PROPERTIES OF RNA INEXTRACTABLE FROM ESCHERICHIA COLI BY PHENOL AT pH 6.0

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Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 57, No. 2, pp. 65-68, February, 1964
Original article submitted February 4, 1963

The work of Georgiev and Mant'eva [2-4, 9] and of Japanese biochemists [10, 13, 14, 15, 17] has shown that when nucleic acids are extracted from animal tissues by the phenol method a certain amount of the RNA does not pass into the aqueous layer but remains in the intermediate layer along with the DNA. During subsequent treatment of the material with alkaline phenol or with phenol containing PAS, some of this inextractable RNA passes into the aqueous layer with DNA. The nucleotide composition of this RNA differs from that of the total RNA of the cell and is closely similar to the composition of DNA. These findings are of great interest in relation to research indicating the existence of natural DNA–RNA complexes [8, 12].

We have attempted to answer three questions: 1) is a similar inextractable RNA present in bacteria; 2) is it bound with DNA, and if so, how; and 3) is this RNA similar in its composition to DNA.

EXPERIMENTAL METHOD

Escherichia coli strain B* was cultivated in flasks containing dried nutrient broth for 18 h at 37°. The resulting culture was used to seed a glucose-mineral medium containing 10^{-1}M phosphate buffer at pH 7.0, 10^{-3} M $MgCl_2$, $1.5 \cdot 10^{-2}$M $(NH_4)_2SO_4$, $2 \cdot 10^{-6}$M $FeSO_4$ in 0.4% glucose solution. Cultivation took place at 37° with intensive aeration for 5 h (observations showed that the end of this period corresponded to the end of the logarithmic phase of growth). The bacterial mass was collected by centrifugation, washed with 0.85% NaCl solution during centrifugation, and stored at −15°.

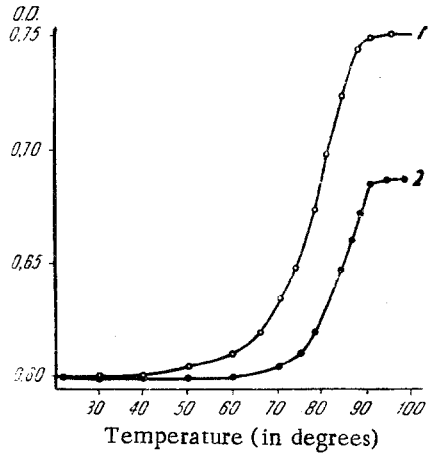
To obtain inextractable RNA the bacteria were lyzed with a 0.5% solution of sodium desoxycholate and treated with phenol by the Kirby–Georgiev method [11]. The aqueous solution obtained as a result of treatment of the material with alkaline phenol (pH 8.3) after preliminary extraction of the RNA with phenol at pH 6.0 contained all the DNA and part of the RNA. This layer was purified by two additional deproteinizations with phenol at pH 8.3, and then washed three times with acidified ether (to remove phenol and traces of protein). The DNA with its admixture of RNA was precipitated with 96% ethanol and redissolved in a 0.001 M solution of NaCl. The concentration of nucleic acids in the extracted preparation was determined spectrophotometrically [7] and the protein concentration by Lowry's method [11].

TABLE 1. Action of RN-ase on Crude Preparation

Split fraction (including free nucleotides)	inRNA	DNA	inRNA / DNA
55.5	7.4	39.2	0.18
50.8	8.4	38.2	0.22
49.0	8.5	39.5	0.21
54.0	5.0	34.0	0.15
46.8	8.5	37.5	0.23
Mean. . 49.2	7.6	37.7	0.20

To determine the strength of the bond between the DNA and inextractable RNA (inRNA) the resulting preparation was treated with nucleases: either with ribonuclease (RNase) alone or with RNase and desoxyribonuclease (DNase) at the same time. In the first case the incubation mixture contained 0.5 mg nucleic acids and 25 μg RNase (manufactured by Reanal, Hungary or Sigma, USA) per milliliter 0.02 M phosphate buffer at pH 7.0; in the second case 50 μg

* Strain E, coli B was supplied by the N. F. Gamaleya Institute of Epidemiology and Microbiology.



Melting-point curves of the preparation, untreated (1) and treated with RNase (2).

DNase (Reanal, Hungary) was added to the mixture and magnesium acetate was added to the medium to give a final concentration of 0.002 M. A parallel series of control tests was undertaken, in which the samples either contained enzyme but were not incubated, or were incubated without enzyme. Incubation continued for 2 h at 37°. Preliminary experiments to study the kinetics of degradation of the preparation by the nucleases showed that no further increase in the optical absorption in the acid-soluble fraction took place after the end of this period.

At the end of incubation the preparation was cooled, and the undigested residue was precipitated with an equal volume of 0.5N $HClO_4$, separated from the acid-soluble portion by centrifugation, washed 3 times with 0.25 N $HClO_4$, dried with alcohol and ether, and hydrolyzed with 0.5 N KOH for 18 h at 37°. The RNA hydrolyzate was separated from the DNA by acidification with $HClO_4$ to a final concentration of 0.2-0.3 M and subsequent centrifugation. The DNA residue was washed 3 times with 0.25 N $HClO_4$. Hence three fractions were obtained: 1) split RNA (plus split DNA in the case of the combined action of RNase DNase); this fraction also included the free nucleotides contained in the preparation; 2) RNA unsplit by RNase, hydryolyzed by alkali (inRNA); 3) DNA fraction not hydrolyzed with alkali (in the case of treatment with both RNase and DNase practically no DNA was present in this fraction). The concentration of nucleic acids in the fractions was determined spectrophotometrically after acid hydrolysis [8].

