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PROTECTIONS BY NON-COPPER METAL IONS AGAINST COPPER-MEDIATED INACTIVATION OF POLIOVIRION RNA OCCURRING AT EXTRACTION OF VIRIONS WITH PHENOL

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Summary. Al^{3+}, Ca^{2+}, Co^{2+}, Cu^{1+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{4+}, and Zn^{2+} were incubated individually with redistilled reagent-grade phenol containing impurities known from previous work to interact with Cu^{2+} to produce a potent inactivator(s) of the transfectivity of naked poliovirion RNA. Only the mixture with Cu^{1+} inactivated the RNA. Tests of each of the 11 non-copper test metal ions mixed with Cu^{2+} before adding the phenol showed that Ca^{2+} and Mg^{2+} do not protect, Co^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} provide moderate protection, and Al^{3+}, Fe^{2+}, Fe^{3+}, and Sn^{4+} give strong protection against the Cu^{2+}-mediated inactivation. Other points of addition of protective metal ion were tested using Fe^{3+}. Strong protection was afforded even when Fe^{3+} was added after synthesis of the inactivator(s) from Cu^{2+} and the active impurities. The relation between Cu^{2+} and the Fe^{3+} was shown to be competitive. The hypothesis that ions compete for semiquinone anion is proposed.

Key words: copper; metal ions; phenol; RNA inactivation: RNA protection

Introduction

Phenol is commonly used for the preparation and isolation of viral nucleic acids from virions and from infected cells. Factors which influence the quality and biological activities of the viral nucleic acids isolated using phenol are an important concern to virology, molecular biology, and in vitro DNA recombinant technology.

The presence of Cu^{2+} during the extraction of RNA from poliovirions using phenol can result in large losses in the transfectivity of the extracted RNA (Dubes and Wegrzyn, 1978*b*). Even trace levels such as 10^{-7} M Cu^{2+}, which can be derived from impurities in or contamination from laboratory materials, can cause large losses of transfectivity.

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The Cu^{2+} by itself does not inactivate the RNA, but requires a cofactor for the inactivation. Cofactor is supplied by impurities in the redistilled reagent-grade phenol used for the extraction. Cu^{2+} and cofactor interact to produce an inactivator(s) of RNA (Dubes *et al.*, 1979*a*, *b*; Dubes *et al.*, 1980).

The work reported herein was done to answer two questions related to the effects of metal ions in this system.

The first question was: Is this role of Cu^{2+} in the production of an inactivator of viral RNA a role which can be played by other metal ions? We selected Cu^{1+} and 11 species of multivalent non-copper metal ions for testing in this regard. These 11 ions were: Al^{3+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{4+}, and Zn^{2+}. Ions of 5 of these metals (Mn, Fe, Co, Ni, and Zn, of atomic numbers 25, 26, 27, 28 and 30, respectively) were selected because of their proximity to copper (atomic number 29) in the periodic table. Ions of the other 5 metals were selected because of their biological importance, common occurrence in laboratory materials, or toxicity.

The second question was: Do the non-copper metal ions have any capacity to protect the RNA against the Cu^{2+}-mediated inactivation?

Part of the results have been published in abstract form (Dubes and Wegrzyn, 1978*b*).

Materials and Methods

Metal chlorides. Reagent-grade metal chloride used were: $AlCl_3 \cdot 6\,H_2O$, $CoCl_2 \cdot 6\,H_2O$, $MnCl_2 \cdot 4\,H_2O$, $NiCl_2 \cdot 6\,H_2O$, $PbCl_2$, and $SnCl_4 \cdot 5\,H_2O$ from J. T. Baker Chemical Co., Phillipsburg, New Jersey; $CaCl_2 \cdot 2\,H_2O$, $FeCl_3 \cdot 6\,H_2O$, and $ZnCl_2$ from Mallinckrodt, Inc., Saint Louis, Missouri; and $CuCl_2$ from Fisher Scientific Co., Fair Lawn, New Jersey. CuCl and $MgCl_2 \cdot 6\,H_2O$ were Fisher certified A.C.S., and $FeCl_2 \cdot 4\,H_2O$ was Fisher certified. Concentrates of the metal chlorides were prepared in deionized distilled water.

