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FRACTIONATION OF RAPIDLY-LABELLED NUCLEIC ACIDS FROM *RHODOSPIRILLUM RUBRUM* USING POLYLYSINE KIESELGUHR COLUMN CHROMATOGRAPHY

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SUMMARY

Poly-L-lysine kieselguhr column chromatography has been used to fractionate a rapidly labelled RNA fraction from *Rhodospirillum rubrum*. Labelled nucleic acid mixtures, prepared from *R. rubrum* grown anaerobically in the light, were fractionated on poly-L-lysine kieselguhr columns. Preliminary results of this study are reported in this paper.

INTRODUCTION

The facultative photoheterotroph *Rhodospirillum rubrum* can be cultured either aerobically in the dark or anaerobically in the light, the two types of growth being manifested in the specific differences in cellular structure and metabolic pathways.

The process of transition from heterotrophic growth to photosynthetic growth, is accompanied by the extensive synthesis of protein pigments and other components of the photosynthetic apparatus. The heterotrophic organism possesses very low levels of pigments and structures necessary for photosynthesis, and when transferred to anaerobic light conditions, is able to grow only after a certain lag period. The organism must synthesise pigments and other components of the photosynthetic organelle during this lag phase, and this may require the synthesis of specific messenger RNA's (mRNA).

YAMASHITA AND KAMEN[1,2] extracted pulse-labelled RNA from *R. rubrum* cells labelled with 0.1 mCi of [^{3}H]uracil per ml of culture for 3 min. They examined and compared the properties of pulse-labelled RNA from cells grown in the dark and light, and found no differences in the base composition of the two RNA's. Methylated-albumin coated kieselguhr column chromatography and sucrose gradient centrifugation showed the pulse-labelled RNA to be associated with the ribosomal RNA. They therefore concluded that if a light-specific messenger RNA were required to initiate photopigment synthesis, it was present in an undetectable amount. However, although they could not detect the existence of a light-specific messenger RNA using hybridisation techniques, they observed a marked increase in uracil incorporation into RNA in the presence of light.

In this paper the fractionation of rapidly-labelled RNA extracted from *R. rubrum* using a poly-L-lysine kieselguhr (PLK) column, devised by AYAD AND BLAMIRE[3–5], was achieved.

PROCEDURES

Strain of bacteria

Rhodospirillum rubrum strain S, was used as the source of nucleic acids.

Culture of micro-organisms

R. rubrum were grown in the medium described by ORMEROD *et al.*[6]. Stock liquid cultures were grown in completely filled screw cap bottles in the presence of light. These were maintained by transferring every 24 h into fresh medium. A 24 h-old culture was used as an inoculum. Anaerobic cultures were grown in conical flasks, three-quarter filled with medium, maintained at 28–30° and illuminated on either side of the flask. Sufficient nitrogen gas was bubbled through to disperse the cells in the culture medium and to maintain an atmosphere of nitrogen in the flask. Cells were grown to the middle of logarithmic phase of the growth cycle to give a very bright red colour.

Preparation of DNA and nucleic acid mixtures

Preparation of DNA. R. rubrum DNA was prepared by the method of MARMUR[7].

Preparation of nucleic acid mixtures. Nucleic acid mixtures were prepared by a modification of DNA preparation as described by MARMUR[7]. Ribonuclease treatment was omitted and nucleic acids were precipitated with cold ethanol (95 %) after standing 2 h at —22°. The precipitate was collected by centrifugation for 5 min at 3000 r.p.m. at 4° using MSE Major centrifuge and dissolved in standard saline citrate. Two more deproteinisations were carried out as described by MARMUR[7] and the nucleic acid mixtures were precipitated with cold ethanol (95 %) and dissolved in standard saline citrate and stored in a concentrated form at 4°.

Preparation of rapidly-labelled nucleic acid mixtures. Rapidly-labelled nucleic acid mixtures were prepared by labelling the cells in the log phase of growth with 0.05 to 1 μCi of [^{3}H]uracil per ml of culture incubated under photosynthetic conditions as described previously. After labelling for the appropriate time, the reaction was stopped by the addition of 0.1 volume of 0.1 *M* sodium azide dissolved in saline–EDTA buffer pH 8, and an excess of crushed-ice. Rapidly-labelled nucleic acid mixtures were then prepared as described previously.

