Sedimentation Properties of Ribonucleic Acid from *Rhodopseudomonas spheroides*

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SUMMARY

Phenol-purified RNA was prepared from *Rhodopseudomonas spheroides* and characterized by sucrose-gradient centrifugation. The patterns obtained indicate that *R. spheroides* possesses a ribosomal RNA complement similar to that of *Escherichia coli*. This is contrary to previous claims that the 23S component is missing. This species of ribosomal RNA was demonstrated to be more labile than the corresponding component in *E. coli* and variations in yields produced by changes in extraction procedures are described.

INTRODUCTION

Although sedimentation profiles of ribosomes obtained from many species of bacteria invariably exhibit 50S and 30S subunits, which yield respectively 23S and 16S RNA molecules (Osawa, 1968), it has been reported (Lessie, 1965) that the facultative photoheterotroph, *Rhodopseudomonas spheroides*, does not contain the 23RNA species, which implies that this bacterium is unique in that it can build all its ribosomes from the 16S species alone. However, some data indicating that *R. spheroides* does in fact contain the usual ribosomal subunits have appeared (Lessie, 1965; Friedman, Pollara & Gray, 1966). In addition, the sedimentation profiles of *Rhodospirillum rubrum* and other facultative photoheterotrophs have revealed both types of RNA with sedimentation characteristics quite similar to those obtained from *Escherichia coli* (Lessie, 1965; Yamashita & Kamen, 1968). In the course of researches on polynucleotide metabolism in *R. spheroides*, we have had occasion to examine RNA composition in this organism, including not only the strain investigated originally but also two others—one of which is a uracil-requiring mutant—and report herewith that in all cases the 23S RNA component can be isolated, but that its lability is pronounced. This fact appears to be the source of the present contradictions in previous reports.

METHODS

Organisms

The strains of *Rhodopseudomonas spheroides* used were: no. 2.4.1 (originally from the collection of Professor C. B. van Niel); another culture of no. 2.4.1 obtained from Dr S. Kaplan; and no. 5-11, a uracil-requiring mutant of strain GA, obtained from Dr J. Lascelles. The *Escherichia coli* used was laboratory strain K12-W3110.

Growth of Organisms

Rhodopseudomonas spheroides was grown photosynthetically, from a 1/10 (v/v) inoculum, in medicine bottles, under strong illumination, at 30 to 32°, in the glutamate medium of Lascelles (1959). For radioactive labelling, the medium was supplemented with 0.5 μ C/2 μ g./ml. of either tritiated or ¹⁴C-uracil. Organisms were grown mostly to about the second half of the logarithmic phase, although on occasion organisms in early stationary phase were used with no apparent effect on results. *Escherichia coli* was grown with forced aeration, at 37°, for about five generations in K medium (Green, 1966), supplemented with 0.5 μ C ¹⁴C-uracil/2 μ g./ml. All organisms were harvested by centrifugation. As explained in the Results section, it was found necessary to avoid storage by freezing and to use these suspensions immediately for RNA isolation and characterization.

Preparation of phenol-purified RNA from Rhodopseudomonas spheroides

Organisms were washed once with the appropriate buffer (see Results) and re-suspended, at about $\frac{1}{10}$ th of their original volume, in buffer containing 10 μ g. DNase (Worthington DNase I, electrophoretically purified) per ml. The suspensions were chilled on ice and passed through a pre-cooled French pressure cell at 5000 to 9000 p.s.i. They were then left at room temperature for 10 to 15 min. for degradation of the DNA. Sodium dodecylsulphate (SDS) was added to a final concentration of 0.5 to 1% and the mixture extracted three times with freshly distilled phenol equilibrated with the buffer used in extraction. The aqueous phase of the last extraction was precipitated with twice its volume of ethanol, in ice, for about one half hour. When the extinction was to be measured, the precipitates were washed twice with ether to remove traces of phenol, then dried with air. The precipitates were dissolved in small volumes of SSC (0.15 M-NaCl, 0.015 M-Na citrate).

