THE USE OF DIMETHYL SULFOXIDE FOR THE CHROMATOGRAPHIC SEPARATION OF mRNAs WITH DIFFERENT LENGTHS OF THE 3'-TERMINAL POLY(A) FRAGMENT

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UDC 577.216

Recently, methods of isolating poly(A)-containing mRNAs based on the formation of hydrogen bonds between the adenylate residues of the untranslated poly(A) sequence at the 3'-terminus of the majority of mRNAs and oligo- and polynucleotides immobilized on inert support has come into wide use [1, 2]. The most specific in this respect is the hybridization of the mRNAs with poly(U) of oligo(dT) sorbed on filters [4] or covalently bound to columns of cellulose [5] or Sepharose [6].

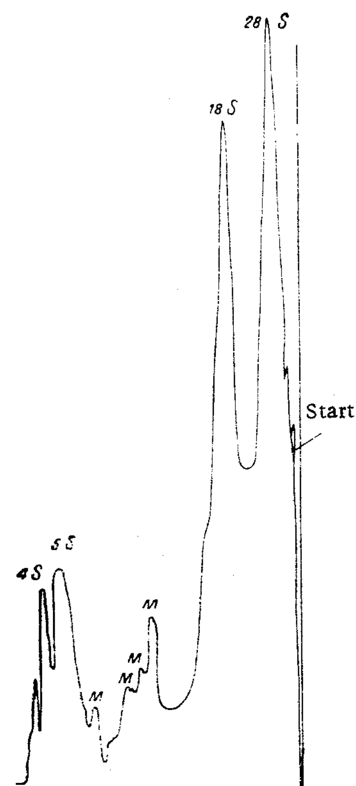
Hybridization is carried out in solutions with a high ionic strength, and elution of the poly(A)-containing mRNAs is, as a rule, performed by the stepwise or linear lowering of the ionic strength, whereupon the mRNAs issue in a broad peak, which leads either to partial losses or to the dilution of the product and complicates further operations with it. In addition, sometimes simple reduction of the ionic strength proves to be insufficient for the elution of all the sample from the column, and therefore it is necessary to carry out elution at relatively high temperatures or to use denaturing reagents in the elution buffers. For example, in the isolation of ovalbumin mRNA on oligo(dT)-cellulose, Roads [7] used elution with a temperature gradient without changing the ionic strength. Under these conditions a correlation was observed between the length of the 3'-terminal poly(A) fragment and the elution temperature. However, in temperature elution a balance has to be struck between the thermal denaturation of the mRNA covalently linked to a column of oligo(dT), on the one hand, and the stability of the complexes, on the other. In view of this, it is more suitable to use denaturing agents in the eluting solutions. Thus, Mollou and Darnell [8] used formamide for this purpose, but the formamide solutions themselves absorb considerably in the ultraviolet region, which complicates the qualitative and quantitative determination of the mRNAs in the eluate fractions.

We have attempted to improve the method of chromatographic isolation of mRNAs containing poly(A) sections by using denaturing reagents in elution buffers. For this purpose we selected dimethyl sulfoxide (DMSO), which is a strong denaturing agent thanks to the polarity of the molecule and its capacity for forming hydrogen bonds with the amino groups of the purines and pyrimidines of the polynucleotide chain. Furthermore, it absorbs insignificantly in the UV region, and the use of DMSO in the phenol-free isolation of RNA does not lead to its degradation.

To isolate the poly(A)-containing RNA we used a preparation of the total plasmatic RNA obtained from polyribosomes by Roads' method [7], which exhibits a typical distribution on electrophoresis in polyacrylamide gel (PAAG) (Fig. 1). Chromatography on poly(U)-Sepharose showed that the yield of poly(A)-containing mRNAs on elution in the cold buffer was lower than on elution in buffer heated to 60°C and in buffer containing DMSO. In the cold, the mRNA was not eluted at all or was eluted in the form of a diffuse peak with an average concentration of 50 μg/ml, and therefore for further operations in the first two fractions, containing only 20% of the poly(A)-containing mRNA sorbed on the column, were taken (Fig. 2b). On elution with hot buffer, the mRNA issued in the form of a compact peak (Fig. 2a) but under these conditions thermal degradation took place both of the covalently bound poly(U) (marked decrease in the capacity of the column on repeated chromatography), and of the mRNA preparation (in 3.5% gel a highly diffuse zone appeared before the 4S RNA). On elution with DMSO-containing buffer, the average concentration of the mRNA amounted to 400 μg/ml. The length of the poly(A) fragments in the eluate fractions (obtained with 40% and 90% DMSO, respectively) was determined after the hybridization with polyuridylic acid in solution followed by the hydrolysis of the hybrids with RNase by electrophoresis in PAAG under denaturing conditions

Institute of Bioorganic Chemistry, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 360-364, May-June, 1978. Original article submitted December 29, 1977.