Preparation of PLK columns

PLK columns were prepared as described by AYAD AND BLAMIRE[3–5]. Nucleic acid mixtures prepared as described previously were fractionated on 5 g PLK columns in which the fractionating layer consisted of 5 g washed kieselguhr treated with 5 mg of polylysine. PLK column was eluted using a linear gradient of 0.4 *M* NaCl containing 0.02 *M* KH_2PO_4, pH 6.7 to 4 *M* NaCl containing 0.02 *M* KH_2PO_4, pH 6.7 (100 ml of each) according to the method of AYAD *et al.*[8]. The flow rate was about 20 ml/h and the extinction of the effluent was continually measured using LKB Uvicord UV absorptiometer (257 nm) and 2 ml or 4 ml fractions were collected using an LKB (Stockholm) fraction collector linked to the Uvicord system.

Following PLK fractionation of nucleic acids (2 mg), each fraction was estimated at 260 nm using Unicam SP 500 spectrophotometer, and the radioactivity was assayed by precipitating the nucleic acids with 5 % ice-cold TCA and filtering onto a glass fibre disc (grade GF/C). The discs, after washing with 5 % ice-cold TCA and cold ethanol (95 %), were dried using an IR lamp. The discs were placed in 5 ml scintillator, (0.4 % 2,5-diphenyloxazole and 0.04 % 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, dissolved in xylene), and counted in the Packard "Tricarb" liquid scintillation spectrometer using three channels with a gain of 60 % and window settings of 50 to 800 in all the three windows.

DNA was estimated by the diphenylamine test[9], RNA by the orcinal test[10] and protein by the method of LOWRY *et al.*[11]. NaCl gradient was measured by using the conductivity cell.

RESULTS

The results obtained when a sample of nucleic acid mixture prepared as described previously was fractionated on PLK column is shown in Fig. 1. Five distinct peaks are obtained, eluting at the salt molarities of 0.4, 1.0, 1.2, 1.75 and 1.9 *M* NaCl. The peak eluting at 0.4 *M* NaCl consists of nucleotides and oligonucleotides of low molecular weight, and most of this material can be removed by dialysis prior to fractionation. Its immediate elution at 0.4 *M* NaCl indicates that it is not retained by the column. When the fractions from each peak are assayed for DNA[9], RNA[10] and protein[11], the results obtained are shown in Fig. 2. The 0.4 and 1.9 *M* NaCl peaks consist of RNA and DNA with some protein respectively. The other three peaks eluting at 1.0, 1.2 and 1.75 *M* NaCl, consist mainly of RNA with very little DNA. Further confirmation of these findings is provided by the elution profiles shown in Fig. 3. They are: (A) *R. rubrum* DNA prepared by the method of MARMUR[7], (B) transfer RNA extracted from yeast and supplied commercially by Sigma Chemical Co., and (C) highly polymerised RNA supplied by B.D.H. Ltd.

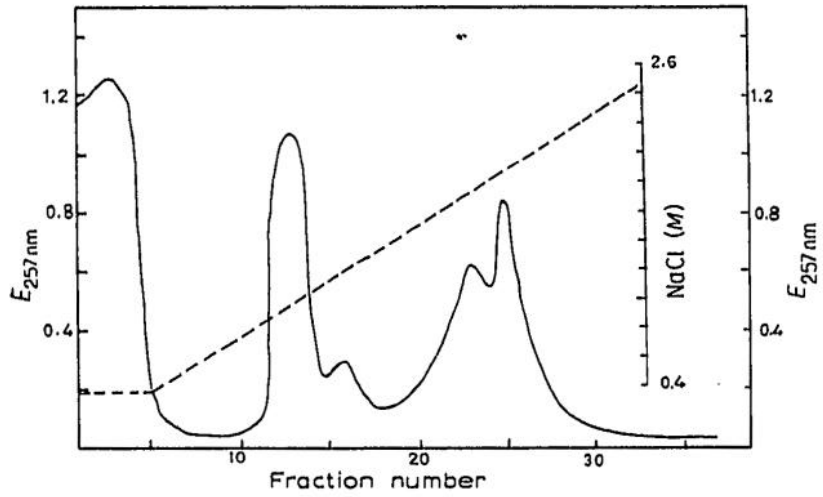

Fig. 1. The continuous elution profile from a 5 g PLK column monitored using an LKB Uvicord UV spectrophotometer of a nucleic acid mixture obtained from *R. rubrum*, using a linear gradient between 0.4 and 4.0 *M* NaCl. 4-ml fractions were collected. E_{257nm} (————) and NaCl molarity (– – – –).

The fractionation of a nucleic acid mixture extracted from a culture incubated for 24 h under photosynthetic conditions as described above in the presence of [^{3}H]uracil, 2 μCi/ml of culture, is shown in Fig. 4. It can be seen that the radioactivity is distributed evenly in all the RNA peaks, this is due to the long period of incubation with [^{3}H]uracil.

