SOME CHARACTERISTICS OF LOWER MOLECULAR WEIGHT ARBOVIRUS RNA'S

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1. Introduction

Cells infected with Sindbis virus contain several forms of viral RNA with sedimentation constants of approximately 42 S, 26 S, 16 to 20 S and 10 to 12 S. The 42 S species is the single-stranded virion RNA [1, 2]. The 26 S RNA is also single-stranded and seems to be an intracellular precursor of 42 S [3]. The polydisperse 16 to 20 S species has the characteristics of both double-stranded and single-stranded RNA and seems to be the "replicative intermediate" [4–7]. Data on the lower molecular weight 10–12 S RNA's are limited and only Poirée [4] and Stollar et al. [8] have described this form. In this paper, some physicochemical characteristics of the 16 S double-stranded RNA and the 10–12 S RNA, which throw some light on their possible physiological significance, are reported.

2. Material and methods

2.1. *Infection of cells and purification of RNA*

Cultures of 11 day-old chick embryo fibroblasts were established in Roux bottles, with Eagle's minimal medium containing 2.9 g/l bacto tryptose phosphate broth and 7.5% calf serum. One day old primary cultures were infected with a 10-fold multiplicity of Sindbis virus, in the presence of 1 μg/ml of actinomycin D. After 1 hr at 20°, 2 mCi of tritiated uridine were added to each bottle and the cultures were incubated at 37° for 2 hr.

RNA was extracted from the infected cells by the method of Bishop et al. [9]: 15 ml of a solution containing 1% sodium dodecyl sulphate and 0.01 M EDTA in 0.05 M sodium acetate buffer (pH 5), was added to each bottle. After mild agitation at 20° for 5 min, the viscous suspension was submitted to two phenol extractions, and the aqueous layer was fully deproteinized with chloroform–isoamylic alcohol (5:1, v/v). The RNA was then precipitated with 2 vol cold ethanol and stored at −20°. The precipitate was redissolved in a small volume of 0.01 M potassium phosphate buffer (pH 6.8).

2.2. *Hydroxyapatite chromatography*

Hydroxyapatite, prepared as indicated by Levin [10] was packed in a column of 2 × 5 cm. RNA's, adsorbed on the column in the solvent buffer, were eluted by increasing the molarity of the potassium phosphate, either stepwise or as a continuous concentration gradient, as shown in fig. 2.

2.3. *Sucrose density gradients*

10 to 30% linear sucrose concentration gradients were prepared in 0.02 M sodium acetate buffer (pH 5). A sample of RNA solution (0.1 ml) was layered on top of the gradient. Sometimes, a known amount of ^{32}P labelled marker ribosomal RNA was mixed with the sample. The RNA was centrifuged in a SW 65 Spinco rotor at 60,000 rpm for 2.5 hr at 4°. Fractions of two drops were harvested on 2 × 4 cm filter papers. Radioactivity was counted by the method of Mans and Novelli [11] in an Intertechnique scintillation spectrometer ABAC SL 40.

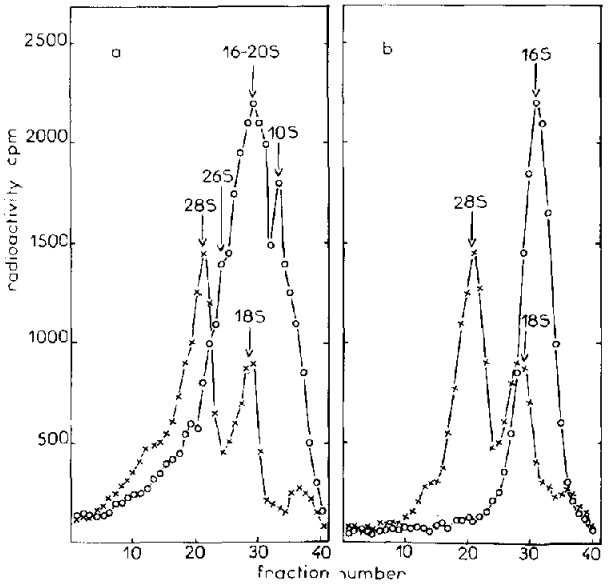

Fig. 1. Sucrose density gradient analysis to RNA from Sindbis virus infected chick embryo fibroblasts. RNA was labelled, extracted and analysed as described in Methods. a) Total extracted RNA after 2 hr labelling (3 hr infection). b) Sedimentation pattern of ribonuclease-resistant core obtained after hydrolysis of the total cellular RNA by pancreatic ribonuclease (4 μg/ml for 20 min at 37°) followed by the isolation of the double stranded RNA and chromatography on hydroxyapatite as indicated in fig. 2. (o—o—o) ^{3}H radioactivity from the actinomycin D resistant, viral RNA. (X—X—X) ^{32}P radioactivity from chick marker ribosomal RNA.