Phenol. Three commercial lots of reagent-grade phenol were used: lot CAZ, liquefied, no preservative, Mallinckrodt, Inc., Saint Louis, Missouri; lot CEX, crystal, 0.15% hypophosphorous acid added as preservative, Mallinckrodt; lot X052, crystal, label noncommittal regarding preservative, Baker and Adamson, Morristown, New Jersey. The commercial phenol lots were redistilled in our laboratory. Before and after redistillation, the lots showed high cofactor activity.

Pre-incubation of metal chlorides with cofactor-containing phenol. Preincubation was in pH 7.3 buffer of the composition: 137 mM NaCl, 2.69 mM KCl, 8.15 mM Na_2HPO_4, 1.46 mM KH_2PO_4. Any later reference to buffer means this buffer. Al^{3+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{4+}, and Zn^{2+} were tested at the highest of the following concentrations not giving a precipitate at any point during the course of the test: 100 μM; 300 μM; 1,000 μM; Ca^{2+}, Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Pb^{2+} were also tested at lower concentrations. Cu^{1+} was tested at 0.1 — 20 μM, and Fe^{2+} at 5 — 100 μM. In tests for protection against Cu^{2+}-mediated inactivation, the Cu^{2+} was at 1 μM, except for one set of tests at 3 μM shown in Fig. 3. The concentrations used in specific experiments are given in the Results section.

The test metal chloride in buffer with or without added $CuCl_2$, at the concentrations indicated above, was shaken at 0 °C with an equal volume of redistilled phenol saturated with water at 0 °C. The aqueous phase was then pre-incubated at 0 °C for about 30 min, then at 23 °C for about 5 min, just before adding the viral RNA. In the special test used for Table 4, the procedure was varied. Ninety-three volumes of the test metal chloride in buffer with or without added $CuCl_2$ and 7 volumes of redistilled phenol saturated with water at 0 °C were mixed, to give a monophasic system. This mixture was then pre-incubated at 23 °C for 95 min, just before adding the viral RNA.

Viral RNA. The Brunhilde strain of type 1 poliovirus was grown and plaqued on sheet cultures of the CLI line (Douglas *et al.*, 1966) of chimpanzee liver cells in commercial plastic 60-mm Petri dishes (Dubes and Wegrzyn, 1978*a*). The poliovirions were purified by differential centrifugation,

followed by sucrose density gradient ultracentrifugation (Dubes, 1974). The purified poliovirions in buffer with about 20% by weight sucrose, were diluted, usually 160-fold, into buffer and extracted once at 0 °C with an equal volume of phenol saturated with water at 0 °C. The extracted virion RNA, in the aqueous phase, was used immediately in the tests of the effects of the selected metal ions. Virion RNA was freshly extracted for each test.

Incubation of viral RNA in pre-incubated mixtures of metal chlorides and cofactor-containing phenol. The extracted virion RNA was diluted 15-fold into the pre-incubated mixture of phenol and test metal ion in buffer with or without added Cu^{2+} at 23 °C. To accommodate the special purpose of the tests for Table 4, the extracted virion RNA was diluted only 2-fold at this step.

In many cases, a sample of the viral RNA-containing mixture was taken immediately. These samples served as tests for effects of the metal ions and phenol on transfection or plaquing. The remainder was incubated at 23 °C. Further samples were taken after pre-determined times, usually 5-90 min, at 23 °C. Each sample was promptly diluted 33 1/3-fold into buffer with added L-histidine hydrochloride and $NaHCO_3$ to bring their concentrations to 300 μM and 7.9 mM, respectively. The histidine stops the inactivation of the RNA (Dubes, 1975, 1980). The $NaHCO_3$ increases the sensitivity of the transfection method (G. R. Dubes, unpublished data). These dilutions were the RNA inocula.