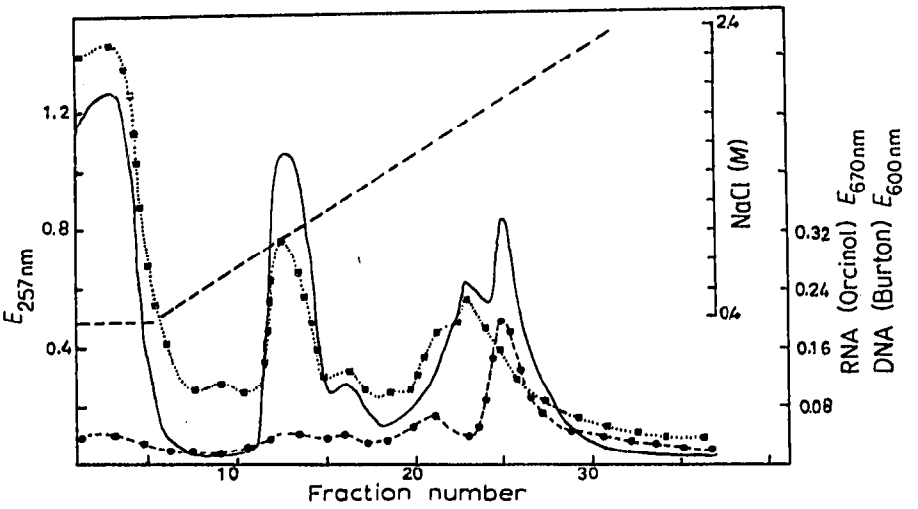

Fig. 2. Samples are taken from a fractionation of a nucleic acid mixture on PLK column (see Fig. 1) and assayed for RNA and DNA. E_{257nm} (———), RNA (■...■), DNA (●---●) and NaCl molarity (– – – –).

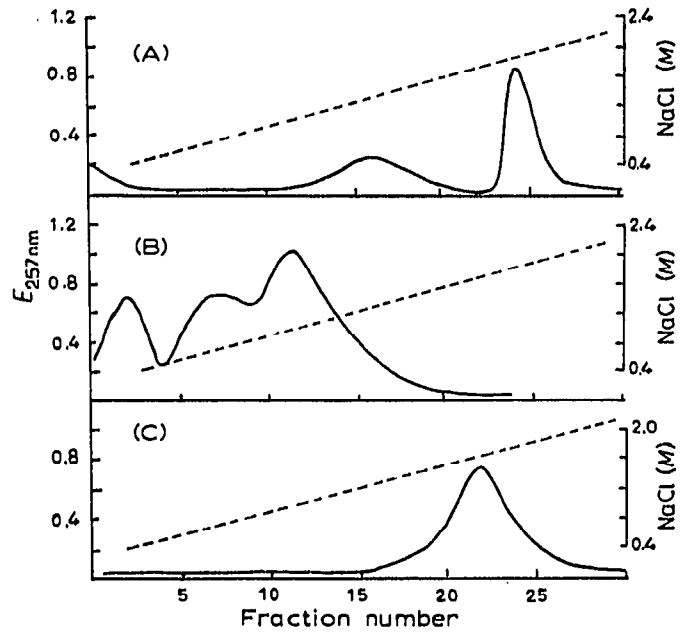

Fig. 3. Continuous elution profile from a 5 g PLK column of (A) DNA isolated from *R. rubrum*, (B) yeast tRNA and (C) highly polymerised RNA. E_{257nm} (———) and NaCl molarity (– – – –).

The elution profiles obtained from fractionation of nucleic acid mixtures on PLK columns, extracted from cultures incubated for 0.5, 1, 5, 10 and 60 min in the presence of [^{3}H]uracil, 0.1 μCi/ml of cultures are shown in Figs. 5, 6, 7, 8 and 9 re-

