In an effort to use the ribonuclease digestion on ribosomes as an approach for analyzing ribosomal function, we attempted to measure the three biological activities of these digested ribosomes. We would like to know if nuclease digestion preferentially impairs one of the three activities. Our results, at the present, do not allow us to make such a distinction mainly because of the relatively large margin of error (15–20%) in the two binding assays (Figures 2 and 3). However, our data do indicate that both the binding of AA-tRNA and mRNA are impaired by nuclease digestion.

As a first step in the attempt to correlate the functions of the ribosomes and the structure of these RNA regions, we have examined the elution profile of the RNA fragments remaining in the 70S ribosomal particle after RNase T_1 treatment. Relatively few fragments were obtained even after considerable treatment with RNase T_1. This observation is in agreement with those of Ehresmann and Ebel (1970) and Santer and Szekely (1971) after RNase T_1 digestion under conditions which would lead to total hydrolysis of the free RNAs.

Finally our data showed that some of the RNA fragments remaining in the ribosomes after enzymatic digestion possessed nonphosphorylated 3′-hydroxyl terminus and thus could be oxidized with sodium periodate followed by reduction with sodium borohydride. These fragments must have been generated from the original 3′-hydroxyl termini of the tRNA molecules, because the action of RNase T_1 should yield 3′-phosphorylated guanylic acid which would not undergo such oxidation–reduction reaction. In addition these termini have been shown to be mainly uridine and thus could not have been produced by RNase T_1. Hence our data indicated that the terminal oligonucleotides of the rRNA molecules lie within the protected area. Our findings that uridine and adenosine were the termini is in agreement with those on the intact 5S, 16S, and 23S rRNA of *E. coli* (McIlreavy and Midgley, 1967; Brownlee *et al.*, 1967).

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Levallorphan-Induced Accumulation of ppGpp in *Escherichia coli*†

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ABSTRACT: Levallorphan, a structural analog of morphine, was found to induce the accumulation of guanosine 5′-diphosphate, 3′-, or 2′-diphosphate in both a relaxed and a stringent strain of *Escherichia coli*.

Starvation for a required amino acid or restriction of the aminoacylation of tRNA causes a severe reduction of RNA accumulation in stringent (*rel*$^+$) but not relaxed (*rel*) strains of *Escherichia coli* (Edlin and Broda, 1968). Several seconds prior to the onset of this so-called stringent response, an unusual nucleotide, guanosine 5′-diphosphate, 3′-, or 2′-di-

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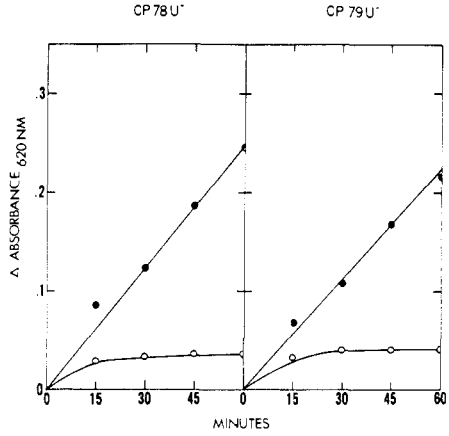

FIGURE 1: Effect of levallorphan on RNA accumulation. CP 78 U⁻ and CP 79 U⁻ were grown in the succinate medium to A_{500} = 0.4 when the cultures were divided into two equal portions and added to culture tubes containing levallorphan (1.54×10^{-3} M final) or sterile H_2O (0 min). Samples were withdrawn and assayed for RNA by the orcinol colorimetric method (Brown, 1946). The left column indicates CP 78 U⁻ (*rel*⁺) and the right CP 79 U⁻ (*rel*). The vertical axis represents the changes in absorbance at 620 nm due to orcinol color (RNA accumulation) divided by the cell densities of samples. Plus levallorphan (○); control without levallorphan (●).

