MESSENGER RNA TURNOVER AND PROTEIN SYNTHESIS IN ACTINOMYCIN INHIBITED ESCHERICHIA COLI

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Actinomycin D, a polypeptide antibiotic having powerful bacteriostatic effects, particularly against gram-positive organisms, is known to attach to guanine sites in the DNA template (Goldberg, et al., 1962) and thereby prevent RNA synthesis in Staphylococci and Neurospora, (Kersten, et al., 1960), B.subtilis (Levinthal et al., 1962) and mammalian cells, (Rabinovitz and Goldberg, 1962) as well as in cell-free systems (Hurwitz, et al., 1962). Since actinomycin D, by thus blocking DNA dependent synthesis of RNA, prevents the reutilization of breakdown products of messenger RNA, it offers a convenient tool for studying the metabolism of pre-existing RNA in these cells.

The use of actinomycin D in vivo has so far been limited to the above-mentioned organisms only, since it is not permeable in a number of other organisms. Thus, it does not normally affect E.coli cells and hence experimental evidence as to the fate of m-RNA in these cells is meagre. Recently, it has been shown that E.coli cells, pre-treated with EDTA, become sensitive to the subsequent action of the antibiotic (Lieve, 1965); hence, this system should prove useful in the study of the following problems:

i) the time constant of decay of pulse-labelled RNA in these cells;

ii) the loss of biological activity of pre-existing messenger for the synthesis of protein; and

iii) the calculation of mean life of messengers specific for the synthesis of particular enzymes.

An attempt has been made here to study the above problems in a E.coli vitamin B_{12}/methionine auxotroph. The results indicate that in these cells, pre-treated with EDTA, C^{14}-uracil incorporation is immediately and completely stopped by actinomycin D and that pulse-labelled RNA decays with a mean half life of 11 - 12 minutes. It has been shown that the incorporation of C^{14}-methionine into protein is stopped after approximately 15 minutes of addition of actinomycin D. However, the use of EDTA-treated cell preparations appears to be limited, since EDTA treatment results in a lowered rate of incorporation of C^{14}-methionine into protein, loss in the ability of the cells to form the enzyme alkaline phosphatase, and a reduced rate of synthesis of β-galactosidase upon induction.

Results and discussion: It is seen from Fig.1 that, in EDTA-treated E.coli cells, RNA synthesis is blocked, immediately and totally, by actinomycin D, when present simultaneously with C^{14}-uracil in the culture. It was ascertained that, in the control culture, actinomycin D has no effect on the incorporation of C^{14}-uracil, the graph for cpm against time in minutes being identical with that for the untreated controls. It can also be seen that the incorporation of C^{14}-uracil into RNA in EDTA-treated cells is less than that in the controls.

Having found that the EDTA-treated cells of E.coli become actinomycin sensitive, we have carried out studies on the fate of rapidly labelled RNA in these cells. Logarithmically growing cells were labelled for 1 minute, 10 minutes and 30

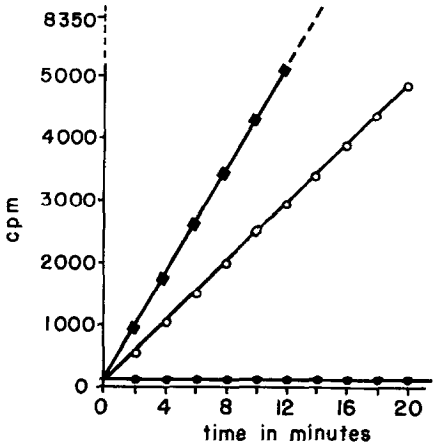

Fig.1. Effect of actinomycin D on RNA synthesis in E.coli cells. E.coli. vitamin B_{12}/methionine auxotroph was grown on the medium of Davis and Mingioli (1950). The logarithmically growing cells were harvested, washed with saline, followed by 0.01 M tris buffer of pH 8.0 and resuspended at a density of 5 x 10^9 cells per ml in 2 ml of 0.033 M tris buffer of pH 8.0. To one flask, 10^{-3} M EDTA was added. The other served as the control. After a 2 minute incubation with shaking, 20.0 ml of growth medium was added, followed, after a further 5 minutes, by 10 µc of uracil-2-C^{14} (specific activity, 11 µc per µmole) to each flask. Half of each culture was transferred to flasks containing actinomycin D (20 µg per ml). Samples were removed at various time intervals into 5% TCA containing 1 mg of unlabelled uracil. The precipitates were repeatedly washed with cold 5% TCA, and radioactivity was determined in a Packard Tricarb Liquid Scintillation Counter (Steinberg, et al., 1958). The counts are expressed per ml culture. o—o, acid precipitable C^{14} count in EDTA treated cells; ■—■, acid precipitable C^{14} count in untreated controls; ●—●, acid precipitable C^{14} count in EDTA treated cells exposed to actinomycin D action.

