EFFECTS OF METHYLATED ALBUMIN ON INFECTIOUS RNA: REVERSIBLE INFECTIVITY LOSS AND RESISTANCE TO NUCLEASE DIGESTION

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Methylated bovine serum albumin (MBSA) has been used as a means of separating different species of RNA. Mandell and Hershey (1960) and Sueoka and Cheng (1962) reported a salt-dependent retention of RNA by a MBSA-"kieselguhr" column and suggested that the RNA complexed with the MBSA. Several other authors reported that nucleic acids could serve as haptenic groups when coupled with various basic proteins, including MBSA (Seaman, et al., 1965; Christian, et al., 1965; Plecia, et al., 1965). In 1966, Takai showed by ultracentrifugation studies that when ØX174 DNA was mixed with ØX174 protein and Ca^{++}, and allowed to incubate for 12 hours, the DNA became resistant to nuclease digestion and sedimented with the protein in a density gradient system. This communication is a report of some investigations on the effect of MBSA on the infectivity and enzymatic digestion of infectious ribonucleic acid (IRNA) from the Trinidad strain of Venezuelan equine encephalitis (VEE) virus.

Methods

The methods used to prepare and assay IRNA were those described by Colón and Idoine (1964). IRNA prepared from primary chick fibroblast tissue culture or embryonated chicken eggs infected with VEE virus was stored at -60 C in 0.02 M phosphate buffer, pH 7.4, containing 10^{-3} EDTA. Methylated albumin was prepared according to the method of Mandell and Hershey (1960) and stored dried or as a 1 per cent aqueous solution. Except where stated otherwise, all dilutions were made in 0.15 M phosphate buffered saline, pH 7.4, containing

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10^{-3} EDTA. When necessary, salt concentrations were adjusted with appropriate volumes of 1.0 or 2.0 M NaCl in the same buffer.

Substances for *in vivo* tests were contained in 0.03 ml and given to mice by the intracerebral (IC) route. All mice that succumbed from infection with IRNA were assayed for intact virus on chick fibroblast monolayers by homogenizing brain tissue as a 1:10 dilution in beef heart infusion broth (BHIB) and subsequent dilution with BHIB. Random plaque-reduction tests by serum neutralization indicated that the deaths resulting from infection with IRNA were specific for VEE virus.

Results

The ability of MBSA to "mask" infectivity of IRNA *in vitro* is shown in Table 1. The data show that the combination of MBSA with IRNA at low salt

TABLE 1

EFFECT OF MBSA ON INFECTIVITY IN CELL CULTURE FROM VEE-IRNA

NaCl Concentration	MBSA Concentration	Infectivity Titer[a] pfu/ml
0.15 M	0	5.2×10^5
0.15 M	0.1%	none[b]
1.0 M[c]	0.1%	5.7×10^5

a. On primary chick embryo monolayer cultures. All samples contained equal amounts (pfu) of IRNA.

b. No plaques were observed at 10^{-2} dilution.

c. Solutions were made 1.0 M 10 min after addition of MBSA.

concentration resulted in masking of infectivity and that all of the original infectivity could be recovered when the salt concentration was increased to 1.0 M. This recovery of infectivity after 10 to 30 minutes incubation at low salt concentration demonstrated the apparent reversibility of complex formation. At 4 C a minimum incubation time of about 10 minutes was required to show

maximum loss of infectivity of IRNA when MBSA was added. All infectivity was recovered within seconds after the mixture was made 1.0 M with NaCl. These observations are suggestive of a slow "annealing" of two large molecules in physiological salt solution and then a rapid denaturation when the salt concentration was suddenly increased to 1.0 M.