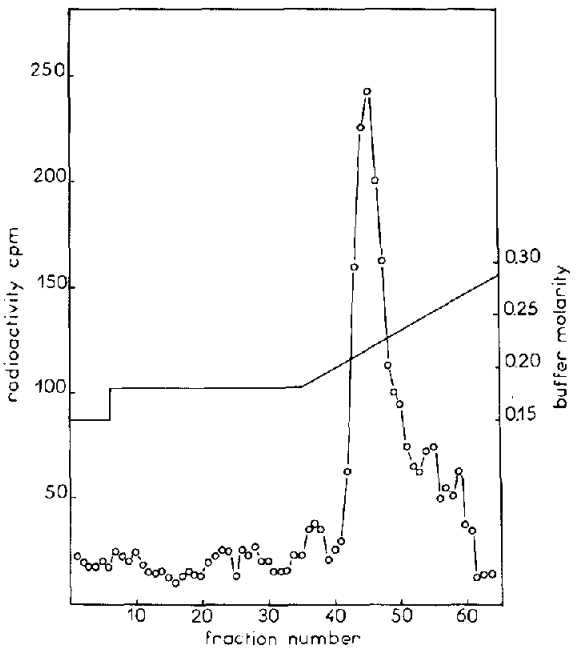

Fig. 2. Chromatography on hydroxyapatite of tritium-labelled RNA from fibroblasts infected with Sindbis virus under actinomycin D treatment. Total cellular RNA has been treated by pancreatic ribonuclease (4 μg/ml for 20 min at 37°) and then loaded on the column. Degraded material eluted at 0.15 M potassium phosphate was rejected. The column was thoroughly washed with 0.18 M potassium phosphate, and a molarity gradient from 0.18 to 0.30 M was applied. 5 ml fractions were collected and acid precipitable radioactivity was measured on 0.1 ml aliquots from each fraction.

3. Results

After 3 hr infection and 2 hr labelling, total RNA extracted from infected cells and analysed on sucrose density gradients showed several peaks of radioactivity corresponding to the intracellular viral RNA species (fig. 1a). After this relatively short infection and labelling time, RNA's with lower sedimentation constants were synthesized, predominantly 16 to 20 S and 10 to 12 S. The 26 S and 42 S RNA's, described after prolonged infection time [6] were hardly present.

If the total RNA was hydrolysed with pancreatic ribonuclease (4 μg/ml for 20 min at 37°) a substantial amount of RNA remained acid precipitable. This ribonuclease resistant RNA was then chromatographed on hydroxyapatite as described by Pinck et al. [11] (fig. 2). Fractions eluted between 0.10 and 0.15 M phosphate buffer were essentially degradation products of very low molecular weight and were rejected. The RNA fraction eluted as a peak at 0.21 M phosphate was further studied. Analysed on sucrose density gradient, this DNA showed one homogeneous peak at 16 S (fig. 1b). 10 to 12 S or heterogeneous 16 to 20 S RNA's were no longer present. That this ribonuclease-resistant 16 S RNA eluted from hydroxyapatite at 0.21 M phosphate [11] was effectively double stranded, was tested by thermal denaturation. Heating up to 110° in a sealed vial was necessary to produce changes in the sedimentation constant of the RNA. After heating for 15 to 30 min at 110° and rapid cooling in dry ice, a single slightly heterogeneous, 10 to 12 S RNA peak was reproducibly seen (fig. 3).