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minutes, with C^{14}-uracil. Actinomycin D was then added and the loss of acid precipitable radioactivity derived from C^{14}-uracil was measured as a function of time of exposure to the antibiotic. It is evident from Fig.2, that, in the presence of actinomycin D, a considerable fraction of rapidly labelled RNA decays. The percentage of radioactive RNA fraction that is labile decreases with the increase in duration of C^{14}-uracil labelling prior to actinomycin D treatment. This is to be expected because of the

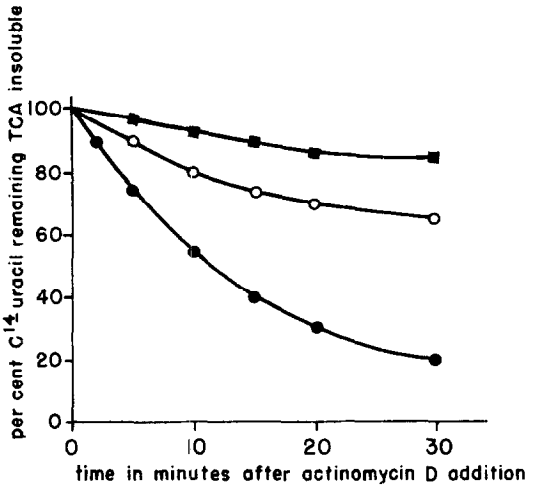

Fig.2. Degradation of pulse-labelled RNA of E.coli in presence of actinomycin D. EDTA-pre-treated E.coli cells were labelled with C^{14}-uracil for 1 minute, 10 minutes and 30 minutes. Details of labelling are as given under Fig.1. The labelling was terminated by addition of actinomycin D (20 µg per ml) and samples were taken at different time intervals for determination of C^{14}-precipitable radioactivity. ●—● pulse of C^{14}-uracil for 1 minute; ○—○ , labelling for 10 minutes; ■—■ , labelling for 30 minutes.

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higher proportion of ribosomal and soluble RNA's in cells labelled for longer time periods.

The half life of pulse labelled RNA in E.coli, which decays upon incubation in actinomycin D, is 11-12 minutes, a value which is much higher than that reported for β-galactosidase m-RNA (Nakada and Magasanik, 1964) or m-RNA of B.subtilis (Levinthal, et al., 1962). It is likely that EDTA, by binding the intracellular ions, interferes with the enzymatic breakdown of pulse-labelled RNA. Cohen, et al., (1961) have provided evidence implicating polynucleotide phosphorylase as the enzyme involved in the breakdown of pulse-labelled RNA in phage-infected E.coli, and the Mg^{++} or Mn^{++} requirement of this enzyme is well known

(Babinet, et al., 1965). The longer mean life for messenger in EDTA-treated cells could thus be explained.

The incorporation of C^{14}-methionine into proteins of EDTA-treated cells compared to the untreated controls, and the effect of actinomycin D in the two cases are shown in Fig.3.

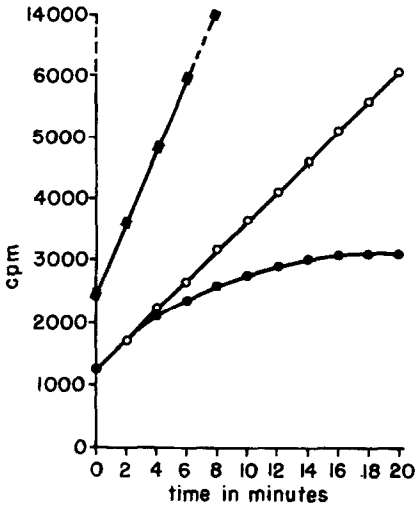

Fig.3. Effect of actinomycin D on methionine incorporation in EDTA-treated E.coli cells. E.coli mutant requiring either vitamin B_{12} or methionine was grown on the medium of Davis and Mingioli (1950). The legarithmically growing cells were harvested, washed with saline, 0.01 M tris buffer of pH 8.0 and resuspended at a density of 5×10^9 cells per ml in 2.0 ml of 0.033 M tris buffer of pH 8.0. To one flask, 10^{-3} M EDTA was added; the other served as the control. After a 2 minute incubation with shaking, 20.0 ml of growth medium was added to each. Incubation was continued for 5 minutes and 10 μc of C^{14}-methionine (methyl-labelled; specific activity 7 μc per μmole) was added. At the end of 5 minutes half of each culture was transferred to a flask containing actinomycin D (20 μg per ml). Samples were removed at indicated time intervals into 5.0 ml of 5% TCA containing 1 mg of unlabelled methionine. The counts in the protein precipitate are expressed per 1.0 ml culture. O–O , incorporation of labelled methionine into protein in EDTA-treated cells; O–O , incorporation of labelled methionine into protein in EDTA-treated cells exposed to actinomycin D action; ■–■ , incorporation of labelled methionine into protein in untreated controls.