The data in Table 2 show the ability of MBSA to reduce reversibly the infectivity of IRNA in suckling and adult mice and in tissue culture. It is

TABLE 2

THE EFFECT OF MBSA ON INFECTIVITY OF VEE-IRNA IN TISSUE CULTURE

Sample	Dose[a]	Age of Mice (days)	Deaths/Total
IRNA	141	21-28	16/58
	24 and 2[b]	5-6	9/9 and 3/9
IRNA-MBSA in 0.15 M NaCl	0	21-28	4/59
	<1 and 0	5-6	0/8 and 0/8
IRNA-MBSA in 1.0 M NaCl	171	21-28	15/60
	N.D.[c]	5-6	N.D.
MBSA	0	All	0
0.15 M NaCl	0	All	0

a. pfu in 0.03 ml, by IC route.

b. Number of plaques at dilutions of 10^{-2} and 10^{-3}.

c. Not done.

clear from these data that the older mice are less susceptible to infection with IRNA in that a dose of 150 pfu killed only 25 per cent of the adult mice whereas only 24 pfu was required to kill all the suckling mice.

The IRNA-MBSA mixture in the presence of 1.0 M NaCl induced severe traumatic reactions in adult mice by the IC route. In the experiment shown here (Table 2), about 12 per cent of the adult mice receiving 1.0 M saline

died within 3-5 minutes of inoculation and were not included in the calculations for infectivity of IRNA.

Since we have observed that MBSA can reversibly inactivate IRNA and it was reported that soluble RNA can be made haptenic (Plecia et al., 1965), it would be expected that the MBSA binds in some way with RNA. It was felt that the demonstration of enzyme resistance would indicate whether or not an actual complex was formed; the indicator being loss of recoverable infectivity of IRNA. Pancreatic RNAase was found to be unsatisfactory whereas micrococcal nuclease (Worthington #NFCP 6612 at 4900 units/mg protein) was observed to be dependent on the presence of a divalent cation and that EDTA could completely inhibit its activity. When yeast RNA was incubated with micrococcal nuclease in the presence of Ca++ and 0.01 M EDTA, no change in the optimal density at 260 mμ could be detected. However in the absence of EDTA a gradual but steady increase in OD_{260} was observed. Furthermore, if EDTA was added to a reaction mixture showing enzyme activity, the reaction was stopped and the OD_{260} remained constant for up to 30 minutes. This provided a way of stopping enzyme activity with EDTA before breaking the MBSA-IRNA complex with 1.0 M NaCl, thus making it possible to detect infectivity of the IRNA in the presence of the nuclease and 1.0 M salt.

The data in Table 3 show that MBSA can prevent the degradation of IRNA by micrococcal nuclease and that infectivity could be recovered upon the addition of EDTA and 1 M NaCl. In the absence of MBSA and EDTA, IRNA was completely destroyed in one minute by the enzyme.

These data indicate that MBSA forms a salt-dependent complex with IRNA that renders the IRNA reversibly noninfectious and resistant to digestion with micrococcal nuclease. The use of biological activity as an indicator of molecular integrity has the obvious advantage of high sensitivity. Refinement of this technique also offers the possibility of controlled enzymatic digestion of RNA by making it possible to "cover" parts of the RNA with MBSA and starting or stopping nuclease activity by the addition of Ca++ or EDTA, respectively,

TABLE 3

EFFECT OF MBSA ON DIGESTION OF IRNA BY MICROCOCCAL NUCLEASE[a]

Time Interval/Reagents Added			Infectivity[b]
Zero min	10 min	20 min	
MBSA	Enzyme+Ca++	EDTA	10^2 pfu/ml
-	-	+	4675
+	-	+	400
-	+	+	<10
+	+	+	469
Enzyme+Ca++	MBSA	+	none
Enzyme+Ca++	No MBSA	-	none

a. The IRNA solution used in all tests contained the same quantity (pfu) of IRNA in 0.15 M NaCl, 0.02 M phosphate buffer, pH 8.0. The final concentrations of reagents added at the time intervals indicated were: MBSA, 0.1%; enzyme, 500 units; $CaCl_2$, 10^{-3} M; EDTA, 10^{-2} M.

b. Mean of four experiments, plaque-forming units on chick fibroblast monolayer cultures. All assays were performed in the presence of 1 M NaCl to regain infectivity that was masked by MBSA.

to the reaction mixture.

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