Enzyme Resistance of Complexes with Ribonucleic Acid with Metals*

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Mercuric salts (one equivalent per 1–2 nucleotide residues) inactivate TMV-RNA and cause a marked shift in its UV absorption band. Silver acetate causes hypochromicity and a small shift, but no significant loss of infectivity. Indium chloride causes no spectral changes, and loss of infectivity only when used in greater amounts. All three metals (one equivalent per two nucleotides) protect the RNA against the action of pancreatic ribonuclease and purified plant nuclease. Infective free RNA can be recovered from the enzyme-treated metal complexes by simultaneous treatment with a chelating agent and bentonite. The extent of protection differs for the metals and enzyme preparations used. In the case of mercury and silver, evidence was adduced that the observed effects were due to the interaction of the metals with the macromolecular substrate, RNA, and not to any inactivating action on the enzymes. Combinations of the various metals cause intermediate effects, and this fact suggests different binding sites for each.

Indications have been obtained that viral RNA may occur intracellularly in a state in which it lacks its protein coat and yet shows greater stability than does free RNA. In attempts to impart such properties to RNA *in vitro*, it was treated with a great variety of agents, and the resultant complexes or mixtures were tested for viral infectivity and enzyme resistance. Of the diamines or triamines tested, spermidine alone showed a consistent effect, in that it inactivated the RNA in a partly reversible manner. However, spermidine did not seem to protect the RNA against the action of nucleases. Of various basic proteins tested, only pancreatic ribonuclease in salt-free media exhibited some of the sought-after properties (Fraenkel-Conrat and Singer, 1960), although its action was for obvious reasons difficult to control. Clear-cut results were obtained, however, with certain metal salts which have in recent years been reported to form complexes with nucleic acids (Katz, 1952; Thomas, 1954; Dove and Yamane, 1960; Yamane and Davidson, 1962a,b [in press]; Aldridge, 1960). Of the metals tested, silver, mercury, and indium salts showed the most pronounced effects in increasing the resistance of TMV-RNA to the pancreatic and tobacco nucleases used. All complexes tested, except the mercuric and the ferrous, were infectious. RNA could be recovered from all but the ferrous complex in metal-free and fully infectious form.

This paper will report some effects of metals, with particular reference to Hg^{++}, In^{+++}, and Ag^{+}, on the infectivity and enzyme resistance of TMV-RNA, as well as on the UV absorption spectrum of the RNA, and stoichiometric considerations derived from these data. Studies on the nature of the interaction of RNA with silver salts, and on some of the physicochemical properties of this complex, will be reported by Dr. M. Daune in a later publication.

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Methods and Materials

TMV-RNA was prepared with phenol and bentonite, as previously described (Fraenkel-Conrat *et al.*, 1961). Analytical grade silver and mercury salts were used. The mercuric and silver salt solutions were unstable and therefore freshly prepared for each experiment. Anhydrous indium trichloride was obtained from the Indium Corporation of American (Utica, N. Y.). Mercury analyses were performed with dithizone after wet combustion, and semiquantitative silver analyses of similar digests by a turbidimetric procedure.

Crystalline pancreatic ribonuclease was the Worthington product. Tobacco leaf ribonuclease was kindly given us by Dr. P. Whitfeld, who had prepared it by a modification of the procedure of Frisch-Niggemeyer and Reddi (1957), using DEAE-cellulose chromatography. The preparation contained approximately 10,000 units/ml. "Crude plant nuclease" represents a filtered and concentrated homogenate (100 g of leaves to 5 ml final volume) of healthy tobacco leaf tissue. The dinucleoside 3′ → 5′ phosphates used were kindly placed at our disposal by Dr. H. Witzel.

Infectivity assays were performed on *N. tabaccum*, var. Xanthi nc, in the customary manner (Fraenkel-Conrat, 1959). Since bentonite was used to remove added enzymes, the more sensitive method of assay of RNA in the presence of bentonite (Singer and Fraenkel-Conrat, 1961), being the more convenient, was used for most experiments. Whenever possible, test solutions were prepared so that the number of lesions per half leaf were similar in both experimental and control solutions (10–50 lesions).