Titration of RNA transfectivity. The transfectivity of the RNA was titrated on DEAE-dextran-sensitized sheet cultures of CLI cells, as described (Dubes and Wegrzyn, 1978*a*). The sensitivity of this transfection method is 24×10^6 plaque-forming units (PFU) per/μg RNA from poliovirions (Dubes and Wegrzyn, 1978*a*).

Tests under nitrogen. Because of the oxidizability of Cu^{1+} and Fe^{2+} by O_2 the deionized distilled water used to prepare the concentrates of CuCl and $FeCl_2$ had been boiled vigorously for 30—40 min to remove dissolved oxygen and then gassed at 23 °C with N_2 for 15—20 min before adding the solid metal chlorides; the concentrates of CuCl and $FeCl_2$ were used only on the day of their preparation. In the tests of effects of CuCl and $FeCl_2$ on RNA transfectivity all solutions used had been freshly gassed at 23 °C with N_2 for 15—20 min and gassing with N_2 was done before and after each experimental manipulation.

Tests for effects of the test metal ions and phenol on transfection or plaquing. Transfection and plaquing by the inocula prepared from the samples taken immediately after adding the viral RNA to the pre-incubated mixture of test metal ion and phenol were compared with transfection and plaquing by control inocula prepared by dilution of the viral RNA into buffer without test metal ion and without phenol. These comparisons showed that the phenol and test metal ions at their inoculum concentrations, which at their highest were 18 mM for phenol, 9 μM for Al^{3+}, Fe^{3+}, Mn^{2+}, Pb^{2+}, and Zn^{2+}, and 30 μM for Ca^{2+}, Co^{2+}, Mg^{2+}, Ni^{2+}, and Sn^{4+}, affected neither transfection nor plaquing. Therefore, the metal ion effects described in the Results section are effects on the transfectivity of the viral RNA, and not effects on the transfection process or on plaquing.

Statistics. For tests or controls where at least one plaque was found, data are expressed as arithmetic mean ± its standard error. Where no plaques were found on n-plates, data are expressed as < 1 plaque per n plates, or $< n^{-1}$.

Results

Tests of the capacity of Al^{3+}, Ca^{2+}, Co^{2+}, Cu^{1+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sn^{4+}, and Zn^{2+} to inactivate naked poliovirion RNA

Each of these 12 test metal ions was tested under conditions where Cu^{2+} mediates strong inactivation of the viral RNA. The test metal ion was pre-incubated with cofactor-containing phenol, and the pre-incubated mixture was tested for capacity to inactivate the viral RNA by incubating the RNA with the mixture at 23 °C. RNA controls were prepared and incubated in the same way except that the test metal ion was omitted. Though varied from experiment to experiment, the duration of incubation of the RNA at 23 °C in a given experiment was the same for the control and the experimental.

Table 1. Tests of the capacity of pre-incubated mixtures of cofactor-containing phenol and twelve species of metal ion to inactivate naked poliovirion RNA

Ion	Concentration μ M[1])	Plaques per plate[2])			
		Incubated RNA			Unincubated RNA control: no test metal ion
		Incubation min[3])	Experiment[4])	Control[5])	
Al^{3+}	300	60	235 ± 4	32 ± 0	179[6])
Ca^{2+}	1,000	30	36 ± 5	56 ± 4	208 ± 46
Co^{2+}	1,000	30	236 ± 44	272 ± 22	364 ± 86
Cu^{1+}	10	30	< 0.5	244 ± 18	
Fe^{2+}	100	30	229 ± 13	167 ± 36	
Fe^{3+}	300	60	144 ± 67	13 ± 1	411 ± 71
Mg^{2+}	1,000	60	26 ± 3	22 ± 6	273 ± 29
Mn^{2+}	300	30	69 ± 22	56 ± 4	208 ± 46
Pb^{2+}	100	60	47 ± 2	32 ± 0	179[6])
Ni^{2+}	1,000	90	144 ± 5	80 ± 7	351 ± 25
Sn^{4+}	1,000	60	205 ± 42	13 ± 1	411 ± 71
Zn^{2+}	300	90	128 ± 20	80 ± 7	351 ± 25