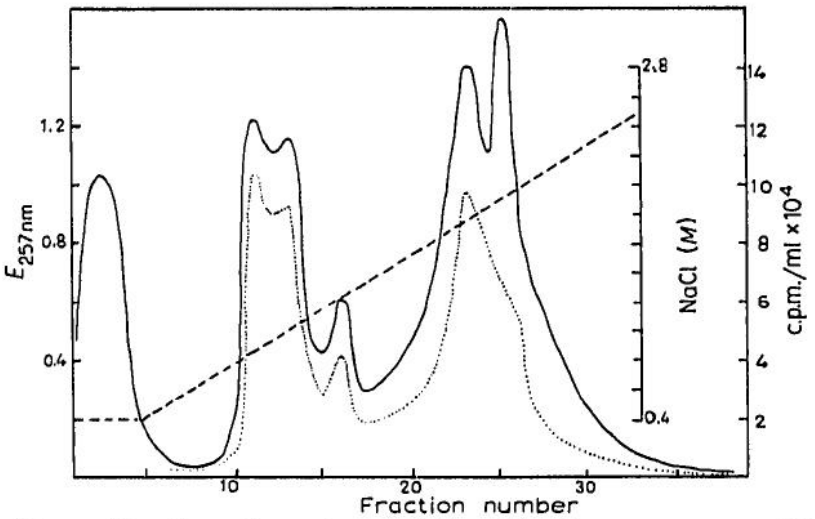

Fig. 4. Fractionation of a nucleic acid mixture on a 5 g PLK column, extracted from a culture of *R. rubrum* incubated for 24 h with [^{3}H]uracil (2 μCi/ml of culture); 4-ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

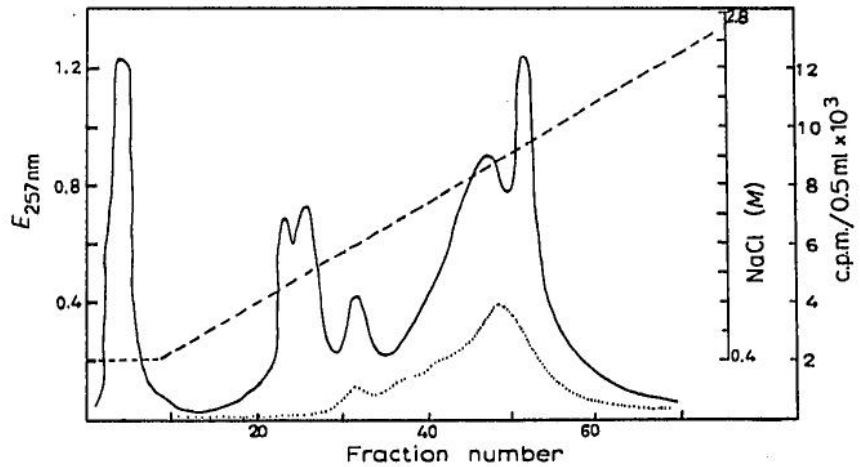

Fig. 5. Fractionation of a nucleic acid mixture extracted from *R. rubrum* pulse-labelled for 30 sec with [^{3}H]uracil (0.1 μCi/ml of culture) on PLK column; 2-ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

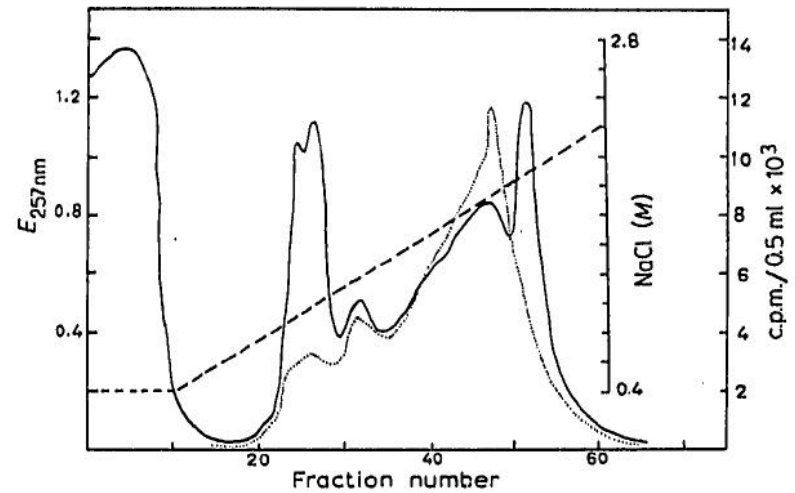

Fig. 6. PLK fractionation of a nucleic acid mixture, extracted from *R. rubrum* pulse labelled for 1 min with [^{3}H]uracil (0.1 μCi/ml of culture); 2 ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