The general experimental procedure was as follows: To aliquots of an RNA solution (about

0.2 mg corresponding to 0.67 μmole of nucleotide) were added 0.2-1.7 μmole of the various metal salts and phosphate buffer (*p*H 7.0) to a final concentration of 10^{-3} M (1 ml total), unless otherwise specified. The infectivity of these solutions, in comparison with that of the metal-free control RNA, was ascertained by the customary assay method (after appropriate dilution in 0.1 M phosphate, without bentonite). Aliquots (usually 0.2 ml) of these solutions were then treated at 37° with the specified amounts of enzymes for the time period indicated. The addition of 0.03 ml 0.1 M ethylenediamine tetracetate (EDTA, versene) and 0.6 ml 6% bentonite suspension freed the RNA from the metals and simultaneously from the enzymes, when these were present. After at least 15 minutes at 0°, these solutions were diluted and assayed in the usual manner, with enough of the bentonite suspension added to the dilution to be assayed to bring it to 1%. While the untreated control RNA was generally tested at 0.5 μg/ml, in the presence of bentonite it was usually assayed at 0.02 μg/ml. The recovery of infectivity upon EDTA-bentonite treatment of the metal complexes before and after enzyme treatment, as well as of the metal-free enzyme treated samples, was expressed as percentage of the infectivity of the control RNA tested under the same conditions, *i.e.*, in the presence of bentonite. All samples were tested at least twice and usually more frequently on six half leaves.

Results[1]

Experiments with Mercuric Salts.—Mercuric chloride is known to have a marked effect on DNA. It greatly shifts the UV absorption band of DNA, and a two-step mechanism has been proposed for its action (Katz, 1952; Thomas, 1954; Yamane and Davidson, 1961). Transforming principle can be recovered from its mercury complex in active form (Dove and Yamane, 1960). When $HgCl_2$ was added to TMV-RNA in 10^{-3} M phosphate (*p*H 7) a similarly marked spectral shift from λ_{max} = 258 to λ_{max} = 274 was noted with an associated increase in OD at 300 mμ (see Fig. 1). In the absence of phosphate these effects were less pronounced. The complete shift was produced when the molar ratio of $HgCl_2$ to RNA nucleotide (*r*) was about 1, but a near complete shift was obtained with r = 0.5. Assay of such complexes showed over 99% inactivation with r = 1, and about 98% inactivation with r = 0.5. Further loss of activity was observed upon storage at −70°.

The concentration of mercury in the test solution, which was usually less than 5 μg/ml, or 0.25 μg per half leaf, was not noticeably toxic at this level, and probably was not as such inhibitory to the pathologic response to the infection. Control experiments in which the mercury salt was rubbed onto the leaves before or after the RNA, or in which the RNA-$HgCl_2$ complex was dissociated prior to assay, showed the expected RNA infectivity. It thus appears justified to conclude that the loss in infectivity can be attributed to the formation of a complex between the RNA and the metal.

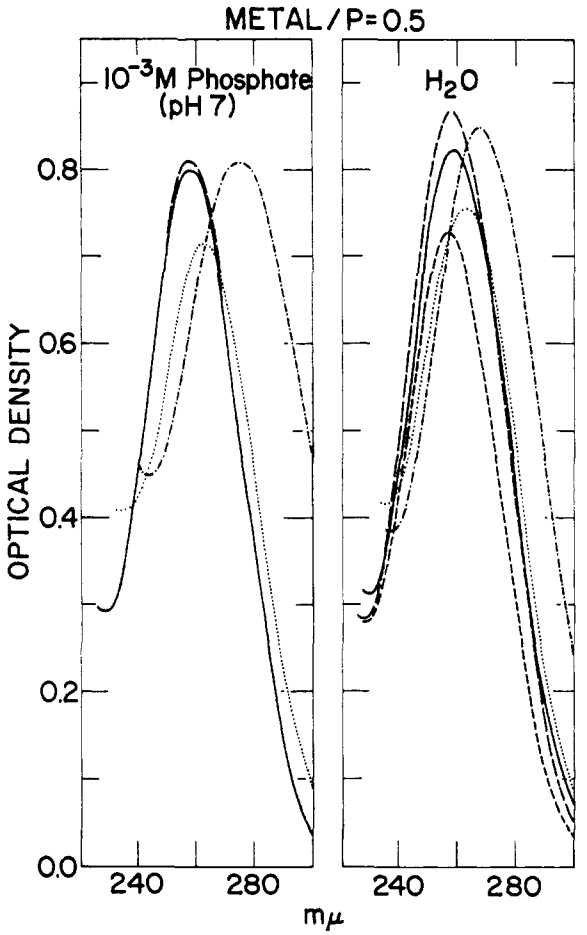

FIG. 1.—UV absorption spectra of TMV-RNA and its metal complexes. ·- ·-·-·-·-·-, Hg^{++}; . . . . . Ag^{+}; ----------, Mg^{++}; ——————, In^{+++}; - - - - - - -, control. Metal/P ratio = 0.5.

