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Note

Fractionation of ^{32}P-labeled animal RNA on Sephadex–Sepharose columns

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Sepharose (agarose) is used for separation of various macromolecules including nucleic acids[1–3]. A mixture of macromolecules is fractionated in this gel according to molecule size. Thus, it was rather surprising to find that at least some small molecules, namely the low-molecular-weight phosphates, were not separated from RNA of high molecular weight, apparently due to the formation of complexes between ^{32}Pi and RNA in the presence of agarose. I report here on the observation that a Sepharose column topped with a layer of Sephadex satisfactorily fractionates the RNA and removes the inorganic phosphate. Moreover, such columns have several characteristics superior to those built with Sepharose alone.

MATERIALS AND METHODS

Materials

Sources of RNA: RNA was obtained either from Ehrlich ascites cells incubated with the radioactive precursor *in vitro* or from mouse livers after the animals were injected 90–120 min before sacrificing with 500 to 800 μCi of ^{32}P.

Ehrlich ascites tumor cells were collected from peritoneal cavities of one or several mice inoculated 7–10 days earlier with the Hamburg strain of this tumor[4]. The cells were washed once in a sterile Hanks solution (Microbiological Associates, Bethesda, MD, U.S.A.) and suspended in an Eagle's medium low in phosphate (Grand Island Biological Company, Grand Island, NY, U.S.A.). Radioactive phosphorus was added to the concentration of 20 μCi/ml and the cells were incubated at 37°C and constant pH as previously described[5]. After 30–120 min of incubation the cells were harvested, lysed, and RNA extracted with hot phenol[6,7]. RNA from livers was extracted in a similar manner, atfer the organs had been washed with saline and minced with scissors.

Several lots of Sephadex G-25 and Sepharose 2B were purchased from Pharmacia, Piscataway, NJ, U.S.A. over a period of almost 10 years. No appreciable differences between lots were observed.

Building of Sepharose and Sephadex–Sepharose columns; chromatography of RNA

Sepharose columns were prepared according to the specifications given by the manufacturer. The combined Sephadex–Sepharose columns were prepared as follows: in most cases, a commercial glass column was used. However, equally good

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results were achieved with a section of bottom-stoppered glass tubing. Several centimeters of Sepharose gel were built and the column material allowed to settle. Sephadex was then carefully added. In most cases, the total length of the Sephadex–Sepharose column bed was about 50 cm and the Sephadex layer occupied some 40% of this length. We have repeatedly noted that in order to achieve a satisfactory separation of various RNA fractions it was necessary to avoid mixing of the two gels when building the column: the borderline between the two should be uniformly sharp and even.

Both types of columns were then equilibrated with a sodium acetate–acetic acid buffer, pH 5.1[7]. Subsequently, we found that a better fractionation of RNA is achieved on Sephadex–Sepharose columns if the ionic strength of the buffer is increased to 0.6 *M* NaCl.

The purified RNA was layered on the top of the column, allowed to enter the column material and then eluted with the same buffer as the one used for equilibration. The effluent was monitored for the presence of UV-absorbing (Uvicord, LKB, Rockville, MD, U.S.A.) and radioactive materials (Ratemeter, Nuclear Chicago, presently Tracor Analytic, Elk Grove Village, IL, U.S.A.). The outputs from these two instruments were automatically recorded. Fractions collected during the experiment were subsequently individually assayed for the presence of ^{32}P and UV absorbing materials.

Base ratio analysis

RNA was precipitated and digested as described before[5,8]. The nucleotides were separated by high-voltage electrophoresis according to Click[9].

RESULTS AND DISCUSSION

RNA extracted from Ehrlich ascites cells or, in other experiments, from mouse livers, was applied to and eluted from a Sepharose 2B column (46 × 2.5 cm). The material that eluted in the void volume had levels of specific radioactivity significantly higher than those which could be expected on the basis of other experiments performed with the same material (*e.g.* chromatography on methyl esterified albumin[5,8,10]). Further experimentation revealed that only a very small percentage of this radioactivity, usually less than one percent, was precipitable by trichloroacetic acid (ACT). This apparent low molecular weight of the bulk of the radioactive material eluted in the void volume was confirmed by still another technique. The RNA was layered on the top of a 5–20% sucrose gradient, centrifuged for 2.5 hours at 4°C and at 45,000 rpm in a Spinco SW 56 rotor and the contents of the tubes collected from the bottom. Under these conditions, ribosomal 18 and 28S RNAs are recovered in the middle of the gradient. In contrast, most of the radioactive material present in our "void volume" RNA samples remained close to the top of the gradient. A substantial portion of this radioactivity diffused smoothly into the gradient. The large excess of the low-molecular-weight, highly contaminating radiophosphorus made subsequent analyses of this RNA material often difficult and unreliable (*e.g.* base composition analysis).

