STUDIES ON s-RNA SYNTHESIS

II. ASSAY AND METHOD OF PURIFICATION OF s-RNA α, β AND γ

E. S. CANELLAKIS AND EDWARD HERBERT

Department of Pharmacology, Yale University Medical School, New Haven, Conn. (U.S.A.) and Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. (U.S.A.)

(Received April 23rd, 1960)

SUMMARY

Methods are described for the assay and for the purification of components of the enzymic system from rat liver which incorporates ribonucleotide units derived from ribonucleoside triphosphates into a soluble form of ribonucleic acid.

INTRODUCTION

The soluble cytoplasmic fraction of rat liver contains "enzyme systems"* which incorporate ribonucleotide units derived from ribonucleoside triphosphates into the s-RNA of the same cell fraction[1-4]. During purification these enzyme systems are fractionated with the s-RNA into three distinct ribonucleoprotein components designated ribonucleoproteins α, β and γ**. Further purification of these ribonucleoproteins results in the separation of the protein component from the s-RNA component. Neither component exhibits enzymic activity when assayed alone, but full activity is restored when the protein and nucleic acid fractions are combined[5]. In the present communication the method of fractionation of these components, as well as the details of the methods of assay, are presented. Fractionation of the individual components is in progress and will be presented at a later date.

METHODS

Assay

General principle: The assay is based on the general reaction:

$$\text{XTP}^* + \text{s-RNA} \longrightarrow \text{XMP-s-RNA} + \text{PP}$$

Abbreviations: s-RNA, soluble ribonucleic acid; DEAE, diethyl amino ethyl; ATP, adenosine triphosphate.

* In order to demonstrate full activity in the reaction under study, both the "protein" and the "RNA" components are required. For simplicity of communication, therefore, we wish to refer to the "protein" component as the enzyme fraction or system and to the "RNA" component as the "s-RNA" or the "RNA" component. Whether this, in effect, is the relationship of the two fractions will be discovered with further understanding of the reaction under study. Since the reactions performed by these fractions are not limited to the incorporation of ribonucleotide units into terminal positions of the RNA, we wish to avoid naming them in more specific terms until they have been defined with greater precision.

** See NOTE ADDED IN PROOF.

where XTP* is a radioactive ribonucleoside triphosphate and XMP is the corresponding radioactive ribonucleoside monophosphate. The total amount of radioactivity incorporated into acid-precipitable material is used as a measure of the extent of reaction. The reaction is stopped by the addition of perchloric acid. A small amount of carrier-protein and s-RNA is added to ensure complete precipitation of the acid-insoluble components in the incubation mixture. Adsorbed acid-soluble radioactivity is removed and the residual acid-insoluble radioactivity is determined.

Procedure

Of the protein of the original extracts (crude material from steps 1–3), 10 to 20 mg should be used per assay tube. The more purified ribonucleoprotein components should be diluted to give an optical density of 20 at 260 mμ, 1 cm light path. The standard incubation mixture consists of 0.8 ml of 0.08 *M* potassium phosphate, pH 7.2, 0.2 ml of the protein fraction (dialyzed against the same buffer), 2 μmoles $MgCl_2$ and 10 mμmoles of the XTP*. Incubation is for 10 min at 37°. The assay tubes are chilled in ice, 0.5 ml of 1.5 *N* $HClO_4$ is added, and this is followed by 0.05 ml of carrier (obtained from step 3 of this procedure). The addition of carrier is unnecessary when material isolated up to step 3 is assayed. After standing in ice for 5 min, the samples are centrifuged. The residue is washed three times with 8 ml of cold 0.2 *N* $HClO_4$. The removal of adsorbed radioactivity may be performed by one of the following two methods.

1. After the last centrifugation the perchloric acid is carefully drained from the residue; the latter is then suspended in 1 ml of 1 *M* $Na(NH_4)HPO_4$ and incubated at 37° for 5 min. The samples are chilled in ice, 0.20 ml of concentrated perchloric acid and 8 ml of 0.2 *N* cold perchloric acid are added. The perchloric acid wash is repeated once more, the excess acid is carefully drained off, 0.5 ml of 5 *N* NH_4OH added and the solution plated on a planchet, dried at 80° and the radioactivity determined.