The RNA could be set free from the complex by a variety of agents. The addition of sodium chloride represents the simplest method (Katz, 1952), but EDTA achieved the same purpose. In each case the typical UV absorption spectrum of TMV-RNA was regenerated, and each treatment restored 50–100% of the original infectivity of the preparation (average of six experiments: 89%). In contrast, monovalent cations and phosphate and other polyanions had no effect, nor did phenol treatment in the presence of phosphate dissociate the complex.

Mercury analyses performed on the alcohol-precipitated and washed RNA indicated that 91, 86, and 83% of the mercury was bound with r = 0.5, 0.75, and 1, while the presence of 0.1 M

[1] For a preliminary report of these results see Singer and Fraenkel-Conrat (1962).

sodium chloride reduced the amount bound to about 20% (with $r = 0.5$).

Reconstitution is generally used in this laboratory to potentiate and stabilize the infectivity of TMV-RNA. However, upon addition of a 20-fold amount of protein to the RNA-mercury complex the mercury appeared to be released and to cause the precipitation of the protein, while the RNA in the supernatant showed its typical spectrum ($E_{max} = 258$ mμ), presumably because no native protein was available for reconstitution. With considerable excess of protein, partial reconstitution of the RNA in active form could be achieved (see Table I).

TABLE I
RECONSTITUTION OF METAL-COMPLEXED RNA

Metal (r)[a]	Protein/RNA[b]	Infectivity After Reconstitution (%)[c]
Hg (0.5)	20	0.007
	200	15
In (0.5)	20	51
	200	43
Ag (0.5)	20	39
	200	47
—	20	64
	200	71

[a] r is the molar ratio of metal salt (if any) to average nucleotide, assuming residue weight = 300. Hg stands for $HgCl_2$, In for $InCl_3$, Ag for CH_3COOAg. [b] Weight ratio (based on OD) employed in reconstitution. [c] Of theoretical maximum for amount of RNA present.

While $HgCl_2$ seemed to reversibly mask the infectivity of TMV-RNA, there remained the question whether it would also protect the RNA against the action of nucleases. To test this, mercury-RNA complexes (about 0.2 mg/ml, 10^{-3} M *p*H 7 phosphate, r varying from 0.3 to 1) and free RNA were treated under various conditions with crude or purified tobacco leaf nuclease, or with pancreatic ribonuclease. To recover free RNA from both types of reaction mixtures these were simultaneously treated with bentonite and EDTA. The former has been shown to adsorb nucleases effectively (Singer and Fraenkel-Conrat, 1961), while the latter complexes with the metals. Free RNA of typical UV absorption properties was thus recovered, and it was found that the material which had been enzyme treated in form of the mercury complex showed up to 40% of the original infectivity while mercury-free RNA retained only on the order of 0.1% of its infectivity after the enzyme treatment (see Tables II, III). Similar results were obtained with mercury over the range of *p*H 5 to *p*H 9 (Table III). Mercuric acetate was as effective as mercuric chloride in reversibly inactivating TMV-RNA and in affording it protection against nucleases (see Table V).

The possibility had to be considered that the protection of the RNA was due to inhibition of the enzyme by the mercury, rather than to protection of the substrate. In a series of experiments designed to test this possibility, the action of the plant nuclease on the small-molecular substrate, adenylyl (3′ → 5′) adenosine (ApA), was used as a measure of its activity. This seemed a feasible approach since the affinity of the mercuric salts appears much higher for macromolecular nucleic acids than for oligonucleotides.[2] The amount of adenosine released, as determined spectrophotometrically after chromatographic separation, proved a convenient measure of enzyme activity. When this activity was measured in the presence either of the mercury-RNA complex or of free RNA, the initial rate of digestion of ApA was quite similar, although after 30–60 minutes the mercury complex exerted some inhibition, possibly owing to a slow transfer of mercury from the RNA to the enzyme. Subsequent experiments on the protection of the complexed RNA were performed in the presence of ApA and under conditions in which, on the basis of the preliminary experiments, the enzyme was expected to remain fully active. This expectation was borne out in that the ApA was degraded to the usual extent during the 15-minute reaction period, and yet the Hg-RNA complex was attacked to a much lesser extent than the free RNA, as demonstrated by the infectivity of the two preparations after removal of mercury and enzymes by bentonite-EDTA treatment (see Table IV).