Preparation of phenol-purified RNA from Escherichia coli

The bacteria were washed once with 5×10^{-3} M-tris-Cl, pH 7.3, containing 10^{-2} M-MgSO₄. They were resuspended in the same buffer at $\frac{1}{25}$ th of their original volume, and lysozyme was added to a concentration of 100 μ g./ml. The bacteria were then frozen and thawed three times, over a period of 40 hr. After the last thawing DNase was added to a final concentration of about 20 μ g./ml., and degradation allowed to proceed at room temperature for 15 min. The preparation was made 1% in SDS and stirred well. It was then extracted three times with freshly distilled phenol equilibrated with the above buffer. The aqueous phase from the last extraction was precipitated with twice its volume of ethanol. After centrifugation, the precipitates were dried with air and dissolved in a small volume of SSC.

Sucrose gradient centrifugation

Linear, 27 ml. gradients of 5 to 20% w/v sucrose in 0.01 M-tris-Cl + 0.1 M-NaCl, pH 7.5, were prepared with the aid of an apparatus similar to that of Britten & Roberts (1960). The gradients were left to equilibrate in the cold (4°) for at least one half hour and up to 24 hr. A small sample (0.05 to 0.2 ml.) of the phenol-purified RNA preparations was layered on top of the gradient and centrifugation performed in

a Model L or a Model L-2 Beckman ultracentrifuge, using the SW 25.1 swinging bucket rotor (24,000 rev./min., 15 hr, $T = 3^\circ$). One ml. fractions were collected from the tubes after puncture in the usual fashion. Radioactive samples were precipitated with cold trichloroacetic acid (TCA), filtered over Schleicher and Schuell type B-6 membrane filters, and counted in a scintillation counter. Unlabelled samples were assayed by their extinction at 260 m μ (nm.).

RESULTS

It was found that *Rhodopseudomonas spheroides* possessed an RNA complement similar to that of *Escherichia coli*, i.e. the ribosomal RNA had two components that corresponded to the 16S and 23S RNAs. However, the 23S RNA was labile with yields varying under different conditions of extraction.

Fig. 1(a). Sucrose gradient pattern of a mixed preparation of *Rhodopseudomonas spheroides* and *Escherichia coli* RNA, in the absence of Mg^{2+} , *R. spheroides* no. 2.4.1 was grown on 3H -uracil; *E. coli* was grown on ^{14}C -uracil. The two cultures were mixed; RNA was phenol purified, precipitated with alcohol and dissolved in SSC. A portion of this preparation was layered on top of a 5–20% (w/v) sucrose gradient in $10^{-2} M$ -tris-Cl, (pH 7.5), + 0.1 M-NaCl. —○—, ^{14}C (*E. coli* RNA); —●—, 3H (*R. spheroides* RNA).

(b) Sucrose gradient pattern of a mixed preparation of *R. spheroides* and *E. coli* RNA in the presence of $10^{-2} M-Mg^{2+}$. The RNA preparation in Fig. 1a was dialysed against $5 \times 10^{-3} M$ -tris-Cl (pH 7.5), + $10^{-2} M-MgSO_4$ and analysed as in Fig. 1a except that the gradient contained also $10^{-2} M-Mg^{2+}$. —○—, ^{14}C (*E. coli* RNA); —●—, 3H (*R. spheroides* RNA)

In one experiment *Rhodopseudomonas spheroides* was grown on 3H -uracil and *Escherichia coli* on ^{14}C -uracil, as described in the Methods section. After measuring the TCA precipitable counts, the two cultures were mixed so as to contain an approximately equivalent amount of the two isotopes, and RNA was extracted from the mixed cultures by the usual procedure (see Methods); the buffer used was 0.005 M-tris-

Fig. 2. For legend see opposite page.

