CONVERSION OF NON-COVALENT INTERACTIONS IN NUCLEOPROTEINS INTO COVALENT BONDS: QUANTITATIVE DETERMINATION OF PROTEIN COVALENTLY BOUND TO POLYNUCLEOTIDES*

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1. Introduction

The investigation of the transformation of specific polynucleotide–protein interactions into covalent bonds requires a rapid and sensitive method of quantitative determination of protein covalently bound to nucleic acids. Usual techniques cannot be employed for this purpose. Since viral proteins easily form strong associates, even the single crosslinks result in the complexing of virtually all protein with viral nucleic acids, as revealed by centrifugation of thermally disrupted virions in gradients of caesium salts or neutral sucrose [1, 2]. Centrifugation in alkaline gradients may lead to a degradation of polynucleotides and splitting of certain types of polynucleotide–protein crosslinks (e.g. see [3, 4]).

Standard techniques for separation of proteins from nucleic acids are based on considerable differences in the coefficients of their biphasic distribution between water and organic solvents [5, 6]. However with a covalently linked nucleoprotein a fraction of nucleic acids may be trapped by protein in the organic phase or in the interphase (e.g. see [7]), and a fraction of proteins may be trapped by nucleic acid in the water phase. However, if an anionite is added to the water phase, all polynucleotides and covalently bound protein must come into the water phase, while all free protein goes to the organic phase irrespective of the extent of the crosslinking.

On the basis of this rationale we developed a simple and sensitive method of quantitative determination of polynucleotide-linked, phenol deproteinisation in a thin layer of DEAE-cellulose.

2. Materials and methods

Bacteriophage MS2 and its RNA were obtained as described elsewhere [8]. To label phage proteins 0.1 M *N*-[^{14}C]acetoxysuccinimide in dioxan with specific activity of 5 mCi/mmole [9] was used. Freshly distilled phenol saturated with 1 M NaCl was used. For the thin layers the 3:1 mixture of microcrystalline cellulose (FND, GDR) and DEAE-cellulose (Reanal, Hungary, 0.6–0.8 mg eq./g) supplemented with 1% luminophor was used. The adsorbent suspension (20 g in 100 ml water) was layered onto 6 × 10 cm plates using an automatic applicator. The resulting layers contained 13 mg of the adsorbent per cm^2.

To count the radioactivity, a required region of the adsorbent was removed from the plate with 3% nitrocellulose solution in 1:1 ether–ethanol mixture [10] and counted in a toluene scintillator in a scintillation spectrometer SL-30 (Intertechnique, France). The regions of UV-absorbance on the plates were detected under a low pressure mercury lamp.

To 3.7 μl of phage suspension in 0.5 M NaCl (59 μg; 0.48 units of A_{260}) 50 μl of 0.05 M carbonate–bicarbonate buffer pH 8.5 and 18.4 μl of the dioxan solution of *N*-[^{14}C]acetoxysuccinimide were added. The mixture was incubated for 30 min and the excess

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reagent was destroyed by adding 5 μl of 1 M Tris–HCl pH 8.5. Simultaneously the solution of 19 μg of MS2 RNA (0.440 units of A_{260}) and the control buffer solution were treated in the same way. After 30 min, the reaction mixture was diluted with 330 μl of 0.05 M Na-phosphate buffer pH 8.5 and 100 μl aliquots were applied to the plates. Each plate was divided into four 1.5 X 10 cm strips and contained 4 samples. The samples were applied by polyethylene capillaries in a moist chamber at 3.5 cm from the bottom of the slide. A bridge of 6 X 20 cm filter paper strip folded 4 times was applied to the top of the slide. Prior to the sample application, the plates were chromatographically wetted up to 4 cm from the bottom. After the application, the plates were chromatographically washed in an open chamber with 8–10 ml of 0.05 M phosphate buffer pH 8.5. This procedure continued to the exhaustion of the buffer and took about 20 hr. The paper bridge was removed, the slide was dried and half of the UV-absorbing spots were picked for radioactivity determination. Then, a new bridge was applied to the slide and developing with phenol was carried out for 24 hr. The bridge was changed and developing was repeated. Then the plate was washed in an excess of methanol, dried and developed in methanol to remove the traces of phenol. Finally, the radioactivity of the spots was determined.

The results are presented in table 1.

3. Results and discussion

The position of RNA and proteins on the plate was determined from the distribution of UV-absorbance and radioactivity respectively. When the *N*-[^{14}C]acetoxysuccinimide-treated RNA (0.1 absorbance units) is applied to the plate the UV absorbance is distributed in a 2–3 mm diameter spot, while with MS2 phage (0.12 absorbance units) it was in a 5–7 mm diameter spot. The dimensions and shapes of the spots did not differ when developed by phosphate buffer and phenol, provided the plate is not permitted to dry during sample application. The spot of an RNA sample developed by phosphate buffer contains an amount of radioactivity scarcely exceeding the control, which is slightly decreased by phenol developing (see table 1). This radioactivity can be partially accounted for by an unspecific adsorption of the label and partially by the acetylation of RNA components.

The radioactivity of phage MS2 recovered within the UV-absorbing spot after developing with phosphate buffer, is almost equal to that revealed in an aliquot of the applied sample by gel-filtration whereas the plate regions above the spot are scarcely active (200–300 cpm over the background). The phenol development of the phage spots results in almost complete removal of the label: the radioactivity of the UV-absorbing spot is decreased to the level slightly higher than in the RNA spots (see table 1) and the activity of the regions above the phage spot is diminished to 50–100 cpm over the background. Taking into account the radioactivity recovered in the spots containing RNA and the control solution one can conlcude that less than 1% of the label is retained in the phage spot after developing with phenol. The prolongation of the phenol development or the raising of temperature to 60°C does not affect this results. Consequently, more than 99% of phage protein is detached from the polynucleotide adsorbed to DEAE-cellulose by the phenol deproteinization.

Thus, the method of phenol deproteinization on TLC plates allows accurate determination of small quantities of protein covalently bound to nucleic acids simultaneously in dozens of samples. The method

Table 1

Radioactivity (cpm) within the application regions on DEAE thin layers of treated with *N*-[^{14}C]acetoxysuccinimide phase MS2 and its RNA.

	Control	RNA*	Phage**
After development of TLC with phosphate buffer	307 ± 24	307 ± 24	25998 ± 553
After subsequent development with phenol	94 ± 10	148 ± 10	321 ± 19

* 0.11 units of A_{260}, 4.7 μg RNA per spot.

** 0.117 units of A_{260}, 4.6 μg RNA, 9.7 μg protein per spot. Mean values from 5–10 experiments are presented.

is applicable to nucleoproteins labelled both in vivo [2] and in vitro [11] particularly to the nucleoproteins in which the polynucleotide–protein crosslinks were induced prior to their chemical labelling.

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