[1]) Concentration in buffer just before extraction with phenol.
[2]) Each row gives the results of a single experiment or the normalized results of two or more experiments. In all cases, the standard error was calculated from the intra-experimental variance.
[3]) The incubations at 23 °C were done under air, except that with Cu^{1+} and Fe^{2+} and their controls were done under N_2, as described in Materials and Methods.
[4]) RNA incubated with phenol and test metal ion.
[5]) RNA incubated with phenol but without test metal ion.
[6]) Due to Petri culture loss, only a lone Petri culture remained for counting plaques.

Twenty experiments of this kind were done. In most experiments, unincubated RNA controls were also included. Some of the results are presented in Table 1. The survival of RNA transfectivity in the controls incubated at 23 °C was 27 % and 75 % after 30 min, 18 %, 3 %, and 8 % after 60 min, and 23 % after 90 min, for the data shown. Results obtained in other experiments not shown in the table also showed significant inter-experiment variation in control RNA survival. When L-histidine, a chelator of Cu^{2+}, was added, this inter-experiment variation disappeared and the RNA transfectivity was stable. It seems likely that the inter-experiment variation observed in control RNA survival without protective agent was due to inter-experiment variation in trace copper impurity in and contamination of the experimental materials used.

Of the 12 test metal ions, only Cu^{1+} caused RNA inactivation. In addition to the data shown in Table 1 for the other 11 ions, data were obtained at lower test metal ion concentrations (Ca^{2+}, Co^{2+}, and Mg^{2+} at 100 μM; Mn^{2+} at 30 μM; Pb^{2+} at 10 μM) and with shorter incubation periods. These additio-

Table 2. Comparison of Cu^{1+} with Cu^{2+} in capacity to mediate inactivation of naked poliovirion RNA

Copper chloride[1])	Phenol	Plaques per plate	
		Exp. C 20	Exp. C 21
None (control)	Yes	156 ± 16	110 ± 25
0.5 μM $CuCl_2$	Yes		46 ± 5
1.0 μM CuCl	No (control)	147 ± 12	145 ± 7
1.0 μM CuCl	Yes	25 ± 1	58 ± 7
1.0 μM $CuCl_2$	Yes	4.0 ± 2	22 ± 6
2.0 μM CuCl	Yes	9.0 ± 3	
2.0 μM $CuCl_2$	Yes	2.0 ± 0	
3.0 μM CuCl	Yes		14 ± 4

[1]) Concentration in buffer just before the extraction with phenol. The viral RNA, after being mixed with the pre-incubated copper chloride solution or with the control without copper chloride, was incubated at 23 °C for 30 min. All of these tests, including the controls, were done under N_2 as described in the Materials and Methods section and were plated at the same dilution.

nal data support the conclusion that these 11 ions did not cause RNA inactivation under the conditions used.

Though these 11 test metal ions showed no RNA inactivating activity in this system, the data of Table 1 suggest that some (Al^{3+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Sn^{4+}, and Zn^{2+}) protected the RNA against the inactivation occurring in the incubated control.

Data comparing Cu^{1+} with Cu^{2+} in capacity to mediate RNA inactivation are shown in Table 2. The tests, which were done under N_2, showed that (*a*) the activity of Cu^{1+}, through high, was still substantially lower than the activity of Cu^{2+} and (*b*) Cu^{1+}, like Cu^{2+}, required cofactor-containing phenol for activity. Though these tests were done under N_2 as described, we cannot be certain that the observed activity of Cu^{1+} was not mediated through oxidation of a portion of the Cu^{1+} to Cu^{2+}.