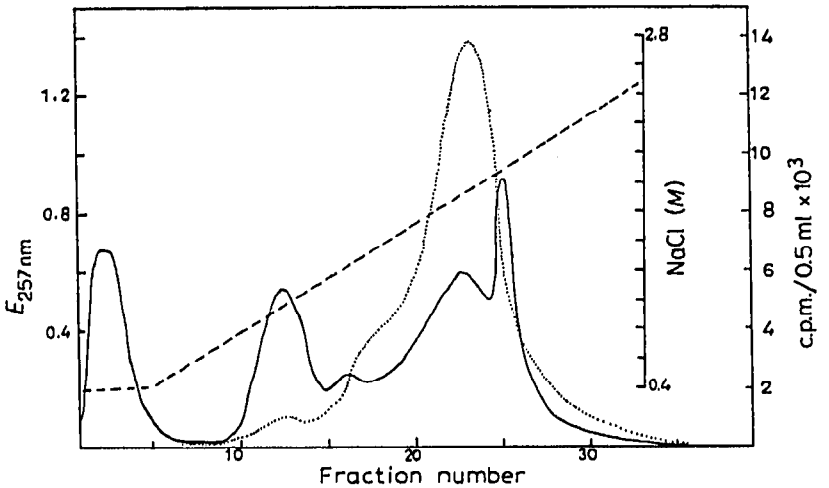

Fig. 7. PLK fractionation of a nucleic acid mixture, extracted from *R. rubrum* labelled for 5 min with [^{3}H]uracil (0.1 μCi/ml of culture); 4 ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

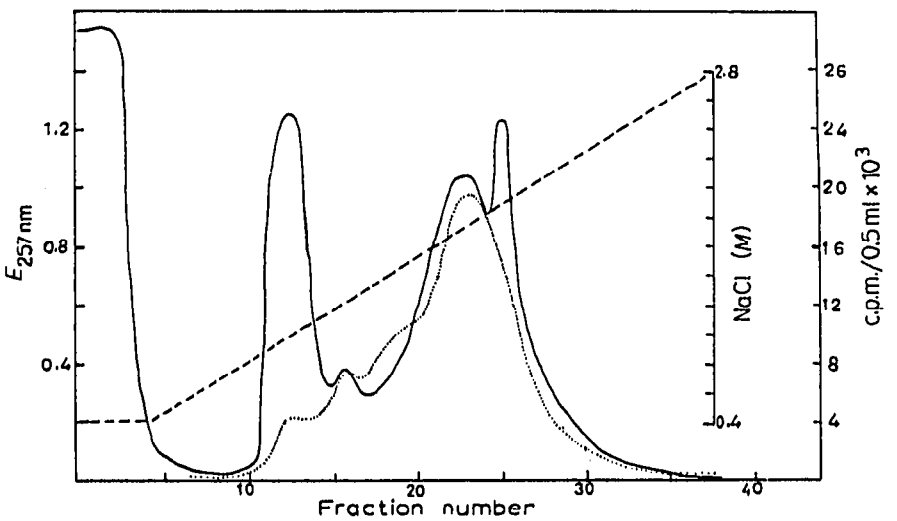

Fig. 8. PLK fractionation of a nucleic acid mixture, isolated from *R. rubrum* labelled for 10 min with [^{3}H]uracil (0.1 μCi/ml of culture); 4 ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

spectively. There is a very marked variation in the distribution of radioactivity in the RNA peaks with the time of incubation.

Proflavine is known to inhibit the synthesis of RNA in *Escherichia coli* in a manner similar to that of actinomycin D in other systems[12,13]. The inhibition of RNA synthesis occurs because actinomycin D complexes with the DNA and subsequently inhibit transcription[14–16]. The synthesis of RNA in viruses is unaffected by the antibiotic[16]. The effect of proflavine on rapidly-labelled RNA in *R. rubrum* was investigated. Total nucleic acid were isolated and fractionated on PLK columns and the results are shown in Figs. 10–12. It can be shown that proflavine is very effective in inhibiting RNA synthesis in *R. rubrum*. During a 1 min exposure to proflavine, at a concentration of 60 μg/ml of culture, in the presence of [^{3}H]uracil (0.05 μCi/ml of culture), the RNA synthesis is inhibited by about 82 %. Moreover, if the cells are

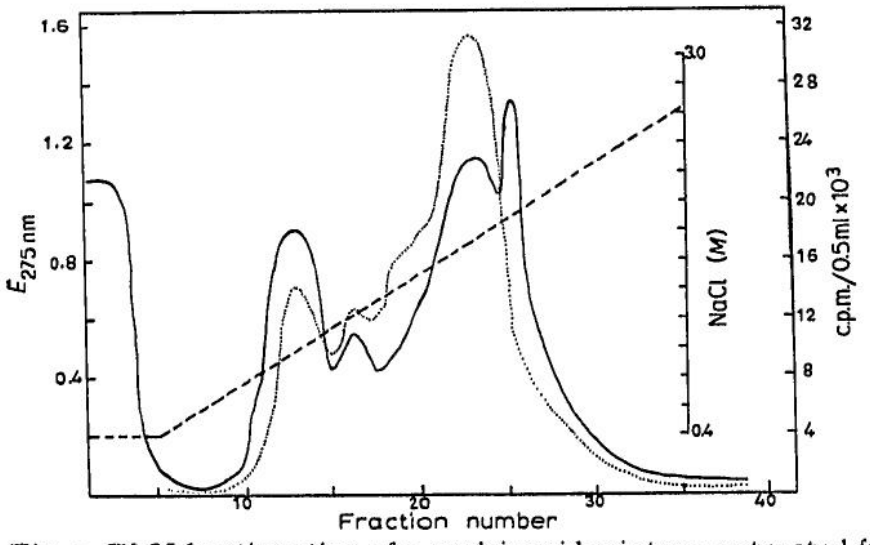

Fig. 9. PLK fractionation of a nucleic acid mixture, extracted from *R. rubrum* labelled for 60 min with [^{3}H]uracil (0.1 μCi/ml of culture); 4 ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