Similar experiments were performed with pancreatic ribonuclease. To check the activity of this enzyme in the reaction mixture, cytidylyl (3′ → 5′) adenosine (CpA) was used as a small molecular substrate. It is evident from the data presented in Table IV that the rate of splitting of CpA is about the same in the presence of infective TMV-RNA and of the mercury-RNA complex. Yet, the infectivity is markedly protected against this enzyme also when the RNA is in form of the complex. It is thus shown that the comparative enzyme-resistance of the mercury-RNA complex is a property of that complex, and not due to inhibition of the enzyme by the mercury.

Experiments with Indium Chloride.—Indium chloride has recently been described as a selective precipitant for nucleic acids (Aldridge, 1960). When this salt was added to TMV-RNA in 10^{-3} M *p*H 7 phosphate in less than equivalent amount per nucleotide (*e.g.*, $r = 0.5$) it caused no obvious precipitation nor did it have any pronounced effect on the UV absorption spectrum of the RNA (Fig. 1). Upon bioassay, the infectivity of RNA was only slightly decreased in the presence of indium ($r = 0.5$) (average of six experiments: 73%), but greater amounts of indium caused progressive inactivation, though always fully reversible by EDTA-bentonite treatment. Enzyme experiments showed that the indium

[2] Dekker, C. A., private communication; see also Pour-El (1960).

TABLE II
EFFECT OF ADDED SALTS ON INFECTIVITY AND ENZYME RESISTANCE OF TMV-RNA

Metal (r)[a]	Infectivity of Complex[b] (%)	Infectivity Recovered After Enzyme Treatment[b]: Ribonuclease (Pancreas)[c] (% of control)[d]	Plant Nucleases[c]: Purified (% of control)[d]	Plant Nucleases[c]: Crude (% of control)[d]
Hg^{++} (0.5)	0.9, 1.7, 2.4[e]	21, 32	40, 27, 34	30, 12, 16
In^{+++} (0.5)	58, 60, 100, 48	34, 26	1.7, 3.8, 4.7	0.1, 0.13, 0.17
(2.5)	0.9	34	9.5	1.1
Ag^{+} (0.5)	99, 125, 84, 52	41, 54	38, 93	0, 0.3
(2.5)	82, 31	87	32	0
Fe^{+++} (0.5)	52, 74	17, 18		
Fe^{++} (0.5)	0	0		
Cu^{++} (0.5)	83, 110	1.9, 4.2		
Mg^{++} (2.5)	89	0.45 (1:20,000)		
Na^{+} (1M)	20	0		
Co^{++} (0.5)	95	0		
Zn^{++} (0.5)	111, 111	0.45, 0.06		
Ni^{++} (0.5)	110	0.25		
None	100	0.06, 0.2 <0.01, <0.008	<0.005, 0.08 0.08	0.07, 0 0

[a] Metal used (if any) and r value (molar ratio of metal salt to average nucleotide, assuming residue weight = 300). In^{+++} stands for $InCl_3$; Ag^{+} for CH_3COOAg; Hg^{++} for $HgCl_2$; Fe^{+++}, Na^{+}, and Mg^{++} for the chlorides; Cu^{++} and Zn^{++} for the acetates; Ni^{++} and Co^{++} for the nitrates; Fe^{++} for $FeSO_4$. [b] Each figure represents the data (averages of at least two bioassays of 6–12 half leaves) of a separate experiment. [c] Figures in parentheses are enzyme/substrate weight ratios, other than those customarily used (namely, 1:1000 for pancreatic ribonuclease, 10 μl purified plant nuclease or 20 μl crude plant extract per 0.3 mg RNA). All enzyme incubations were at 37° for 15 minutes. [d] Control infectivity is that of the non-metal-containing, non-enzyme-treated RNA, after same bentonite and EDTA treatment as used for the enzyme treated samples, all simultaneously assayed in presence of 1% bentonite. RNA when complexed with mercury, silver, or indium and then freed of these metals showed within the error of the assays the original infectivity, whether tested with or without bentonite. [e] RNA mercury complexes progressively lost infectivity upon storage at −70°. The infectivities listed are therefore the results of the first assay, rather than averages of several tests.