phosphate (ppGpp) (Cashel and Kalbacher, 1970) begins to accumulate above the basal levels found in growing cells, whereas such an appreciable increase is not observable in relaxed strains (Cashel, 1969; Cashel and Gallant, 1969). Recently, it has been shown that ppGpp accumulates in stringent strains under various physiological conditions (carbon, nitrogen, and sulfur starvation) (Boquet *et al.*, 1971; Edlin and Donini, 1971), demonstrating that the ppGpp accumulation is not specific to the stringent response. In addition, during carbon shift-down or glucose starvation, relaxed as well as stringent strains accumulate ppGpp concomitantly with the inhibition of RNA accumulation (Harshman and Yamazaki, 1971; Lazzarini *et al.*, 1971; Winslow, 1971), indicating that its accumulation is not a specific property of stringent strains.

Levorphanol and levallorphan, structural analogs of morphine, are known to predominantly inhibit rRNA synthesis (Roschenthaler *et al.*, 1969; Simon and Van Praag, 1964a,b; Simon *et al.*, 1970a,b). Recently Boquet *et al.* (1971) reported that levallorphan causes accumulation of ppGpp in a stringent strain but not in a relaxed strain of *E. coli* and a greater inhibition of labeled uracil incorporation in the stringent strain than in the relaxed strain. Thus they concluded that the *rel* gene is directly involved in inhibition by levallorphan of RNA accumulation and that levallorphan triggers a regulation process mediated by the product of the *rel* gene. However, these results apparently conflict with the results of Simon and Van Praag (1964a) who reported that RNA accumulation in a relaxed and stringent strain of *E. coli* are similarly inhibited by levorphanol whose action is the same as that of levallorphan (Roschenthaler *et al.*, 1969). We suspected that the observed difference could possibly be accounted for by some minor alteration in experimental conditions. Simon and Van Praag (1964a) used succinate as a carbon source in the growth medium, whereas Boquet *et al.* (1971) used glucose. The present paper reports that levallorphan causes a similar accumulation of ppGpp in a relaxed as well as a stringent strain of *Escherichia coli* grown in the succinate medium of Simon and Van Praag (1964a).

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*). The uracil auxotrophs were derived in our laboratory from CP 78 and CP 79, which are isogenic except at the *rel* locus (Fiil and Friesen, 1968). Stringency and relaxedness of the uracil auxotrophs were found to be the same as their parent strains as determined by the effect of amino acid starvation on RNA accumulation which was measured by both orcinol colorimetric method and [^{14}C]uracil incorporation.

The cells were grown in a low phosphate medium buffered by 0.05 M Tris (pH 8.0), supplemented with 0.5% sodium succinate (in succinate growth medium) or 0.4% glucose (in glucose growth medium) as a carbon source and with 0.2% salt-free Casamino acids as sources of carbon and amino acids as described by Simon and Van Praag (1964a). In addition, thiamine (10 μg/ml) and uracil (20 μg/ml) were added. Bacteria were grown at 37° on a gyrotory water bath shaker. Absorbance at 500 nm (A_{500}) was taken as cell density of bacterial cultures. Under the present growth conditions, both strains entered their stationary phase of growth after the cell density reached A_{500} of 1.0.

Assay of ppGpp. Carrier-free [^{32}P]orthophosphate (Atomic Energy of Canada, Ltd.) was added to the cultures to a final specific activity of 270 mCi/mmole. The intracellular phosphate pools were found to be equilibrated within 10 min as judged by the labeling of the GTP and ATP pools. After 16-min preincubation with [^{32}P]phosphate, the culture was divided into two portions. To one portion, levallorphan tartrate (a gift from Hoffmann-La Roche Limited) was added to a final concentration of 1.54×10^{-3} M. Samples were withdrawn at various times from the untreated and treated cultures and were assayed for MS I (ppGpp) and MS II (Cashel and Gallant, 1969) by formic acid extraction and thin-layer chromatography as described previously (Cashel, 1969).