2. The residue is washed once with 95 % ethanol and the tubes are carefully drained. After the addition of 2.0 ml of 10 % NaCl, the mixture is neutralized using phenol red as an internal indicator and heated for 30 min at 100°. The samples are chilled in ice and centrifuged. The residue is washed once with 0.5 ml of 10 % NaCl. To the combined supernatant fluid and wash, 2.5 volumes of cold 95 % ethanol are added. After standing for 2 h at —20°, the samples are centrifuged and the residue is washed once with 95 % ethanol. The tubes are drained by inversion on a paper towel. The sediments are dissolved in 0.5 ml of water, plated on planchets, dried and the radioactivity determined.

Enzyme fractionation

A summary of the purification method, with the pertinent data, is presented in Table I.

Step 1. Preparation of the rat liver extract: The method is described for approx. 130 g of rat liver. This amount of tissue (obtained from 9 rats weighing 450–500 g each), when blended, fills completely the centrifuge cups of a Spinco Model L Preparative Ultracentrifuge, Head No. 30.

* In the crude extracts, 5 to 10 times this amount of XTP* should be used because of the extensive degradative reactions which occur. In addition, an ATP-generating system consisting of phosphoenolpyruvate and pyruvate kinase or of creatine kinase phosphate and creatine kinase should be added.

The livers of the rats are excised, chilled in ice, minced with scissors and homogenized in 2.5 volumes of a sucrose–$MgCl_2$–KCl solution (0.35 *M*–0.004 *M*–0.025 *M*) for 30 sec in a Waring blendor at top speed. The homogenate is then centrifuged for 1 h at 85,000 × *g*. The supernatant liquid is decanted and diluted with one volume of cold water.

Step 2. Ammonium sulfate fractionation: Solid ammonium sulfate is added to the level of 55 % saturation over a period of 10 min to the solution of *Step 1*. This is then allowed to equilibrate with stirring during a period of 30 min and centrifuged at 15,000 × *g* for 10 min. To the supernatant fraction, solid ammonium sulfate is again slowly added up to 85 % saturation. This suspension is centrifuged after 30 min of equilibration and the 55–85 % ammonium sulfate precipitate saved. This precipitate, which contains 80–90 % of the total enzyme activity of the initial liver extract, is suspended in 0.08 *M* potassium phosphate buffer, pH 7.2, to give a final volume of 100 ml and dialyzed overnight against two 4-l changes of the same buffer.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE

	*Specific activity**	*Yield* %	*RNA*** *mg*	*mg Protein**** / *mg RNA*
Soluble cytoplasm	5–10	100	45	100
Ammonium sulfate (0.55–0.85)	30–50	80	23	60
pH 5-precipitate	100–150	60	12	15
Hydroxylapatite	500–750	50	10	1
DEAE-cellulose "protein"	750	50	—	100
"RNA"	—	—	8–10	0.05

* The specific activity is expressed as the number of micromicro-moles of [^{14}C]AMP incorporated into s-RNA, with 1.0 mg protein, in 6 min at 25°.

** RNA was determined by an orcinol method[4].

*** Protein was determined by the procedure of LOWRY[7].

Step 3. Precipitation at pH 5.2: The 55–85 % ammonium sulfate fraction is brought to pH 5.2 with 2 *N* acetic acid with constant stirring at 0°. After an equilibration time of 10 min this suspension is centrifuged, the precipitate is suspended in 20 ml of 0.08 *M* potassium phosphate buffer pH 7.2. and dialyzed overnight against 4 l of 0.02 *M* potassium phosphate, pH 7.2. This material contains 20 % of the protein, 60 to 80 % of the RNA and 80 % of the activity present in the 55–85 % ammonium sulfate fraction. The E_{260}:E_{280} is approximately 1.3 indicating a protein to RNA ratio of approximately 10:1.

Step 4. Fractionation on an hydroxylapatite column[6]: The dialyzed solution of *Step 3*, is diluted to 200 ml and added to a hydroxylapatite column, 20 × 2.5 cm, which has been equilibrated previously with 0.001 *M* potassium phosphate, pH 7.2. If the hydroxylapatite has been properly graded, a flow rate of 2 to 4 drops per minute is possible without the application of pressure. The elution of the enzymically active fractions may be carried out in one of two ways.