complex was also considerably more resistant to attack by the pancreatic ribonuclease than the free RNA (Table II, III). But indium protected the RNA much less effectively against degradation by crude plant juice. Against the purified plant nuclease indium afforded good protection when used at higher concentration (*e.g.*, r = 2.5). No consistent differences were noted whether the digestibility of the complex was studied at *p*H 5 or 7. Reconstitution proceeded normally upon addition of a 20-fold amount of TMV protein to the indium complex (Table I).

Experiments with Silver Acetate.—The addition of silver acetate to TMV-RNA (10^{-3} M *p*H 7 phosphate) caused marked hypochromicity, with near-maximal effect, *i.e.* −17%, being approached upon addition of 0.5 to 1.0 mole Ag/nucleotide. The absorption maximum shifted from 258 to 262 mμ (Fig. 1). No significant loss of infectivity was observed with this amount of silver, and little inactivation was seen even with 5 or 10-fold this amount of silver. Treatment of the silver complex with pancreatic ribonuclease caused little loss of infectivity under conditions leading to over 99.9% inactivation of free RNA. The protection by silver, like that by indium, was less efficient at *p*H 9 than at *p*H 5 or 7, and it was more marked against the purified plant ribonuclease than against the enzyme mixture present in crude plant juice (Tables II, III). The observation that the digestion of CpA by pancreatic ribonuclease proceeded normally in the presence of the silver-RNA complex indicated that silver, like mercury, exerted its effect under the test conditions by complexing the macromolecular substrate, and not by inhibiting the enzyme (Table IV). Reconstitution proceeded almost normally, the silver becoming transferred from the RNA to a small amount of protein precipitating from solution in the course of the reaction (Table I). Semiquantitative silver analyses showed that the 0.5 or 2.5 moles of added silver (per mole P) was removed slowly by dialysis (0.2 mole remaining after 48 hours), but that only about 0.02 mole remained after 24 hours of dialysis against EDTA.

Miscellaneous Experiments.—The three metals investigated have the one feature in common that they protect TMV-RNA against the attack by nucleases, but they differ in most other regards. Only mercury inactivates the RNA, only indium causes no major change in the UV absorption characteristics of the RNA, only silver does not tend to precipitate it from solution. It appeared nevertheless possible that the interaction of bases or the aggregation of the RNA, phenomena which were caused by one or the other of the metals.

TABLE III
EFFECT OF *p*H ON ENZYME RESISTANCE OF RNA-METAL COMPLEXES

Metal (*r*)[a]	*p*H of Complex Formation and of Enzyme Treatment	Infectivity of Complex[b] (%)	Infectivity Recovered After Enzyme Treatment[b]			
			Ribonuclease (Pancreas)[c]		Plant Nucleases	
			1:500	1:1000	Purified[c]	Crude[c]
			(% of Control)[b]		(% of Control)[b]	
Hg (0.5)	5	1.5[d]	18			21
	7	1.3		19		31
	9	2.2	20			18
In (0.5)	5	86	42		1.3	3.5
(2.5)	5	1.4			67	2.1
(0.5)	7	91		16	5.7	0.23
(2.5)	7	0.3			60	4.5
(0.5)	9		2.4			
Ag (0.5)	5	110	20, 29		0.7	0.17
(2.5)	5	78			30	0.3
(0.5)	7	72		36, 47	119	1.1
(2.5)	7	47			128	1.7
(0.5)	9		2, 13			
None	5	107	0.003, 0.006		0.07	0.02, 0.04
	7	100		0.03, 0.003	0.07	0.02, 0.02
	9	100	0.006, 0			0

[a] Metal used (if any) and *r* value (molar ratio of metal salt to average nucleotide, assuming residue weight = 300). [b] Each figure represents the data (averages of at least two bioassays of 6–12 half leaves) of a separate experiment. Control infectivity is that of the non-metal-containing, non-enzyme-treated RNA, after same bentonite and EDTA treatment as used for the enzyme treated samples, all simultaneously assayed in presence of 1% bentonite. RNA when complexed with mercury, silver, and indium and then freed of these metals showed within the error of the assays the original infectivity, whether tested with or without bentonite. [c] Tests with pancreatic ribonuclease at *p*H 5 and 9, away from the optimum, were performed at twice the enzyme concentration as those at *p*H 7, which is near the optimum. Of the plant nucleases the same amounts were used as indicated in Table II, footnote *c*. [d] Mercury-containing RNA loses infectivity upon storage, so that these figures represent the first assay, rather than an average of repeated assays, as do all other data.

