II was accumulated and in fact the addition of NaCl to the arginine starved stringent cells resulted in the rapid disappearance of MS II; (3) whereas the accumulation of MS nucleotides induced by amino acid starvation is not dependent on nascent RNA synthesis, the NaCl-induced accumulation of MS I exhibited a marked dependence on RNA synthesis; (4) NaCl induced a substantial accumulation of MS I in the presence of chloramphenicol which is known to completely suppress MS I accumulation during amino acid starvation. Thus, it appears that the mechanism governing the NaCl-induced MS I accumulation is different from that involved during amino acid starvation or restricted aminoacylation of tRNA.

The correlation between the extent of RNA degradation and the level of MS I accumulated, though interesting, does not provide information as to the mechanism of NaCl-induced MS I accumulation. It is entirely possible that these phenomena are independent of each other. It is possible that NaCl at a high concentration causes the inhibition of uptake of amino acids and uracil which are required by the strains used in the present experiment. The resulting uracil starvation might then trigger RNA degradation (Lazzarini *et al.*, 1969). However, it is known that neither amino acid starvation nor uracil starvation causes the accumulation of MS I in relaxed strains (Cashel and Gallent, 1969; our unpublished data). The very transient nature of the NaCl-induced MS I accumulation and the concomitant degradation or cessation of RNA accumulation may be related to the rapid plasmolysis and deplasmolysis which occur in *E. coli* cells when placed in hypertonic NaCl solutions.

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Nearest-Neighbor Frequencies of Mitochondrial Deoxyribonucleic Acid in Mouse Liver*

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ABSTRACT: Nearest-neighbor frequencies of mouse liver mitochondrial DNA have been determined and compared to the dinucleotide frequencies of nuclear DNA from the same tissue. The results show that the distribution of dinucleotides in mitochondrial DNA is decidedly less random than in any other double-stranded DNA species studied to date.

There are three methods available at present for gaining an insight into the primary structure of a DNA molecule: the determination of isostichs as worked out in Chargaff's laboratory over the past 20 years (for review, see Chargaff, 1968), the analysis for nearest-neighbor frequencies introduced by Josse *et al.* (1961), and recently the application of the fingerprint method by Murray (1970) to DNA hydrolysis products following enzymic digestion. Even though the date of sequencing a DNA molecule, that Chargaff (1968) places in the 21st century, has hardly been brought any closer by these methods, they are still valuable tools for comparing either DNA preparations from different sources, or the same DNA molecules prior to and following the effect of various agents or processes on the genome. Furthermore, the smaller the size of the DNA molecule the less utopian a study of this nature is. mtDNA[1] seems to be one of the smallest functional DNA molecules known in living organisms. Its size as well as the possibility of being studied under identical conditions as its nuclear counterpart render mtDNA a comparatively attractive molecule for sequence studies. Furthermore, such studies might even contribute to answering the question of the phylogenetic origin of mitochondria as Cummins *et al.* (1967) have suggested.

In the present paper data are presented for the nearest-neighbor frequencies of all possible dinucleotides in mt- and nDNA of mouse liver.

Materials and Methods

Preparation of Nuclei and Mitochondria. Nuclei and mitochondria were isolated as previously described (Georgatsos *et al.*, 1970) from the livers of ether anesthetized mice of the pure inbred strain C3HAvy/HeSy. All livers were examined histologically by Dr. T. Sirmakesian of this Institute. The authors are aware that the trustworthiness of the results depends mainly on the total absence of nDNA in the preparations of mtDNA. Consequently extreme care was taken in the preparation of the mitochondrial fractions which were further processed only when the total absence of nuclei was

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[1] Abbreviations used are: mtDNA and nDNA, mitochondrial and nuclear DNA, respectively.