Tests of capacity of 11 metal ions to protect naked poliovirion RNA against the inactivation mediated by Cu^{2+}

Each of the eleven non-copper test metal ions was tested by mixing it with deliberately added Cu^{2+} before the extraction with phenol. The capacity of the preincubated mixture to inactivate the viral RNA was measured. The results obtained with all of these test metal ions except Ni^{2+} and Zn^{2+} are shown in Table 3. The RNA was incubated at 23 °C for 30 min in the experimental and in two of the three kinds of controls. The data showed that strong protection of the viral RNA was afforded by Al^{3+}, Fe^{2+}, Fe^{3+}, and Sn^{4+}, moderate protection by Co^{2+}, Mn^{2+}, and Pb^{2+}, and no protection by Ca^{2+} and Mg^{2+}.

The results obtained with Ni^{2+} and Zn^{2+} are shown in Fig. 1 as the effects of these two test metal ions on the kinetics of inactivation of the viral RNA.

Table 3. Tests of the capacity of nine species of metal ion to protect naked poliovirion RNA against inactivation mediated by Cu^{2+}

Ion	Concentration[1] μM	Plaques per plate[2])			
		$CuCl_2$[3]) plus test metal ion incubated[4])	Incubated controls		Unincubated control[5]) no $CuCl_2$ and no test metal ion
			$CuCl_2$[3]) alone	No $CuCl_2$, no test metal ion	
Al^{3+}	300	148 ± 12	6.5 ± 1.5	130 ± 26	250 ± 22
		86 ± 10	<0.5	67 ± 25	190 ± 50
Ca^{2+}	1,000	5.0 ± 1.0	6.0 ± 2	228 ± 30	318 ± 32
		2.5 ± 0.5	9.5 ± 1.5	288 ± 32	452 ± 36
Co^{2+}	1,000	40 ± 19	6.5 ± 1.5	130 ± 26	250 ± 22
		10 ± 1.0	<0.5	67 ± 25	190 ± 50
Fe^{2+}	5*	37 ± 1.0	4.0 ± 1.0	90 ± 24	
	20*	258 ± 18	22 ± 4	243 ± 45	
	20*	152 ± 38	4.0 ± 1.0	90 ± 24	
	100*	247 ± 7	22 ± 4	243 ± 45	
Fe^{3+}	20*	67 ± 20	4.0 ± 1.0	90 ± 24	
	50*	90 ± 51	22 ± 4	243 ± 45	
	300	147 ± 17	<0.5	86 ± 14	370 ± 23
		212 ± 54	<0.5	106 ± 6	389 ± 102
Mg^{2+}	1,000	2.0 ± 1.0	6.0 ± 2	228 ± 30	318 ± 32
		16 ± 4	9.5 ± 1.5	288 ± 32	452 ± 36
Mn^{2+}	300	89 ± 2	9.5 ± 1.5	288 ± 32	452 ± 36
		30 ± 1.5	6.5 ± 1.5	130 ± 26	250 ± 22
		46 ± 2	<0.5	67 ± 25	190 ± 50
Pb^{2+}	100	25 ± 2	6.5 ± 1.5	130 ± 26	250 ± 22
		12 ± 0.5	<0.5	67 ± 25	190 ± 50
Sn^{4+}	1,000	244 ± 64	6.0 ± 2	228 ± 30	318 ± 32
		377 ± 70	9.5 ± 1.5	288 ± 32	452 ± 36

[1]) In buffer just before the extraction with phenol.
[2]) For each test metal ion at a given concentration, each row gives the results for a separate experiment.
[3]) At 1 μM in buffer just before the extraction with phenol.
[4]) Incubation of the viral RNA was at 23 °C for 30 minutes.
[5]) No incubation after adding the viral RNA.
* These tests of iron cations and the corresponding controls were done under N_2.