Cl, (pH 7.3) + 0.01 M-MgSO₄ ("TM"). Part of the final preparation (in SSC) was analysed on a sucrose gradient (Fig. 1*a*). Another portion was dialysed against TM buffer and then analysed on a sucrose gradient containing 10⁻² M-MgCl₂ (Fig. 1*b*). The resulting fractionation pattern clearly revealed RNA complements similar to those of *E. coli*, i.e. three peaks corresponding to the 4S, 16S and 23S species. Moreover, *R. spheroides* RNA, like *E. coli* RNA, sedimented more rapidly in the presence of Mg²⁺, but the three components were again clearly visible. However, the ratio of 23S to 16S RNA in *E. coli* was approximately 2:1, as expected for an equal number of 50S and 30S ribosomes (because 23S RNA has twice the molecular weight of 16S RNA), while for *R. spheroides* the ratio was lower. Since all operations were carried out on the mixed cultures, it was apparent that 23S RNA in *R. spheroides* was more labile.

In the course of a large number of RNA extractions from the three different cultures of *Rhodopseudomonas spheroides* used, the relative amounts of 23S and 16S ribosomal RNA were found to vary, ranging from no apparent 23S to a ratio of 23S/16S of 1.7 in the best preparation obtained (see Fig. 2*a-e*).

The phenol-purified RNA preparations degraded randomly with time when stored at 4°. At -20° they were more stable, but a decrease in the 23S to 16S ratio was still noted. It appeared that the 23S RNA was sensitive to extraction conditions. Further experiments were performed to evaluate the effects of different parameters in the extraction procedure.

(*a*) *Effect of Mg²⁺ concentration.* As this factor was known to affect the 23S to 16S ratio in *Escherichia coli* (Midgley, 1965*a*), we tried extractions in 5 × 10⁻³ M-tris-Cl, (pH 7.3), buffer with varying amounts of Mg²⁺ from none up to 10⁻² M-MgSO₄. In general, slightly higher yields of 23S relative to 16S were obtained in the absence of Mg²⁺, but the over-all yields of RNA extracted in this manner were from 2 to 10 times lower than when Mg²⁺ was present. Therefore, when it is not essential to have intact 23S RNA, as in preparations used for hybridization assays, we recommend extraction in the presence of Mg²⁺.

(*b*) *Phenol.* It was found essential for both *Rhodopseudomonas spheroides* and *Escherichia coli* that phenol be freshly distilled. Various methods for storage of distilled phenol were tried, such as maintenance at 4° or -10°, presence or absence

Fig. 2. Sucrose gradient patterns of several preparations of RNA from *Rhodopseudomonas spheroides*. The gradient was 5 to 20 % (w/v) sucrose in 10⁻² M-tris-Cl, (pH 7.5) + 0.1 M-NaCl. ¹⁴C labelled RNA from *E. coli* was prepared separately and was used in some of the experiments as a marker.

(*a*) *R. spheroides* suspension used for RNA extraction had been stored in frozen state for several months.

(*b*) *R. spheroides* suspension used was kept suspended in 0.005 M-tris-Cl, (pH 7.5) + 10⁻² M-MgSO₄, at 4°, for 48 hr, before breaking it and extracting the RNA.

(*c*) RNA from *R. spheroides* 5-11, a uracil auxotroph obtained from Dr J. Lascelles. —○—, ¹⁴C (*E. coli* RNA); —●—, ³H (*R. spheroides* RNA).

(*d*) RNA from *R. spheroides* no. 2.4.1 obtained from Dr S. Kaplan. —○—, ¹⁴C (*E. coli* RNA); —●—, ³H (*R. spheroides* RNA).

(*e*) One of the better RNA preparations obtained from the culture of *Rhodopseudomonas spheroides* no. 2.4.1 that was used in most of this work. —○—, ¹⁴C (*E. coli* RNA); —●—, ³H (*R. spheroides* RNA).

of buffer, use of nitrogen and avoidance of light. None of these practices, however, singly or together, proved to be entirely reliable, and it was concluded that phenol should be freshly distilled and used within 24 hr.