It was noted that removal of ^{32}Pi from the samples of crude RNA extract by pre-filtration on Sephadex G-25 prevented the formation of complexes between the

phosphates and the RNA. It became obvious that building a column composed of Sepharose topped with Sephadex may achieve the same purpose.

Fig. 1 shows the elution pattern of RNA chromatographed on such a combined Sephadex–Sepharose column. The first peak contained the high-molecular-weight DNA-like RNA which, during centrifugation in a sucrose density gradient, preceded both the ribosomal RNA bands (Table I). RNA with a base ratio and mobility in sucrose gradients characteristic for ribosomal RNA was found around fraction 28; the material between these two peaks had the base ratio intermediate between DNA-like and ribosomal. Its composition changed from one lot of RNA to another, depending on the conditions of incubation at the time of labeling with ^{32}P (ref. 5). The large peak of radioactivity between fractions 30 and 40 was for more than 99% not precipitable by TCA and presumably contained mostly inorganic phosphate. Its position in the eluate was independent from the other RNA peaks and was related to the length of the top layer of Sephadex: the deeper this layer was, the later the ^{32}Pi peak was recovered in relation to RNA fractions.

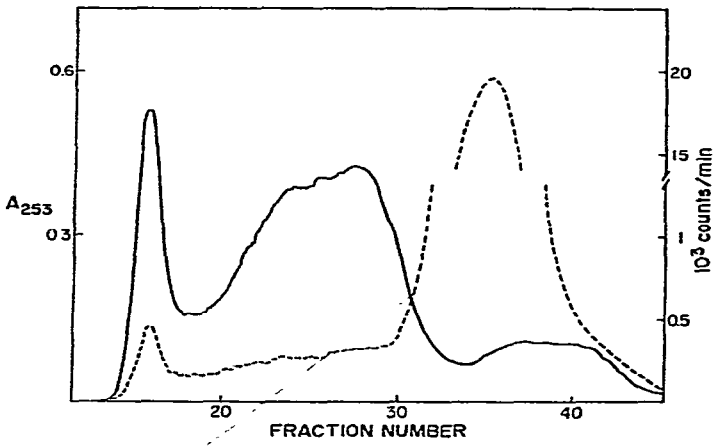

Fig. 1. Elution diagram of ^{32}P-labeled RNA from Ehrlich ascites tumor cells. The Sephadex–Sepharose columns (30 cm of Sepharose 2B, covered with 18 cm of Sephadex G-25, diameter: 2.5 cm) was loaded with approximately 2 mg of RNA and eluted with the 0.6 *M* NaCl solution buffered with pH 5.1 acetate buffer[7]. The effluent was monitored for the presence of UV-absorbing (continuous line) and radioactive materials (broken line) as described in the Materials and methods section. Fractions of 200 drops were collected and base ratio analysis was carried out on the material contained in some of these fractions (Table I).

TABLE I

BASE RATIO ANALYSIS OF RNA ELUTED IN THE VOID VOLUME AND AROUND THE "RIBOSOMAL RNA" PEAK (SEE FIG. 1)

C, A, G and U: cytidylic, adenylic, guanylic and uridylic acids, respectively.

Fractions	*C*	*A*	*G*	*U*
15–17	22.2	27.9	20.9	28.9
27–29	30.0	19.2	29.7	21.2

The Sephadex–Sepharose columns are useful for chromatography of other nucleic acids, including bacterial RNA and DNA from various sources (see, for example, ref. 11). Serum and other body fluids may also be fractionated. Such

columns are easy to build and to operate: they are resistant to the changes in column bed size and to shrinkage due to excessive pressure and/or flow-rate, normally seen with Sepharose. A column may be used repeatedly for several months and even years. The results are reproducible and meaningful comparisons can be made between analyses performed over extended periods of time. For the last 10 years, *i.e.* since we started using the combined Sephadex–Sepharose columns, our laboratory applies them to routine fractionation of cellular macromolecules such as nucleic acids and large proteins.

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