TABLE IV
ACTIVITY OF NUCLEASES TOWARD SMALL SUBSTRATES IN PRESENCE OF RNA-METAL COMPLEXES

Substrate[a]	*p*H	Enzyme (37°, 15 min.)	Adenosine Formed (%)	Infectivity Recovered After Enzyme Treatment[b] (%)
RNA + Hg (0.75), CpA (1.5)	7	Pancreas RNase 1:1000	4.5	10
RNA + Hg (0.75), CpA (1.5)	7	1:20	32	0.23
RNA + CpA (1.5)	7	1:1000	4.2	0.3
RNA + CpA (1.5)	7	1:20	35	<0.005
RNA + Hg (0.5), CpA 1.5	5	Pancreas RNase 1:1000	9	39
RNA + Hg (0.5), CpA 1.5	5	1:100	18	11
RNA + CpA (1.5)	5	1:1000	9	0.3
RNA + CpA (1.5)	5	1:100	14	0.1
RNA + Ag (0.5), CpA (1.0)	7	Pancreas RNase 1:200	25	4.9
RNA + Ag (0.5), CpA (1.0)	7	1:50	37	0.18
RNA + CpA (1.0)	7	1:200	26	0.0
RNA + CpA (1.0)	7	1:50	34	0.0
RNA + Hg (0.5), ApA 1.5	5	Crude plant enzymes 2 μl	13	21
RNA + Hg (0.5), ApA 1.5	5	5 μl	8	6
RNA + ApA (1.5)	5	2 μl	12	0.2
RNA + ApA (1.5)	5	5 μl	5	0.2

[a] Figures in parentheses represent molar ratios of metals and small molecular substrates to RNA-nucleoti residue. [b] Percentage of non-enzyme-treated control, assayed under same conditions. de

TABLE V
EFFECT OF COMBINED METALS ON INFECTIVITY AND ENZYME RESISTANCE OF RNA

Metal (r)[a]	Infectivity of Complex[b] (% of Control)	Infectivity Recovered After Treatment[b] with Pancreatic Ribonuclease		
		1:1000	1:5000 (% of Control)	1:20,000
Hg (0.5) + Mg (10)	27, 27	2.2, 0		
Mg (10) + Hg (0.5)	40, 40	0.8, 0.8		
Hg (0.5) + Mg (0.5)	9, 11	24, 40		
Mg (0.5) + Hg (0.5)	15, 8	52, 48		
Ag (0.5) + Mg (10)	60	0.5		
Mg (10) + Ag (0.5)	98	0.5		
Ag (0.5) + Mg (0.5)	59, 110	34	56	58
Mg (0.5) + Ag (0.5)	48, 110	48	44	55
Ag (0.5) + Hg (0.5)	0, 0[c]	3.5, 10		
Hg (0.5) + Ag (0.5)	0	5.9		
Hg (0.5)	1.3, 1.5[c]	27, 37		
Ag (0.5)	42, 45	27, 66		
Mg (0.5)			0.08	
Mg (10)	65, 78, 53	0.08, 0, 0	0.08	0.3
No added metal	(100)	0 (<0.01)		

[a] r is the molar ratio of metal salt to average nucleotide, assuming residue weight = 300. All metals used were in the form of the acetates. [b] Each figure represents the data (averages of at least two bioassays of 6-12 half leaves) of a separate experiment. Control infectivity is that of the non-metal-cotaining, non-enzyme-treated RNA, after same bentonite and EDTA treatment as used for the enzyme-treated samples, all simultaneously assayed in presence of 1% bentonite. RNA when complexed with mercury, silver, or indium and then freed of these metals showed within the error of the assays the original infectivity, whether tested with or without bentonite. [c] RNA mercury complexes progressively lost infectivity upon storage at −70°. The infectivities listed are therefore the results of the first assay, rather than averages of several tests.

were responsible for the protection. To test the effect of aggregation or precipitation, RNA was treated with pancreatic ribonuclease in molar sodium chloride and in 0.1 M magnesium chloride, conditions which cause hypochromicity and precipitation or aggregation. In both of these states, the RNA was considerably more susceptible to enzymatic degradation than under the standard conditions used in this study (Table II).