Stepwise procedure: This procedure yields three distinct ribonucleoprotein peaks designated ribonucleoproteins α, β and γ in their order of elution from this column.

These are eluted at the salt concentrations indicated in Fig. 1. The material in the peak tubes has ratios of $E_{260}:E_{280}$ of 1.6 to 1.9, which indicate a high nucleic acid content.

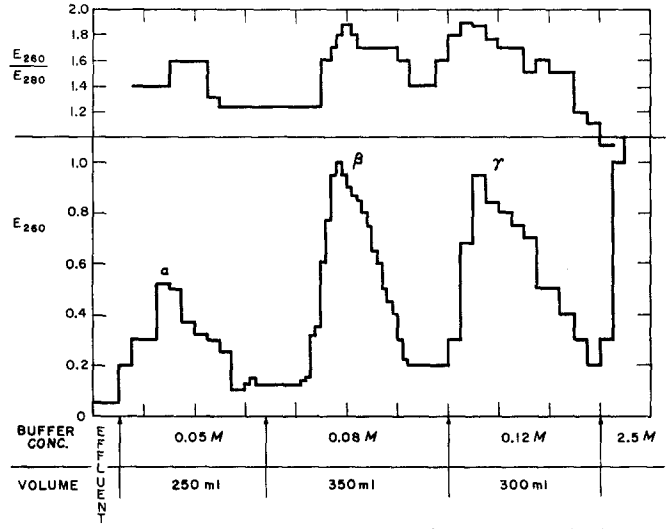

Fig. 1. Stepwise procedure for fractionation of s-RNA α, β and γ on an hydroxylapatite column.

Linear gradient elution. The linear gradient is performed with 300 ml of 0.01 *M* potassium phosphate, pH 7.2, in the mixer and 300 ml of 0.25 *M* potassium phosphate, pH 7.2, in the reservoir. This gives an adequate separation of the three peaks and has the advantage of being much faster than the stepwise procedure. It is advisable to pool the β and γ peaks obtained by this method because of the possibility of cross-contamination. If the column is further eluted with 2.5 *M* potassium phosphate, pH 7.2, as indicated by the arrow in Fig. 2, a large amount of heme proteins may be eluted. This high salt elution also serves to prepare the column for another fractionation.

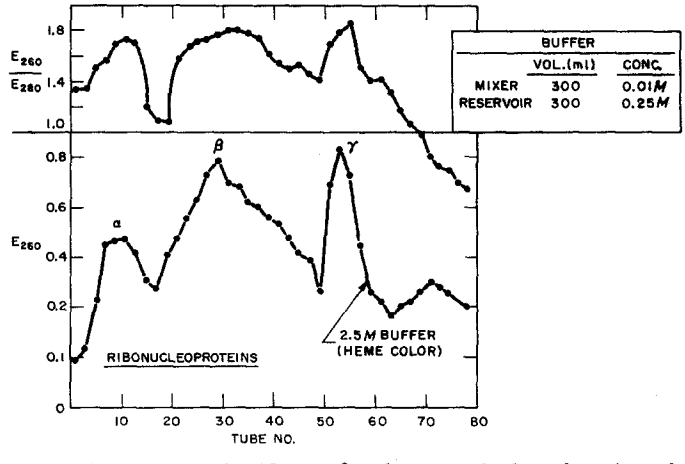

Fig. 2. Linear gradient elution of s-RNA α, β and γ on an hydroxylapatite column.

The three fractions with $E_{260}:E_{280}$ ratios of 1.6 to 1.9 may be pooled separately and lyophilized. The powders obtained are suspended in a small amount of water and dialyzed against 0.08 *M* potassium phosphate, pH 7.2, or against 0.001 *M* potassium phosphate, pH 7.2; the 0.08 *M* phosphate solution is used for assay and the 0.001 *M* phosphate solution for further fractionation.