Results

The effect of levallorphan on cellular RNA accumulation was studied in CP 78 U⁻ (*rel*⁺) and CP 79 U⁻ (*rel*) which were grown in essentially the same medium as that described by Simon and Van Praag (1964a) containing succinate and Casamino acids as carbon sources. Our medium was buffered with Tris instead of triethanolamine. Such replacement does not affect the action of levorphanol (Simon *et al.*, 1970a). Figure 1 shows that upon the addition of levallorphan, RNA accumulation (as determined by the orcinol method) in both CP 78 U⁻ (*rel*⁺) and CP 79 U⁻ (*rel*) were equally inhibited. Thus this result is consistent with those reported by Simon and Van Praag (1964a). The effects of levallorphan on the ppGpp accumulation, [^{14}C]uracil incorporation into RNA, and cell growth were then investigated. The results are shown in Figure 2. The [^{14}C]uracil incorporation would not only provide the data comparable to that of Boquet *et al.* (1971), but also provide more detailed kinetic data than the orcinol assay. To ensure that the specific activity of the intracellular UTP approximated that of the extracellular uracil, the RNA labeling mixture was added 16 min prior to the addition of levallorphan. The addition of levallorphan to the cultures resulted in a rapid reduction in the rate of uracil incorporation in both CP 78 U⁻ and CP 79 U⁻. In both strains growth was markedly inhibited by levallorphan. The most interesting fact, however, is that levallorphan induced the accumula-

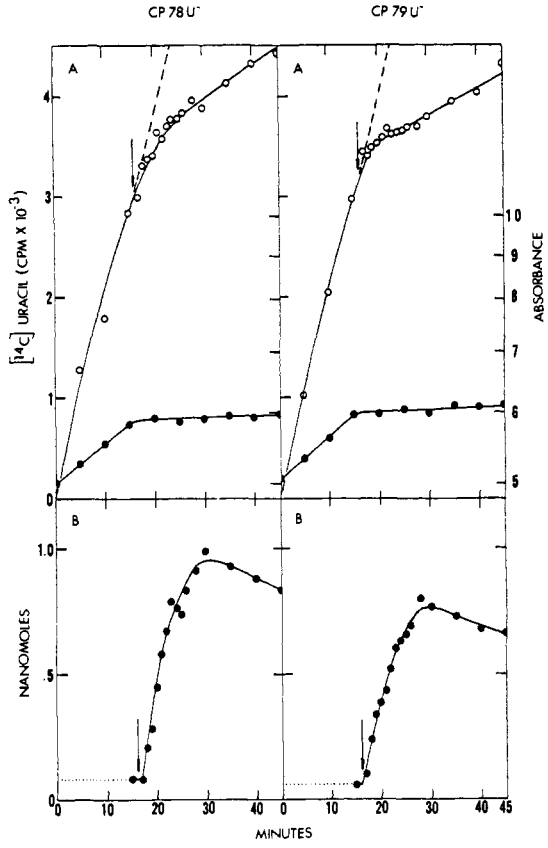

FIGURE 2: Effect of levallorphan on growth, [^{14}C]uracil incorporation, and ppGpp accumulation in the succinate medium. CP 78 U$^-$ and CP 79 U$^-$ were grown in the succinate medium to A_{500} = 0.5. Each culture was divided into three portions. The first portion was mixed with one-tenth the volume the RNA labeling mixture which consisted of [^{14}C]uracil (5 μCi/ml), uracil (100 μg/ml), and cytosine (200 μg/ml). To the second portion, [^{32}P]orthophosphate was added to yield a final specific activity of 270 mCi/mmole. The third portion was used for the assay of cell density. Zero time indicates the time of addition of the labels, [^{14}C]uracil or [^{32}P]orthophosphate. At various times, samples were withdrawn and assayed for labeled RNA according to the filter paper disk method (Bollum, 1968) and for ppGpp as described previously (Cashel, 1969). Levallorphan was added to a final concentration of 1.54 $\times$ 10^{-3} M at 16 min after the addition of the respective labels, as indicated by the arrows. The left column indicates CP 78 U$^-$ (*rel*$^+$) and the right, CP 79 U$^-$ (*rel*). Part A indicates [^{14}C]uracil incorporation (○) and cell density (●). The dashed lines indicate the incorporation of [^{14}C]uracil in the control without the addition of levallorphan. Part B indicates the amounts of phosphate incorporated into ppGpp expressed as nmoles/ml of culture of A_{500} = 1.0.