0009-3130/78/1403-0299$07.50 © 1978 Plenum Publishing Corporation



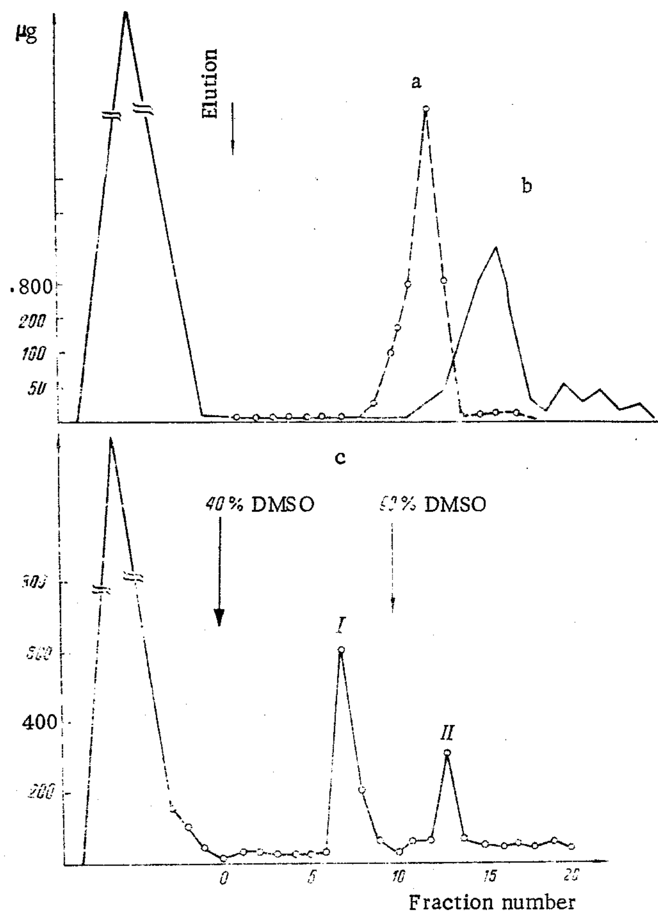


Fig. 1   Fig. 2

Fig. 1. Electrophoretogram of a preparation of the total cytoplasmatic RNA. The unstained gel (2.75%) was scanned in a Hitachi-365 instrument.

Fig. 2. Chromatography of mRNAs on a column of poly(U)-Sepharose: a) elution with hot buffer; b) with cold buffer; c) with DMSO-containing buffer. Elution (v) was performed successively with 40 and 90% DMSO of 50 mg to total cytoplasmatic RNA, and the yield of poly(A)-containing mRNAs was 2.3%. The lengths of the poly(A) fragments in fractions (I) and (II) were determinded separately.

using a mixture of 5S and 4S RNAs as markers. On elution with 40% DMSO, mRNAs issued from the column with poly(A) fragments having lengths averaging 50-70 AMP residues/mole of mRNA and of heterogeneous composition. On electrophoresis under denaturing conditions, the fragment appeared in the form of a broad band with a mobility somewhat greater than that of the 4S RNA. In some cases, the bands corresponding to the tRNA and to the poly(A) fragment practically fused. In the fractions obtained by elution with 90% DMSO, the length of the poly(A) residues was 180-200 nucleotides (diffuse band in the gel higher than the 5S RNA).

Thus, RNAs having longer 3'-terminal sequences are eluted at higher concentrations of DMSO, which can apparently serve as a criterion for their separation, since the remainder of mRNAs has no appreciable influence under these conditions. It must be mentioned that such a separation according to the length of the poly(A) fragment as applied to a homogeneous preparation of mRNAs will permit a check on the hypothesis of the dependence of the template activity of mRNAs on the length of the 3'-terminal sequence of a mRNA consisting of the poly(A) fragment.