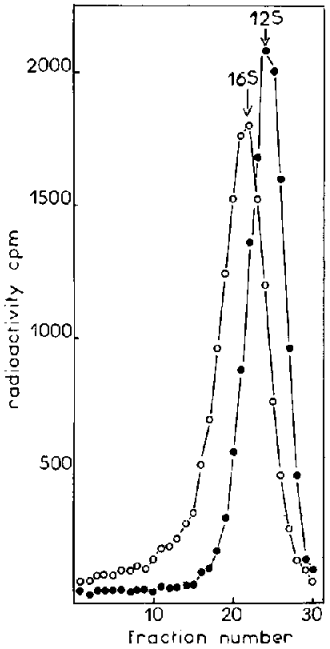

Fig. 3. Sucrose density gradient analysis of ribonuclease resistant, hydroxyapatite chromatographed viral RNA. (o—o—o) ^{3}H radioactivity from the undenatured RNA. (•—•—•) ^{3}H radioactivity from the RNA denatured by heating for 20 min at 110° followed by fast cooling in dry ice.

4, Discussion

Cells infected with Sindbis virus for rather short periods produce predominantly 10 to 12 S and 16 to 20 S viral RNA's. At least part of the 16 to 20 S RNA's behave as the presumptive "replicative intermediates" described in the case of several arboviruses [4–7] Treatment by ribonuclease either of the whole cellular RNA's, or of the isolated 16 to 20 S RNA's, left a single homogeneous 16 S RNA peak. The ribonuclease resistant RNA was eluted from hydroxyapatite in 0.21 M phosphate buffer. Elution at this molarity is characteristic of RNA's with developed secondary or double stranded structures [12]. Similar elution properties from BD cellulose have been observed for the replicative form of Semliki Forest virus [13]. The reverse is found for methylalbumin–silicic acid chromatography, since single stranded RNA's are stronger adsorbed [14].

Thermal dissociation at 110° of the 16 S RNA yields 10 to 12 S RNA. Such high melting temperatures have previously been reported for viral double-stranded RNA's [7].

Thus, we could observe the 10 to 12 S RNA both after thermal dissociation from a double stranded viral RNA, and as a single-stranded, ribonuclease-sensitive RNA present among the intracellular viral RNA's. Few investigators have called attention to this lighter RNA form [4, 8], and opinions as to its function differ.

We suggest that the 10 to 12 S RNA obtained as a dissociation product is the "negative strand" playing a template role for transcription of the positive messenger strands. After destruction by ribonuclease of the dangling nascent strands from the replicative intermediate only the replicative form is left behind. Then, by thermal denaturation, the nascent positive fragments, hydrogen-bonded to the negative strand (perhaps to a double strand forming a triple stranded replicative form) are lost as very short or acid soluble fragments. The free 10 to 12 S RNA found in the whole RNA extract would represent the finished replication product.

This interpretation implies that positive 10 to 12 S chains are produced as monocistronic messengers for translation, which must then be secondarily bound together, end by end, to form 26 S and 42 S viral RNA's.

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References

[1] P. Dobos and P. Faulkner, J. Virol. 4 (1969) 429.
[2] B.M. Arif and P. Faulkner, J. Virol. 9 (1972) 102.
[3] K.L. Cartwright and D.C. Burke, J. Gen. Virol. 6 (1970) 231.
[4] J.C. Poirée, "Contribution à l'étude des acides nucléiques d'un Arbovirus", Thèse de Doctorat en Pharmacie, Marseille 1971.

[5] Z. Ben-Ishai, N. Goldblum and Y. Becker, J. Gen. Virol. 2 (1968) 365.
[6] J.G. Levin and R.M. Friedman, J. Virol. 7 (1971) 504.
[7] R.M. Friedman, J. Virol. 2 (1968) 547.
[8] V. Stollar, T.E. Shenk and D. Stollar, Virol. 47 (1972) 122.
[9] J.M. Bishop, W.E. Levinson, N. Quintrell, D. Sullivan, L. Fanshier and J. Jackson, J. Virol. 42 (1970) 182.
[10] O. Levin, in: Methods in Enzymology, Vol. 5, eds. S.P. Colowick and N.O. Kaplan (Academic Press New York, 1962) p. 27.
[11] R.J. Mans and G.D. Novelli, Arch. Biochim. Biophys. 94 (1961) 48.
[12] G. Bernardi, in: Methods in Enzymology, Vol. 21, eds. S.P. Colowick and N.O. Kaplan (Academic Press New York, 1971) p. 95.
[13] R. Stern and R. Friedman, J. Virol. 4 (1969) 356.
[14] T. Sreevalsan, R.Z. Lockart, Jr., M.L. Dodson, Jr. and K.A. Hartman, J. Virol. 2 (1968) 558.