In EDTA-treated cultures containing actinomycin D, the incorporation proceeds at nearly the same rate as in the controls without actinomycin D addition for about 4-6 minutes, after which, it is rapidly slowed down. There is a definite though small incorporation of methionine up to about 15 minutes. Actinomycin D was found to have no effect on amino acid incorporation in cells not treated with EDTA. It can also be seen from Fig.3 that EDTA-treated cells incorporate amino acid at a much lower rate compared to the untreated controls. The same trend of lowered synthesis of protein is observable in the

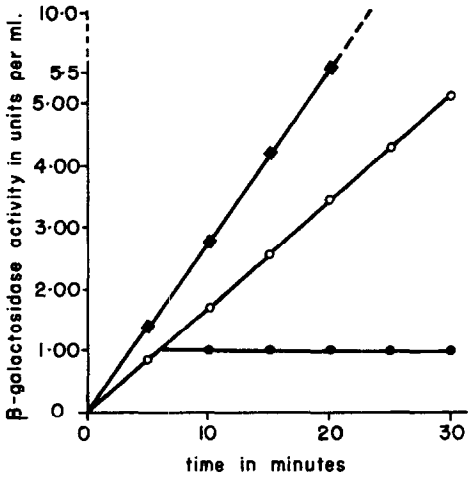

Fig.4. β-galactosidase induction in EDTA-treated E.coli cells E.coli cells grown on the medium of Davis and Mingioli (1950) with addition of 0.25 per cent glycerol as carbon source, were suspended in 0.033 M tris buffer of pH 8.0 at a density of 5×10^9 cells per ml. EDTA treatment was the same as described earlier. After a 10 minute incubation in the growth medium isopropylthiogalactoside (4×10^{-4} M in final concentration) was added. At the end of 5 minutes, half of each culture was transferred into flasks containing actinomycin D (20 μg per ml). Samples were taken at different time intervals into chloramphenicol-containing tubes (50 μg per ml). The samples were toluenised and assayed for β-galactosidase activity by the o-nitrophenyl β-D galactoside procedure (Roshizky et al., 1960). o—o , β-galactosidase activity in EDTA-treated cells; ●—● , β-galactosidase activity in EDTA-treated cells exposed to actinomycin D action; ■—■ , β-galactosidase activity in untreated controls.

case of induced formation of β-galactosidase in EDTA-treated cells and the messenger function for its synthesis decays with a decay constant of about 5-6 minutes (Fig.4), which is a much lower rate compared to the figures arrived at by other workers (Kepes, 1963; Nakada and Magasanik, 1964).

Another enzyme that has been investigated is alkaline phosphatase, which could be induced strongly in this mutant, by growing the cells in a medium devoid of phosphate (Pradhan and Sreenivasan, 1962). Thus, when E.coli cells grown for 18 hours on the medium of Davis and Mingioli (1950), are transferred to the same medium devoid of phosphate, alkaline phosphatase activity increases linearly with time up to 4 hours, such synthesis of the enzyme being de novo (Girija et al., 1964). However, if the cells

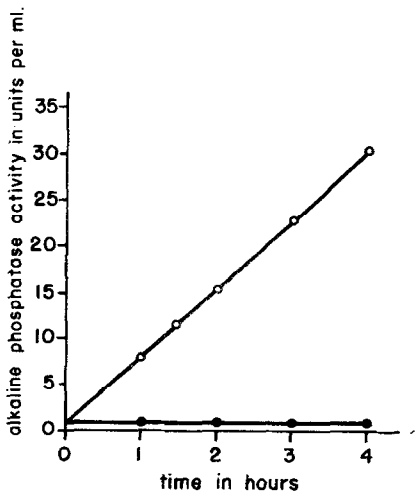

Fig.5. Alkaline phosphatase formation in E.coli vitamin B_{12}/methionine auxotroph. Cells were grown for 18 hours on the medium of Davis and Mingioli (1950) and then transferred to the above medium without phosphate. EDTA treatment of the cells was as described previously. Alkaline phosphatase activity was determined by the rate of hydrolysis of p-nitrophenyl phosphate (Pradhan and Sreenivasan, 1962).
●—● , alkaline phosphatase activity in EDTA-treated cells.
o—o , alkaline phosphatase activity in untreated controls.

are treated with EDTA prior to transfer to phosphate-deficient medium, the enzyme remains repressed (Fig.5).

It was pointed out by Lieve (1965), who also observed a decrease in the rate of synthesis of β-galactosidase by EDTA-treated cells, that the decrease may be due to the metabolite glycerol present as a result of lowered growth of the cells in EDTA-treated culture. It has been shown by Mandelstam (1961) and Magasanik (1961) that, when growth in E.coli is inhibited, β-galactosidase is repressed by carbon sources which do not hinder its formation during normal growth. The fact that the formation of alkaline phosphatase, which is not subject to this kind of repression, is blocked in EDTA-treated cultures and the inhibition of amino acid incorporation into protein observed in EDTA-treated cells, suggest that EDTA must be interfering, in a more general way, by blocking the RNA polymerase system, which is dependent on the presence of Mg ions. It is relevant to note that interconversions among the various polymerase activities have been demonstrated by treatments which are known to disrupt protein structure, EDTA treatment being one of them (Lee Huang and Cavalieri, 1965).

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