EXPERIMENTAL

Preparation of the Polyribosomes (PRSs). The experiments were carried out with random-bred rats weighing 120-150 g. After decapitation, the livers were washed twice with isolating medium: 0.25 M sucrose in buffer A (Tris-HCl, 0.25 M; KCl, 0.25 M; $MgCl_2$, 0.005 M; mercapto-

ethanol 0.005 M) containing poly(vinyl sulfate) (PVS, 150 μg/ml). All the operations were performed at 2-4°C. Homogenization was carried out in isolation medium to give an approximately 10% suspension in a Potter homogenizer with a Teflon pestle. The nuclei and mitochondria were removed at 16,000 rpm for 20 min. The polyribosomes were isolated from the clarified postmitochondrial supernatant by Palmiter's method [9]. The supernatant was lyzed with Triton X-100 in a final concentration of 2%, being incubated in an ice bath for 30 min, and then the concentration of $MgCl_2$ was brought up to 0.1 M. After incubation for 3 h the precipitate (PRSs) was collected by centrifuging at 5500 rpm for 30 min and was suspended in buffer A containing PVS to a concentration of 5 mg/ml. The suspension was layered on a cushion of 2 M sucrose and was centrifuged at 40,000 rpm for 4 h with a Al 42.1 rotor (Spinco, L-5-75). The precipitates of the PRSs were rinsed out with buffer A without sucrose and were suspended in the same buffer to a concentration of 3 mg/ml.

Isolation of the Total RNA. The RNA was isolated from the PRSs obtained by deproteination, and the suspension was mixed with a solution 8 M in LiCl, 8 M in urea, and 4 mM in EDTA (V:V = 1:1) and incubated in the cold overnight. The precipitate was collected by centrifuging at 16,000 rpm for 10 min and was dissolved in buffer B (Tris-HCl, 40 mM, ph 7.0; sodium acetate, 20 mM; EDTA 5 mM; NaDDS, 1%) and the total RNA was precipitated with ethanol at V:V = 1:2, with several reprecipitations. All the solutions for the isolation of the RNA from the PRSs were treated with diethyl pyrocarbonate followed by its elimination [9]. The RNA from the cytoplasmatic PRSs was checked by electrophoresis in tubes [10]. The size of the tubes was 0.6 × 7 cm and they contained 2.75% of PAAG, with a current of 5 mA per tube for 5 h in Tris phosphate buffer pH 7.6. The gels were fixed in 10% acetic acid and were scanned over their lengths on a Hitachi spectrophotometer (see Fig. 1).

Chromatographic Isolation of Poly(A)-containing mRNA. The total cytoplasmatic RNA was chromatographed on a column 0.7 × 2.2 cm containing poly(U)-Sepharose [6]. The preparation was deposited in buffer C (Tris-HCl, pH 7, and EDTA, 20 mM; NaDDS, 0.05%; NaCl, 0.7 M; in a concentration of 2 mg of RNA/ml), the column having previously been equilibrated with the same buffer. The unbound RNA was washed out with buffer C until only the background appeared. Elution was carried out with: 1) buffer D (Tris-HCl, 20 mM; EDTA, 20 mM: NaDDS, 0.05%), 2) buffer D heated to 60°C, and 3) with 40% and 90% solutions of DMSO in buffer D, successively. The yield of poly(A)-containing mRNA was determined spectrophotometrically. The fractions form peaks 1 and 2 (Fig. 2c) were hybridized separately with a small excess of polyuridylic acid and were incubated in buffer at 40°C in a shaking machine. The solution was hydrolyzed with RNase A [11] in 0.02 M Tris-HCl buffer, pH 7.2, containing 50 μg/ml of enzyme at 37°C for 30 min, after which the reaction was stopped with 10% dichloroacetic acid. The acid-insoluble fraction was deproteinated by Perry's method [12] to remove ribonuclease residues. This led to the partial dilution of the preparation, and therefore it was concentrated to 2 mg/ml in polyethylene glycol and was analyzed by electrophoresis in PAAG under denaturing conditions. The electrophoresis of the RNA was carried out in 3.5% gel containing 6 M urea. The samples were deposited in buffer E (50% urea; NaCl, 25 mM; EDTA, 4 mM; pH 7.0) after they had first been heated to 60°C for 3 min for denaturation. The gels were fixed with 10% acetic acid and were stained with Methylene Blue. The molecular weights of the fragments were determined by comparison with the mobilities of markers, for which purpose the 5S and 4S RNAs of rat liver obtained by salting out from the total cytoplasmatic RNA with 2 M NaCl followed by dialysis were used.