To study the character of the bond between DNA and inRNA and of the structure of the residue after the action of RNase, melting-point curves of the preparations treated and untreated with RNase were plotted. The curves were obtained in a 0.001 M NaCl solution, the concentration of nucleic acids being 50 μg/ml, by means of a type SF-4 spectrophotometer and thermostatic dish, between temperatures of 20 and 100°.

The nucleotide composition of the RNA residue not digested by RNase was determined after alkaline hydrolysis. The nucleotides were separated by two-dimensional chromatography on paper (Leningrad "slow") consecutively in two systems: 1) ethanol–butanol–ammonium acetate buffer 1 M at pH 3.5 (4 : 1 : 2) and 2) isobutyric acid, saturated with water and adjusted to pH 3.5 with ammonia. The nucleotides were estimated quantitatively by spectrophotometry of the eluates of the stains [5]. A parallel series of determinations was made of the composition of the DNA of the preparation after hydrolysis with 70% $HClO_4$ at 100°. The bases were separated chromatographically in a system consisting of: methanol–concentrated HCl–water (7 : 2 : 1) and their concentrations were determined spectrophotometrically.

EXPERIMENTAL RESULTS

The preparation extracted at pH 8.3 contained an unexpectedly large amount of RNA – about 50% of the total nuclear matter of the preparation (Table 1). Evidently the RNA of E. coli is not completely extracted with phenol at pH 6.0, which may be attributable to the higher content of RNA in this microorganism. The preparation also contained a small quantity of free nucleotides, passing into the acid-soluble fraction without treatment with nucleases. This amount differed from one preparation to another, and usually amounted to 10-20% of the total quantity of nucleic acids. The preparation contained 3-5% of protein (by weight, from nucleic acids).

It is clear from Table 1 that a certain proportion of the RNA was insensitive to the action of RNase; it accounted on the average for 7.6% of the total quantity of nucleic acids and free nucleotides in the preparation and 20% of their DNA content. As a result of the combined action of RNase and DNase on the preparation, practically the whole of its DNA was digested and an extremely small amount of RNA (2-3%) remained.

It is interesting to note the large quantity of RNA unsplit by RNase in proportion to DNA $\frac{\text{inRNA}}{\text{DNA}} = \frac{1}{5}$, whereas after combined treatment with nucleases the preparation was almost completely split. It may be postulated from these results that the DNA in the preparation in some way protected the RNA from the action of the RNase. Similar results were obtained by Bonner with the chromatin of pea shoots [8].

To investigate the nature of the bond between DNA and inRNA and to determine the structure of this residue melting-point curves of the crude preparation, not treated with RNase, and of the residue after treatment with RNase

TABLE 2. Nucleotide Composition of Total RNA, inRNA, and DNA (molar percentages of nucleotides)

Index	G	A	C	T(U)	$\frac{G+C}{A+T(U)}$	$\frac{Pur}{Pyr}$
DNA	25.0	25.2	25.6	24.4	1.02	1.01
inRNA	26.2	27.2	22.5	23.7	0.96	1.15
RNA	30.7	26.0	24.1	19.2	1.21	1.31 (8)

were plotted (see figure). The width of the melting-point curve of the crude preparation (ΔT) was 50° and the melting point (T_m) was 77°. The width of the melting-point curve of the preparation after treatment with RNase (ΔT) was 20° and its melting point 83°. It is clear from these results that after treatment with RNase the preparation became less heterogeneous (the width of the curve decreased) on account of the digestion of the free portion of the RNA, uncombined with DNA, by RNase. This part of the RNA melted more easily, and after it had been removed the melting point of the residue was raised. The shape of the melting-point curve of the preparation after treatment with RNase was similar to the shape of the melting-point curve of the pure DNA from E. coli under similar conditions.

It may be supposed from the preliminary results of ultracentrifugation that the residue remaining after the action of RNase consists of one component only. Comparison of the results of ultracentrifugation, melting-point determination, and sensitivity to attack by enzymes suggests that inRNA forms a stable spiral complex with DNA, having a structure similar to that of DNA. The results of the determination of the nucleotide composition of RNA unsplit by RNase and of DNA from E. coli (Table 2) were compared with the data of the nucleotide composition of the total RNA from E. coli previously obtained by A. S. Spirin [6]. It is clear from Table 2 that inRNA is more similar in its composition to DNA than to the greater part of the RNA. It may be concluded from these results that the fraction of RNA combined with DNA is to some extent complementary to it, and is possibly synthesized on it. These considerations suggest that this RNA fraction under investigation may be compared with information RNA. The final solution of this problem must await further research.

SUMMARY

In the treatment with phenol at pH6 not all the RNA is extracted from E. coli; some RNA is extracted and some is extracted with alkaline phenol together with DNA. A proportion of this RNA is not split by ribonuclease in conditions in which the usual RNA is split. The ratio of RNA nonsplit by RNase to DNA is 1 : 5. By combined action of ribonuclease and desoxyribonuclease on the preparation studied, both RNA and DNA were split completely. The melting point curve of residue after the action of ribonuclease points to the existence of a polymeric structure of DNA-like type. RNA, unsplit after the action of ribonuclease is related to the intermediate type and is more similar in this respect to DNA, than to the main RNA mass.

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