(c) *Previous history of cells.* Little or no 23S RNA was obtained from organisms that had been frozen for several months or kept for 48 hr at 4° in the extraction buffer. In this context it is to be noted that previous negative findings involved the use of frozen organisms (Lessie, 1965).

DISCUSSION

The characteristics of *Rhodopseudomonas spheroides* RNA may be correlated with those of *Escherichia coli* ribosomal RNA. Thus, studies of the latter have shown that, when extraction conditions are not optimal (Midgley, 1965*a*), or upon mild alkaline treatment (Midgley, 1965*b*), 23S RNA can be degraded, producing predominantly RNA of the 16S class. However, *E. coli* 23S RNA exhibits only one 3'-terminal residue (Midgley, 1965*c*) and one 5'-terminal residue (Takanami, 1967) per molecule, indicating it is not a simple dimer of the 16S RNA. Our extractions of RNA from mixed cultures of *R. spheroides* and *E. coli* produce *E. coli* ribosomal RNA in almost the theoretical ratio, whereas the 23S component from *R. spheroides* is obtained in relatively low yield. This observation might argue for some unusually fragile linkage(s) in the *R. spheroides* 23S RNA which may be particularly susceptible to nuclease attack. Observations on the liberation of various non-covalently bound enzymes from mitochondrial membranes by freezing and thawing (Egger & Rapoport, 1963) support our suggestion that previous negative findings (Lessie, 1965) were due at least in part to the use of frozen cells. One may note also that in these negative experiments even the *E. coli* RNA preparation exhibited a ratio of 23S to 16S of 1:1 instead of the theoretical 2:1. We may conclude that the composition of ribosomal RNA in *R. spheroides* is much like that of *E. coli* and other bacteria and requires no supposition of uniqueness, at least in sedimentation behaviour.

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REFERENCES

- BRITTEN, R. J. & ROBERTS, R. B. (1960). High resolution gradient sedimentation analysis. *Science, N.Y.* **131**, 32.
- EGGER, E. & RAPOPORT, S. (1963). Role of non-covalent bonds for the holding, activation and release of mitochondrial enzymes. *Nature, Lond.* **200**, 240.
- FRIEDMAN, D. I., POLLARA, B. & GRAY, E. D. (1966). Structural studies of the ribosomes of *Rhodopseudomonas spheroides*. *J. molec. Biol.* **22**, 53.
- GREEN, M. H. (1966). Inactivation of the prophage Lambda repressor without induction. *J. molec. Biol.* **16**, 134.
- LASCELLES, J. (1959). The synthesis of enzymes concerned in bacteriochlorophyll formation in growing cultures of *Rhodopseudomonas spheroides*. *J. gen. Microbiol.* **23**, 487.
- LESSIE, T. G. (1965). The atypical ribosomal RNA complement of *Rhodopseudomonas spheroides*. *J. gen. Microbiol.* **39**, 311.

- MIDGLEY, J. E. M. (1965*a*). Effects of different extraction procedures on the molecular characteristics of bacterial ribosomal ribonucleic acid. *Biochim. biophys. Acta* **95**, 232.
- MIDGLEY, J. E. M. (1965*b*). Studies on the structure of 23S ribosomal ribonucleic acid from *Escherichia coli*. *Biochim. biophys. Acta* **108**, 348.
- MIDGLEY, J. E. M. (1965*c*). The estimation of polynucleotide chain length by a chemical method. *Biochim. biophys. Acta* **108**, 340.
- OSAWA, S. (1968). Ribosome formation and structure. *A. Rev. Biochem.* **37**, 109.
- TAKANAMI, M. (1967). Analysis of the 5'-terminal nucleotide sequences of ribonucleic acids. I. The 5'-termini of *Escherichia coli* ribosomal RNA. *J. molec. Biol.* **23**, 135.
- YAMASHITA, J. & KAMEN, M. D. (1968). Observations on the nature of pulse-labelled RNA from photosynthetically or heterotrophically grown *Rhodospirillum rubrum*. *Biochim. biophys. Acta* **161**, 162.