SUMMARY

A modified method for the chromatographic isolation of poly(A)-containing mRNA on columns of poly(U)-Sepharose is proposed. By elution with DMSO-containing buffer, the poly(A)-mRNA is separated into fractions with different lengths of the 3'-terminal poly(A) fragment.

A correlation has been found between the lengths of the poly(A) and the concentration of the DMSO in the elution buffer.

LITERATURE CITED

1. V. S. Gaitskhoki, Itogi Nauki i Tekhniki, Ser. Mol. Biol., 7 (1976).
2. V. I. Spiridonova, Itogi Nauki i Tekhniki, Ser. Mol. Biol., 4 (1975).
3. L. A. Osterman and P. S. Sverdlova, Mol. Biol., 11, No. 1, 237 (1977).
4. R. Sheldon, C. Jurale, and J. Kates, Proc. Nat. Acad. Sci. USA, 69, 417 (1972).
5. M. Edmonds, M. Vauhan, and H. Nakazato, Proc. Nat. Acad. Sci. USA, 68, 1336 (1972).

6. V. Lindberg and T. Persson, Eur. J. Biochem., 31, 246 (1972).
7. R. S. Roads, J. Biol. Chem., 250, No. 20, 8086 (1975).
8. D. R. Mollou and I. E. Darnell, Biochemistry, 12, 2324 (1973).
9. R. D. Palmiter, Biochem. J., 13, 17 (1974).
10. U. E. Loening, Biochem. J., 113, No. 1, 131 (1969).
11. J. Bishop, M. Rcobach, and D. Evans, J. Mol. Biol., 85, 75 (1974).
12. Perry R., La Torre, and D. Kelley, Biochem. Biophys. Acta, 262, 220 (1972).

pH DEPENDENCE OF THE KINETIC PARAMETERS OF NITROTYROSYL-ASPARAGINASE

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UDC 577.15.022

In a number of papers devoted to the study of the enzyme L-asparaginase the question is discussed of the possible localization of the catalytically important hydroxy group of the tyrosine residue in the active center of the enzyme [1-3]. The change in the nature of the pH dependence of the activity when the tyrosine residues are subjected to selective chemical modification leading to a change in the pK value of the phenolic hydroxyl shows its direct participation in the catalytic act [2]. In the present paper we consider the influence of the pH on the kinetic parameters of the hydrolysis of L-asperagine.

The values of the Michaelis constant, K_M, for the hydrolysis of L-asparagine catalyzed by nitrotyrosyl-asparaginase, obtained by using three buffer solutions in the range of pH values from 4.50 to 8.85 are given in Fig. 1. The observed threefold increase in K_M takes place at pH values of 5.0-5.5 at which nitrotyrosyl-asparaginase shows its optimum activity in the reaction under consideration [2].

It followed from a comparison of the kinetic parameters of the reaction obtained by using modified and native L-asparaginase (K_M = 0.008 mmole; k_{cat} = $0.41 \cdot 10^3$ min^{-1}; pH 8.0) that the nitration of the tyrosyl residue leads to a marked increase in K_M (by more than an order of magnitude) and an insignificant change in k_{cat} (Fig. 2). It follows from Fig. 2 that the catalytic constant k_{cat} changes little with a change in the composition of the buffer in the given pH range.

Thus, since the modification of the tyrosine residue of the enzyme with tetranitromethane leads to a marked change in K_M, it may be assumed that the tyrosine residue participates in the formation of the enzyme—substrate complex.

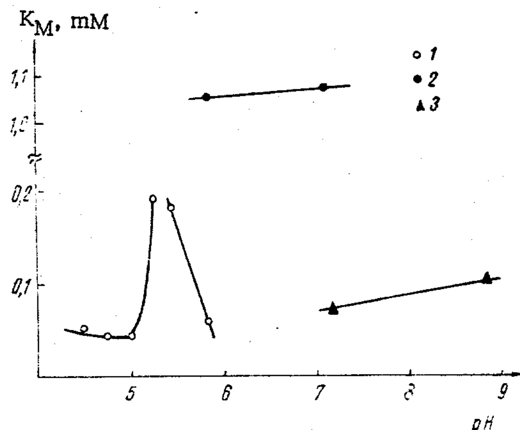

Fig. 1. Dependence of K_M on the pH: 1) sodium acetate buffer; 2) universal buffer; 3) potassium phosphate buffer.

Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 364-367, May-June, 1978. Original article submitted January 26, 1978.

 0009-3130/78/1403-0302$07.50 © 1978 Plenum Publishing Corporation