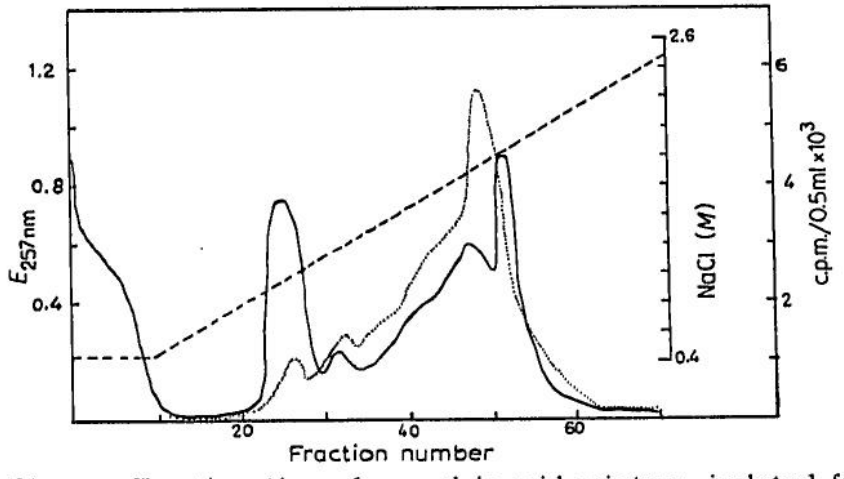

Fig. 10. Fractionation of a nucleic acid mixture, isolated from *R. rubrum* incubated for 1 min with [^{3}H]uracil (0.05 μCi/ml of culture), on PLK column; 2 ml fractions were collected. E_{257nm} (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

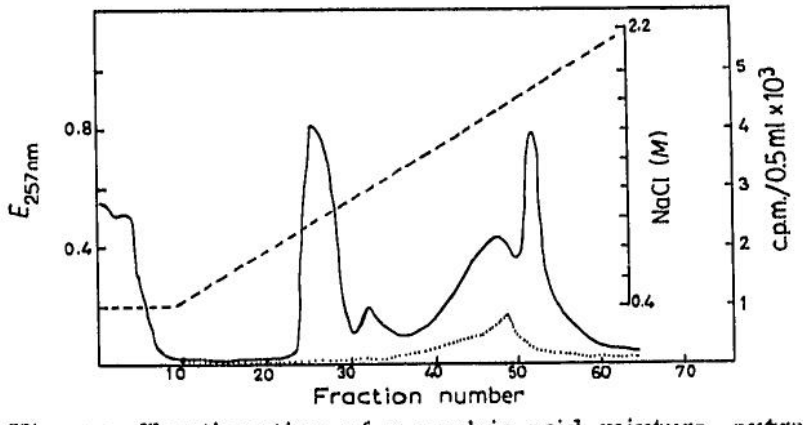

Fig. 11. Fractionation of a nucleic acid mixture, extracted from *R. rubrum*, incubated with [^{3}H]-uracil (0.05 μCi/ml of culture) and proflavine (60 μg/ml), on PLK column; 2 ml fractions were collected. $E_{257\,nm}$ (———), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

incubated for 5 min with the same concentration of proflavine and then labelled for 1 min with [^{3}H]uracil (0.05 μCi/ml of culture), the RNA synthesis is totally suppressed. This suggests that the cells pretreated with proflavine for 5 min, have almost entirely lost their ability to synthesise RNA, as the cells are unable to incorporate any radioactivity into RNA species.