Step 5. Fractionation on a DEAE-cellulose column: The fractions dialyzed against 0.001 *M* phosphate may be separated on a DEAE-cellulose column (N,N-diethylaminoethylcellulose) into a "protein" component and an "RNA" component. Fig. 3 shows the results obtained when 10 ml of the combined fractions of ribonucleoproteins β and γ were added to a 2 cm × 1 cm DEAE column. The elution may be carried out in a stepwise method by the combined use of potassium phosphate buffer, pH 7.2, and sodium chloride solutions of the indicated molarities. The $E_{260}:E_{280}$ ratios of each fraction presented in the same figure indicate that a rather sharp fractionation

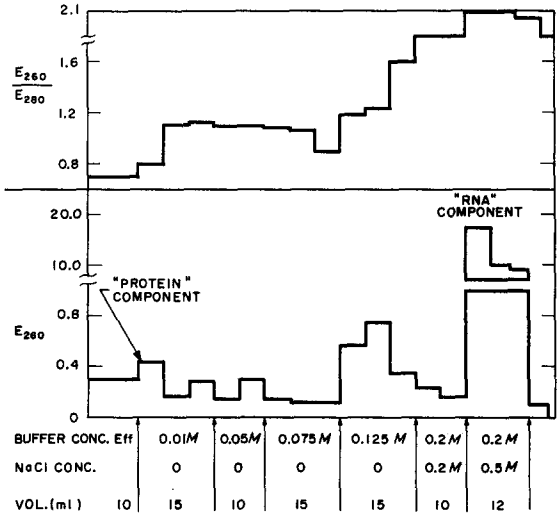

Fig. 3. Stepwise procedure for fractionation of protein from RNA on DEAE column.

of the "RNA" from the "protein" component has been achieved. The u.v.-absorbing material that comes through the column in the first 15 ml contains about 50 % of the total protein ($E_{260}:E_{280} = 0.70$ to 0.75) recovered from the column. With increasing salt concentrations several smaller intermediate peaks appear ($E_{260}:E_{280} = 1.0$ to 1.2) and then a large sharp peak appears ($E_{260}:E_{280} = 2.03$ to 2.10) which accounts for over 90 % of the orcinol-reacting material added to the DEAE column. After lyophilization, the material in the peak tubes may be stored in the dry state or the fractions may be assayed after dialysis against 0.08 *M* potassium phosphate, pH 7.2.

General properties

Some of the enzymic properties of the "protein" and of the "RNA" components have already been described. In addition, they appear to contain some pyrophosphatase and phosphatase activity. The ribonucleoprotein fractions α, β and γ obtained from the hydroxylapatite column are free of amino acid-activating enzymes[4],

when assayed by the pyrophosphate-ATP exchange technique in the presence and absence of amino acids.

Lyophilized powders of all fractions described are stable for several months at $-20°$.

NOTE ADDED IN PROOF

s-RNA α, β, and γ are referred to as ribonucleoproteins for the following reasons: (1) the protein and the RNA fractionate together during an extensive purification process, (2) upon continuous gradient elution from the hydroxylapatite columns three well defined and distinct RNA-protein components can be distinguished, (3) when these isolated components are rechromatographed on an hydroxylapatite column each fraction is eluted with the same molarity of salt as before and (4) each component contains its own s-RNA and ribonucleotide incorporating enzyme. The exact type of physical relationship that exists between these fractions will be best elucidated by detailed enzyme studies.

(Received October 24th, 1960)

ACKNOWLEDGEMENTS

E. S. C. wishes to acknowledge the excellent technical assistance of Mr. C. NIXON JR., and the financial support of the American Cancer Society and the National Institutes of Health of the U.S. Public Health Service. E. H. wishes to acknowledge the invaluable technical assistance of Mrs. H. WILSON and the financial support of the National Science Foundation. One of us (E. S. C.) is a Senior Research Fellow of the U.S. Public Health Service.

REFERENCES

1 E. S. CANELLAKIS, *Biochim. Biophys. Acta*, 25 (1957) 217.
2 M. EDMONDS AND R. ABRAMS, *Biochim. Biophys. Acta*, 26 (1957) 226.
3 L. I. HECHT, P. C. ZAMECNIK, M. STEPHENSON AND J. SCOTT, *J. Biol. Chem.*, 233 (1958) 954.
4 E. HERBERT, *J. Biol. Chem.*, 231 (1957) 217.
5 E. S. CANELLAKIS AND E. HERBERT, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 170.
6 I. W. SIZER AND W. T. JENKINS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. V, New York, Academic Press, in preparation.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.