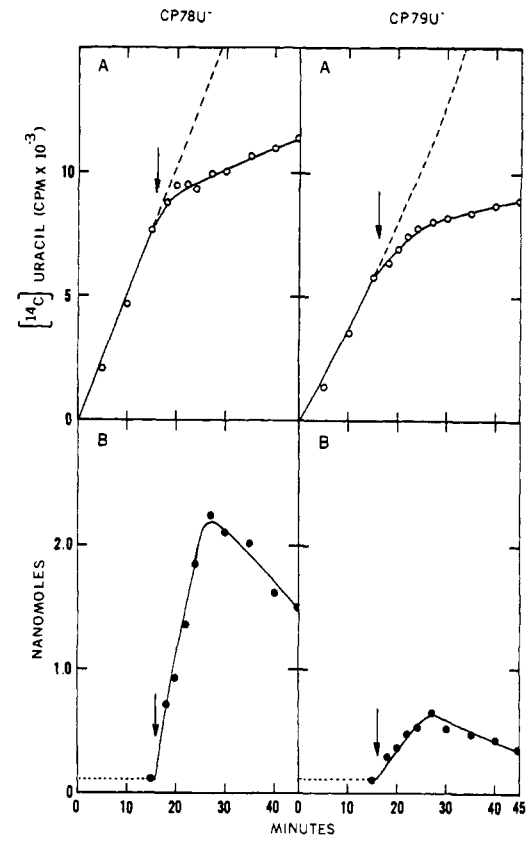

FIGURE 3: Effect of levallorphan on [^{14}C]uracil incorporation and ppGpp accumulation in the glucose medium. CP 78 U$^-$ and CP 79 U$^-$ were grown in the glucose medium to A_{500} = 0.5. Each culture was divided into two portions. The first portion was used for the assay of uracil incorporation and the second portion for the assay of ppGpp as described in Figure 2. Zero time indicates the time of addition of the RNA labeling mixture or [^{32}P]orthophosphate. Levallorphan was added to a final concentration of 1.54 $\times$ 10^{-3} M at 16 min as indicated by the arrows. The left column indicates CP 78 U$^-$ (*rel*$^+$) and the right, CP 79 U$^-$ (*rel*). Part A indicates [^{14}C]uracil incorporation (○). The dashed lines indicate [^{14}C]uracil incorporation in the control without the addition of levallorphan. Part B indicates the amounts of phosphate incorporated into ppGpp expressed as nmoles/ml of culture of A_{500} = 1.0.

tion of ppGpp in the relaxed and stringent strain. In both strains the kinetics of ppGpp accumulation was quite similar. Within 2 min after the addition of levallorphan, ppGpp began to accumulate and reached the maximum level (approximately 20% higher in CP 78 U$^-$) at approximately 10 min after the inhibited rate of uracil incorporation was established. In the absence of levallorphan, no increase in the level of ppGpp was observed during the period of the experiment. Although amino acid starvation of CP 78 U$^-$ causes a large increase in the level of another guanosine nucleotide, MS II as well as ppGpp (unpublished) just as with CP 78 (Cashel and Gallant, 1969), levallorphan did not cause an appreciable increase in the level of MS II above its basal level.