The Ni^{2+} effected moderate decrease in the rate of RNA inactivation; the Zn^{2+} effected a greater decrease.

Can a protective metal ion protect when it is added only after the inactivator(s) has already been synthesized from Cu^{2+} and the dihydric phenol impurities? This question was addressed by testing two other points of addition of protective metal ion; with both of these points of addition, synthesis of inactivator(s) was allowed to take place before the addition of protective metal ion. For these tests, Fe^{3+} was selected as the protective ion. Fe^{3+} was directly added either to (*a*) the inactivator(s) produced by pre-incubation of a mixture of Cu^{2+} and cofactor-containing phenol, or (*b*) the

viral RNA; and the degree of protection afforded by the Fe^{3+} against the Cu^{2+}-mediated inactivation was measured (Table 4). Strong protection was afforded by Fe^{3+} added at either point.

In summary, we see that (*a*) strong protection of the viral RNA against Cu^{2+}-mediated inactivation was afforded by Al^{3+}, Fe^{2+}, Fe^{3+}, and Sn^{4+}, moderate protection by Co^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}, but no protection

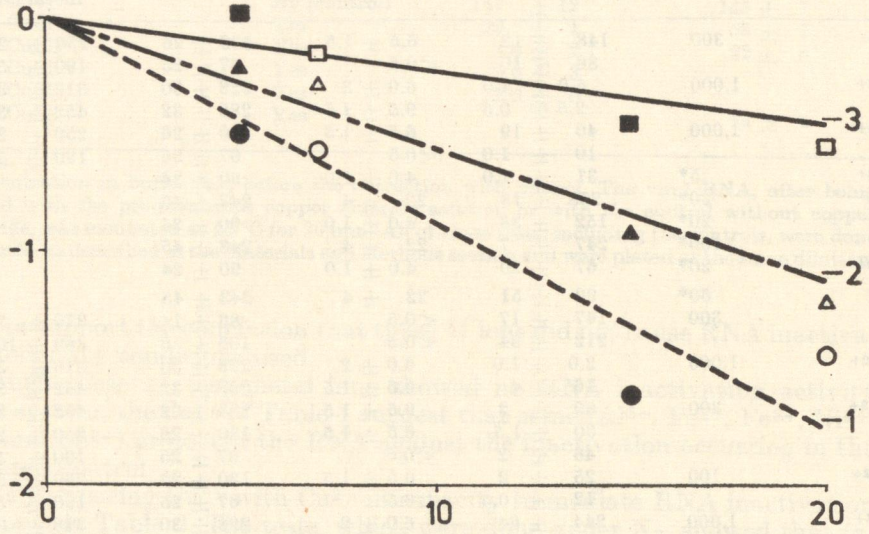


Fig. 1.

Protection of viral RNA by Ni^{2+} at 1,000 μM and Zn^{2+} at 300 μM against inactivation mediated by Cu^{2+} at 1 μM

Curve 1 — Cu^{2+} alone (circles); curve 2 — Cu^{2+} plus Ni^{2+} (triangles); curve 3 — Cu^{2+} plus Zn^{2+} (squares).

Solid and open symbols, data from separate experiments.

Abscissa: time of incubation of RNA at 23 °C (min); ordinate: $\log_{10}$ surviving fraction

by Ca^{2+} and Mg^{2+}, and (*b*) protection could be afforded even when the protective metal ion was added after the inactivator(s) has been synthesized.

Competition between Fe^{3+} and Cu^{2+}

Do the protective metal ion and Cu^{2+} show a competitive relationship? Specifically, if the Cu^{2+} concentration is increased, will a higher Fe^{3+} concentration be required to maintain a given level of protection of the viral RNA? Results with deliberately added Cu^{2+} at 0, 1, and 3 μM are shown in Fig. 2. Various concentrations of Fe^{3+} were tested pre-mixed with each Cu^{2+} concentration before extraction with phenol.