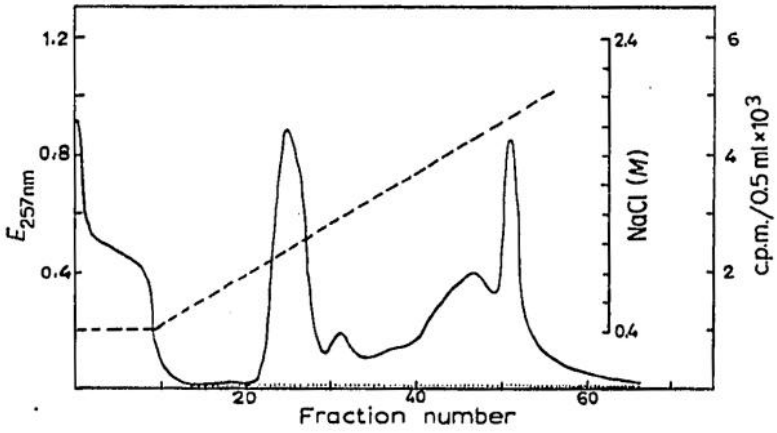

Fig. 12. Fractionation of a nucleic acid mixture, isolated from *R. rubrum* treated for 5 min with proflavine (60 μg/ml) and incubated with [^{3}H]uracil (0.05 μCi/ml) for 1 min, on PLK column; 2 ml fractions were collected. E_{257nm} (————), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

PLK fractionation of rapidly-labelled RNA of treated and untreated cultures of *R. rubrum* with proflavine (Figs. 10–12), shows that at the end of the treatment the bacteria have lost most of their endogenous supply of "rapidly renewable RNA". The RNA species which is very rapidly labelled in the absence of proflavine must be at least partly equivalent to messenger RNA, the effect of proflavine on *R. rubrum* could be to degrade the rapidly-labelled RNA species and to stop the transcription of RNA by complexing with the DNA molecule.

The effect of actinomycin D (which is known to inhibit RNA synthesis in *Bacillus subtilis*)[21] on rapidly-labelled RNA in *R. rubrum* was also studied, and it was found that this antibiotic (at a concentration of 1 μg/ml of culture) had very little effect on RNA synthesis.

It has been reported that in *B. subtilis* cells during exposure to chloramphenicol, they produce large quantities of RNA, while protein synthesis ceases[22]. The effect of chloramphenicol, at a concentration of 20 μg/ml of culture, on RNA synthesis in *R. rubrum* cells was investigated in the presence of [^{3}H]uracil (0.05 μCi/ml of culture) and the results are shown in Fig. 13. It can be seen that the RNA synthesis was inhibited by about 25 %. This finding agrees with the results of YAMASHITA AND KAMEN[2]. However, the effect of proflavine on the rapidly-labelled RNA is very much less in the presence of chloramphenicol as shown in Fig. 14.

DISCUSSION

Fractionation of nucleic acids extracted from *R. rubrum* on PLK columns, shows five distinct peaks, eluting at salt molarities 0.4, 1.0, 1.2, 1.75 and 1.9 *M* NaCl. The peak eluting at 1.9 *M* NaCl is identified as DNA by the diphenylamine test[9],

and also by the fractionation of standard *R. rubrum* DNA prepared by the method of MARMUR[7] (Fig. 3A). Peaks eluting at 1.0, 1.2 and 1.75 *M* are identified as RNA by the orcinol test[10]; these peaks are further identified as transfer RNA and ribosomal RNA by the fractionation of yeast transfer RNA and *E. coli* ribosomal RNA (Figs. 3B and C).

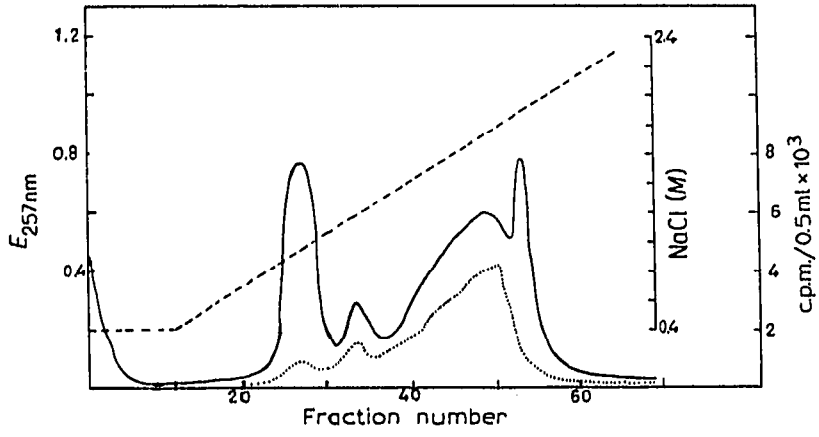

Fig. 13. Fractionation of a nucleic acid mixture extracted from *R. rubrum* treated with chloramphenicol (20 μg/ml) for 5 min and incubated with [^{3}H]uracil (0.05 μCi/ml) for 1 min, on PLK column; 2 ml fractions were collected. E_{257nm} (————), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