Both the strains Boquet *et al.* (1971) used and our strains are auxotrophic for both uracil and amino acids. The observed difference is then likely ascribed to the fact that they used glucose instead of succinate in the medium described by Simon and Van Praag (1964a). In fact, when succinate was replaced by glucose in our medium, a differential effect of levallorphan on ppGpp accumulation in CP 78 U$^-$ and CP 79 U$^-$ was observed (Figure 3B). The maximum amount of ppGpp accumulated in CP 78 U$^-$ was 3.6 times greater than that in CP 79 U$^-$. This amount was approximately twice greater than the maximum amount of ppGpp accumulated in CP 78 U$^-$ grown in the succinate medium (Figure 2B). As in the succinate medium (Figure 2A), levallorphan caused a similar reduction in the rate of uracil incorporation in both strains when grown in the glucose medium (Figure 3A).

Discussions

The present results clearly indicate that levallorphan can induce the accumulation of ppGpp in a relaxed as well as a stringent strain, when they are grown in the succinate medium of Simon and Van Praag (1964a). Therefore, the cellular capacity to produce ppGpp is not directly dependent on the

allelic state of the *rel* gene. This conclusion is substantiated by our previous results that a relaxed as well as a stringent strain produces similar quantities of ppGpp during carbon source shift-down (Harshman and Yamazaki, 1971) and by exposure to hypertonic NaCl solutions (Harshman and Yamazaki, 1972). Similarly, we can conclude, from the data in Figure 1, that the *rel* gene is not necessarily involved in inhibition by levallorphan of RNA accumulation.

Although the mode of action of levallorphan is not yet fully understood, its primary effect appears to be on the permeability of *E. coli* (Simon *et al.*, 1970b). Since the strains used in the present experiment are auxotrophic for both amino acids and uracil, it is possible that levallorphan inhibits the uptake of these nutrients, which might account for the observed inhibition of growth as well as RNA accumulation. However, it is known that neither amino acid starvation nor uracil starvation causes the production of ppGpp in the relaxed strain (Cashel and Gallant, 1969; our unpublished data). On the other hand, it is possible that levallorphan causes, through its membrane effects, the inhibition of the uptake of carbon sources. The ensuing carbon source starvation would in turn result in the accumulation of ppGpp in both strains (Lazzarini *et al.*, 1971). The comparison of the data in Figure 2B and 3B may suggest that levallorphan causes a much greater inhibition of amino acid uptake in the glucose medium than in the succinate medium. The higher level of ppGpp in the stringent strain grown in the glucose medium would then reflect a response to amino acid starvation, superimposed on carbon source deprivation.

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Comparative Studies of the Carbohydrate-Containing Components of 3T3 and Simian Virus 40 Transformed 3T3 Mouse Fibroblasts†

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ABSTRACT: Glucosamine-labeled glycoproteins and glycopeptides of normal and SV-40 transformed 3T3 mouse fibroblasts have been analyzed by a combination of acrylamide gel electrophoresis and chromatography on Bio-Gel P-10. Controls were included for the possible influence of cell growth rate on these measurements. Though the normal and transformed cell lines examined have demonstrable differences in overall carbohydrate content, the glycoproteins and glycopeptides of a bulk membrane fraction of the two lines could not be distinguished by comparison of profiles of glycoproteins on acrylamide gel electrophoresis or by profiles of glycopeptides on Bio-Gel P-10 chromatography. Elution of well-defined glycoprotein peaks from acrylamide gels, followed by chromatography of glycopeptides from the peak fraction also failed to reveal differences. Several transformation mechanisms are suggested which would result in cells of altered carbohydrate content but with similar or identical glycoproteins and glycopeptides.

Transformed cells are characterized by loss of growth control(s) present in normal cells. This loss of control leads *in vivo* to metastasis and invasive and unlimited growth. *In vitro* transformed cells exhibit failure of contact inhibition and thus pile up in multilayered growth. These characteristics of transformed cells may well depend on chemical alterations present in malignant cell membranes. For instance a purified glycoprotein (wheat germ agglutinin) aggregates transformed cells but not normal or nontransformed cells (Burger and

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