Table 4. Protection of viral RNA by Fe^{3+} added at different points

Point of addition of Fe^{3+}	Pre-incubation of RNA at 23 °C[1])	Pre-incubation of inactivat-or(s) of buffer control[3])	Incubation of RNA at 23 °C after adding in-activator(s) or buffer control[3])	Plaques per plate	Protection[4])
No Fe^{3+} added (controls)	None	Buffer control (no inactivator)	None (control)	364 ± 16	
		60 min	30 min	2.0 ± 1.0	
	10 min	60 min	30 min	1.0 ± 0	
To RNA	10 min with Fe^{3+} at 100 μM	60 min	30 min	123 ± 25	34 %
	10 min with Fe^{3+} at 200 μM	60 min	30 min	184 ± 22	50 %
To inacti-vator (s)	None	60 min with Fe^{3+} at			
		100 μM	30 min	161 ± 2	44 %
		200 μM	30 min	232 ± 94	64 %

[1]) Pre-incubation of the viral RNA just before mixing it with preincubated inactivator (s).

[2]) Inactivator(s) was formed by incubating $CuCl_2$ at 2 μM together with cofactor-containing phenol at 560 mM in buffer at 23 °C for 95 min. One volume of buffer without Fe^{2+} or with Fe^{3+} to give the indicated concentrations was then added, and the incubation was continued for 60 min.

[3]) The viral RNA, pre-incubated or not pre-incubated as indicated, was mixed with an equal volume of the pre-incubated inactivator(s) and the mixture was then incubated at 23 °C for 30 min. Inactivation of the viral RNA was terminated by diluting into buffer with histidine, and the remaining RNA transfectivity was titrated.

[4]) The percentage (P) of the RNA protected by the added Fe^{3+}, e. g., for the bottom row $P = (100)(232\text{-}2)/(364\text{-}2) = 64$.

When Cu^{2+} was added at 1 or 3 μM, it was not possible to protect all of the RNA by increasing Fe^{3+} concentration. In other words, there was a component of the Cu^{2+}-mediated inactivation not protectable by Fe^{3+}. During the 30-min incubation used, this component accounted for the inactivation of about 37% of the RNA when Cu^{2+} was at 1 μM and about 68% of the RNA when Cu^{2+} was at 3 μM.

The component of the Cu^{2+}-mediated inactivation protectable by Fe^{3+} did show a competitive relation between the two kinds of metal ion. The two large crosses of Fig. 2 show the points of half protection against this component; these points are at 60 μM Fe^{3+} for 1 μM Cu^{2+}, and 216 μM Fe^{3+} for 3 μM Cu^{2+}. The two ratios $60/1 = 60$ and $216/3 = 72$ are close, and the difference between them can be considered as largely due to statistical error and to some arbitrariness in drawing the two curves. We suggest that the percentage protection against the Cu^{2+}-mediated inactivation component protectable by Fe^{3+} may be simply a function of the ratio of Fe^{3+} concentration to Cu^{2+} concentration.

Discussion

The two most significant results of this research are (*a*) that only copper ions of the various species of metal ions tested interact with cofactor(s) in phenol to yield inactivator(s) of the naked viral RNA, and (*b*) that the ions of several metals protect against the copper-mediated inactivation.

Together with copper in group 1 b of the periodic table of the elements are silver and gold. Tests of the capacity of ions of silver and gold to interact with cofactor(s) to yield inactivator(s) of RNA would be of especial interest.