Preliminary sedimentation data obtained by Dr. M. Daune suggest that little if any aggregation occurs under conditions where silver and indium effectively protect the RNA against enzymes. In homogeneity and S values (about 70% of the OD is due to uniformly sedimenting material of about 30 S) no significant differences were detected between these complexes and the original RNA. Mercury, however, greatly increased the sedimentation rate. These observations thus indicate that aggregation does not account for the enzyme protection afforded by some of the metals investigated, which may well act by entirely different mechanisms.

To test the specificity and reversibility of the observed enzyme protection, EDTA was added to the RNA-metal complexes prior to the 15-minute enzyme incubation period, rather than together with the bentonite at the end of this period. Under these conditions the enzyme caused complete inactivation of the RNA, which must be regarded as evidence that all three metals are released by the RNA under the influence of this chelating agent.

Silver has been reported to increase the melting point (T_m) of RNA as detected by the increasing OD upon heating under standard conditions (Yamane and Davidson, 1962a). The effect of the presence of silver on the rate of thermal inactivation (at 70°) of TMV-RNA was therefore studied. No consistent effect on the inactivation rate was observed with r = 0.5, but a 5-fold of this amount of silver caused a marked increase in this rate.

The diversity of effects on the UV absorption spectrum and the infectivity of RNA observed with silver, mercury, and magnesium suggested itself as a tool to probe some questions pertaining to binding sites and or relative affinities of these metals. Using each at similar molar concentrations (r = 0.5 to 2) it was observed that the red shift in the absorption band produced by silver was not abolished by magnesium and occurred also if the silver was added after the magnesium. The hypochromic effect was further decreased by the combination of the two metals (Ag: −19%, then Mg: −22%; and Mg: −20%, then Ag: −25%, total decrease as compared to the absorbance of the original RNA in H_2O).

When mercury was added after magnesium, the hypochromic effect of the latter was partly reversed (−20% → −12%), and the marked red shift characteristic of the mercury complex was noted (A_{max} 257 mμ → 272 mμ). Addition of the magnesium after the mercury caused only a slight increase in hypochromicity (−2.5% → −5%).

Since magnesium is known to activate the enzymatic degradation of RNA, it seemed of interest to study the effect of combinations of this ion with silver and mercury, which independently protect against nuclease action. Thus complexes of RNA with equal amounts of both silver and magnesium, or of mercury and magnesium, regardless of their order of addition, seemed to show enzyme resistance similar to RNA complexed with silver or mercury alone. When magnesium or silver were present in marked excess ($r = 10$) they lessened the inactivating action of limited amounts of mercury ($r = 0.3$ to 0.5) (Table V). Mercury analyses indicated that the RNA retained almost all of the added mercury ($r = 0.3$ to 2.0) after two alcohol precipitations, even if treated with silver ($r = 0.5$) or magnesium ($r = 10$) before or after addition of the mercury. It would appear from all these observations that in RNA the binding affinities and sites for mercury, silver, and magnesium are comparatively independent of one another.

A preliminary survey of the effects of a few other metals (Table II) showed that most divalent ions seemed to resemble the alkali earth metals in causing hypochromicity and affording no appreciable protection against enzymes (Co^{++}, Ni^{++}, Zn^{++}). Combinations of these with magnesium had no additive effects on the UV absorption spectrum of the RNA. Cu^{++} showed a slight hyperchromic effect and afforded some enzyme protection under the test conditions ($r = 0.5$, pH 7, pancreatic ribonuclease; about 3% of activity remaining, compared to 50% with silver). The affinity of the RNA was higher for Cu^{++} than for Mg^{++}, but silver showed its usual effect on the spectrum when added after the Cu^{++}. Fe^{++} was the only metal to cause irreversible inactivation of the RNA, quite in contrast to Fe^{+++}, which caused little immediate loss of infectivity and afforded considerable protection of the RNA against the action of pancreatic ribonuclease. However, ferric salts caused a time-dependent inactivation of TMV-RNA and decrease of its absorbance at 260 mμ.

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