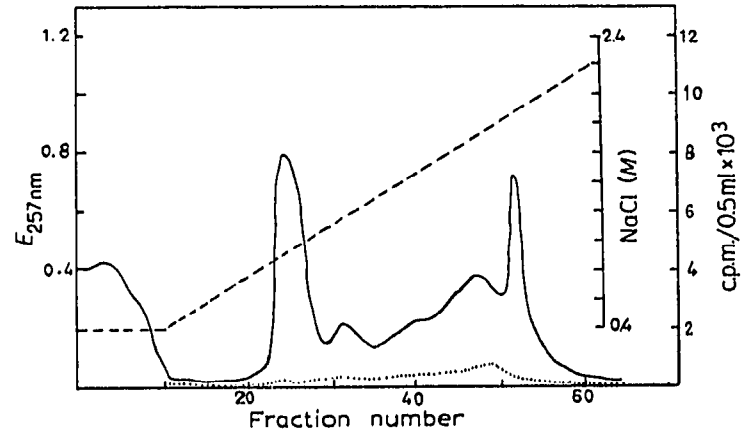

Fig. 14. Fractionation of a nucleic acid mixture, extracted from *R. rubrum* treated with chloramphenicol (20 μg/ml) and proflavine (60 μg/ml) for 5 min and incubated with [^{3}H]uracil (0.05 μCi/ml) for 1 min, on PLK column; 2 ml fractions were collected. E_{257nm} (————), ^{3}H-activity (· · · · · ·) and NaCl molarity (– – – –).

Previous studies on rapidly-labelled RNA in *R. rubrum* have shown that a major portion appears in the region of ribosomal RNA using sucrose gradient centrifugation and methylated albumin kieselguhr columns (MAK)[1,2]. The results reported here show that the rapidly-labelled RNA appears in the region of ribosomal RNA in a PLK fractionation, after a 30 sec exposure to [^{3}H]uracil. Incubation for 1 min however, results in the [^{3}H]activity appearing in the transfer RNA region. Moreover, the lack of coincidence between the radioactivity and the extinction profiles of the peaks indicates that uracil was first incorporated into RNA species other than the main ribosomal and transfer RNA's.

HURWITZ *et al.*[23] reported that proflavine exerts an action similar to that of actinomycin D and inhibits both enzymatic reactions leading to RNA and DNA synthesis. Furthermore, they reported that in the case of proflavine the DNA synthesis is more sensitive than RNA synthesis. SOFFER AND GROS[24] have studied the effect of proflavine and dinitrophenol (DNP) on the synthesis of RNA in *E. coli* and concluded that proflavine is more specific with respect to its inhibition of DNA transcription *in vitro* and effective in degrading the rapidly-labelled RNA *in vivo*. The residual RNA in the proflavine- or DNP-treated cells has lost a significant proportion of its capacity to stimulate amino acid incorporation *in vitro* into protein. However, the protein synthesising machinery remains intact after treatment with DNP, and the inhibition of growth caused by this drug is reversed almost immediately after removal of DNP.

GROS *et al.*[12] observed that if the duration of the radioactive pulse prior to DNP of proflavine treatment in *E. coli* is increased, the percentage of rapidly-labelled RNA fraction decreases and they suggested that the ribosomal RNA and transfer RNA are conserved in DNP- or proflavine-treated cells. In agreement wtth these findings it was observed that when *R. rubrum* cells were treated with proflavine for 5 min, before the addition of [^{3}H]uracil, they lost their ability to incorporate any radioactive uracil into RNA species. The RNA from cells treated with proflavine for 1 min in the presence of [^{3}H]uracil, however, have lost a significant proportion of its rapidly-labelled RNA indicating that RNA synthesis is blocked. Rapidly-labelled RNA synthesised in the absence of proflavine may represent partly a messenger RNA, the only known biologically active RNA *in vitro*[19,20]. The effect of proflavine on *R. rubrum* could be to degrade the rapidly-labelled RNA species and to stop the transcription by complexing with the DNA. There also appears a slow loss of ribosomes from the cells treated with proflavine. COST AND GRAY[18] have observed similar effects of proflavine on DNA synthesis in *Rhodopseudomonas spheroides*. YAMASHITA AND KAMEN[1,2] observed a rapid decrease in the incorporation of [^{3}H]uracil into RNA species after the addition of proflavine to a culture of *R. rubrum* cells.

It is evident from the results presented here that a rapidly-labelled RNA fraction isolated from *R. rubrum* exists and can be fractionated using a PLK column. This RNA species is shown to be degraded by treating the culture with proflavine. Work is in progress to characterise further the rapidly-labelled RNA fraction in the anaerobically grown cells and also to examine and compare its properties with the rapidly-labelled RNA fraction in the aerobically grown cells.

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