Recently, a high performance liquid chromatographic investigation identified two impurities in reagent-grade phenol as catechol and hydro-

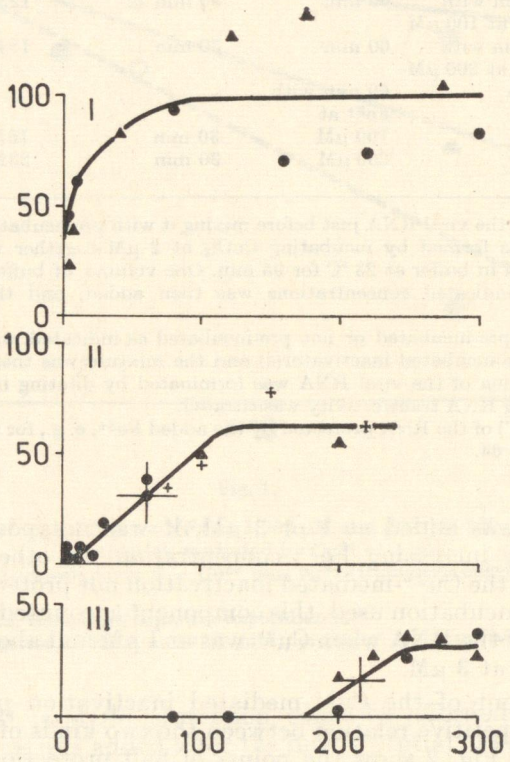
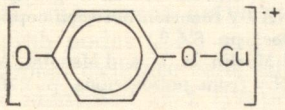


Fig. 2.

Effect of Cu^{2+} on the concentration of Fe^{3+} required to protect the RNA
I — No Cu^{2+} added; II — 1 μM Cu^{2+} added; III — 3 μM Cu^{2+} added. The two large black crosses indicate the concentration of Fe^{3+} required to protect half the plateau value of surviving RNA. For a given panel, symbols of different shape indicate results from different experiments. The indicated concentration of Cu^{2+} and Fe^{2+} were the concentrations in buffer just before the extraction with phenol. The viral RNA was incubated at 23 °C for 30 min.
Abscissa: $FeCl_3$ concentration (μM); ordinate: RNA survival (%)

quinone (Masoud and Dubes, 1979, 1980). Catechol and hydroquinone have cofactor activity (Dubes *et al.*, 1979*b*). Thus, it is now clear that at least part of the cofactor activity of reagent-grade phenol is due to catechol and hydroquinone impurities.

Recent investigations aimed at gaining evidence on the nature of the proximate inactivator(s) synthesized from Cu^{2+} and hydroquinone have indicated that the inactivator(s) (*a*) is synthesized in parallel with the oxidation of hydroquinone to p-benzoquinone under air, (*b*) is neither Cu^{1+} nor p-benzoquinone, and (*c*) is unstable (Dubes and Masoud, 1980). These characteristics and other recent evidence are consistent with the hypothesis that proximate inactivator is the free radical complex of cupric ion and p-benzosemiquinone anion described by Khudyakov *et al.*, (1978):



Such a hypothesized structure for proximate inactivator suggests that the protective metal ions may protect by competing with Cu^{2+} for the p-benzosemiquinone anion. Protection by metal ion added after the synthesis of proximate inactivator could be by the same mechanism, since the cupric complex is expected to be in dynamic equilibrium with Cu^{2+} and the p-benzosemiquinone anion.

This hypothesized mechanism of protection suggests the following explanation for the Cu^{2+}-mediated inactivation component not protectable by Fe^{3+}: We now know that the active reagent-grade phenol contains hydroquinone and catechol, as noted above, as well as possibly ten other phenols as impurities (Masoud and Dubes, 1980). Hydroquinone, catechol, and possibly some of the other impurities are active cofactors. We suggest that the semiquinone anions from the different cofactors may have different affinities for Fe^{3+} and that the Cu^{2+}-mediated inactivation component not protectable by Fe^{3+} is due to a complex of Cu^{2+} with a semiquinone anion for which Fe^{3+} competes poorly or not at all.

The virologist who wants to use phenol to obtain preparations of high-quality poliovirion RNA may wish to pay especial attention to the kinds of metal ions in his working materials, particularly to the presence of copper, and to the purity of the phenol he uses. Whether similar attention should be paid to these factors in using phenol to prepare other kinds of nucleic acids is presently unknown.

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