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Thermal Inactivation of Oral Polio Vaccine: Contribution of RNA and Protein Inactivation

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Heating the Sabin strains of poliovirus at 42 to 45°C caused inactivation, loss of native antigen, and release of the viral RNA (vRNA). The loss of virion infectivity exceeded the loss of vRNA infectivity (as measured by transfection) by roughly 2 $\log_{10}$. Pirodavir inhibited the loss of native antigen and RNA release and reduced the loss of virion infectivity to the same level as the loss of vRNA infectivity. Thermoinactivation thus involves an RNA and a protein component, and pirodavir protected only against the latter.

A major drawback of the oral polio vaccine (OPV), as used in the World Health Organization's Expanded Programme on Immunization, is that it requires the utilization and maintenance of a well-functioning cold chain, because of its lack of thermal stability. Consequently, the quest for a more stable OPV is one of the priorities of the World Health Organization.

Currently, manufacturers are using either $MgCl_2$ or sucrose in a buffered medium as a stabilizer for OPV. Although these compounds do increase the vaccine's thermostability, the search for more efficient stabilizers and formulations remains worthwhile.

An approach which seemed to be promising was the use of capsid-binding compounds as stabilizers. Among these are flavans and chalcones (5, 15), as well as several pyridines (16), isoxazoles (20, 27), and pyridazine derivates (1, 3). For most of these compounds, it has been shown that they bind into the same hydrophobic pocket located at the base of the canyon (2, 8, 24). All these compounds in various degrees inhibit viral replication (antiviral activity) and prevent thermodenaturation (stabilization effect). Experiments showed that the two effects were independent, both resulting from the binding of the drug to the viral capsid (2, 3, 21).

Recently, a large screening program was set up to test 240 pyridazinamines for their ability to protect the antigenicity and infectivity of OPV against thermal denaturation. Seven compounds stabilized the antigenicity of all three vaccine (Sabin) strains and inactivated the viral particles in a way that was reversible by dilution. Out of these, R 77975 or pirodavir, was selected for further studies. It was shown that pirodavir stabilized the infectivity of the three vaccine strains and particularly that of the most thermolabile one, the Sabin type 3 strain. Unfortunately, the protection did not exceed that of the usual stabilizer, 1 M $MgCl_2$ (4).

An unexpected finding was that pirodavir stabilized the antigenicity of virus particles even after they had lost their infectivity. This finding was surprising in view of previous data which related the loss of infectivity to that of native (N) antigenicity (14).

The aim of this paper is to explain the mechanism of the loss of infectivity of the pirodavir-stabilized virions. As will be shown, the biological inactivation under those circumstances is at the RNA level.

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MATERIALS AND METHODS

Virus. Monovalent bulk virus preparations used for the production of OPV were kindly provided by Smith Kline Beecham Biologicals, Rixensart, Belgium, and used as seed to produce [^{3}H]uridine- or [^{35}S]methionine-labeled virus in two passages (21). The virus was purified by sucrose gradient ultracentrifugation. The 160S material was collected and kept at −80°C.

Pirodavir. Pirodavir, also called R 77975, i.e., ethyl-4-[3-[1-(6-methyl-3-pyridazinyl)-4-piperidinyl]ethoxy] benzoate (for the formula, see reference 4) was synthesized by the Janssen Research Foundation. Stock solutions (10 mg/ml) of pirodavir were made in dimethyl sulfoxide and diluted in phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 9.5 mM sodium phosphate, pH 7.3).

Hot phenol extraction. A phenol-chloroform-isoamylalcohol (25:24:1) mixture was prepared as described elsewhere (18). To extract the viral RNA (vRNA) from the virus, an equal volume of the hot mixture (56°C) was added to the virus suspension. The organic and the aqueous phases were mixed by vortexing for 5 min. Phase separation was achieved by centrifugation for 30 min at 130,000 × *g*. The aqueous phase was collected. The A_{260}/A_{280} ratio of the vRNA preparations was measured and found to be ca. 2.0, as expected of pure RNA. Whenever necessary, the RNA was concentrated by the ethanol precipitation method (18). The vRNA was stored at −80°C. To avoid external RNase activity, all solutions and containers were autoclaved, and vanadyl-ribonucleoside complexes (18) were added to the virus sample to a final concentration of 10 mM. The vanadyl-ribonucleoside complexes were also added in each further step. The yield of recovery of RNA from the virions was determined. The virus and RNA concentrations were measured spectrophotometrically, assuming $E^{1\%}_{260}$ = 81.6 for virus (9) and $E^{1\%}_{260}$ = 250 for RNA (18). The recovery of RNA from the virus was found to be in the range of 90 to 100%.

Sucrose gradient ultracentrifugation analysis of viral particles and vRNA. All sucrose density gradients were prepared in RSB buffer (10 mM Tris, 10 mM NaCl, 1.5 mM $MgCl_2$, HCl to pH 7.4). For the analysis of the virus, samples were layered onto a 15 to 30% sucrose gradient and centrifuged for 2 h 15 min at 160,000 × *g* and 4°C in a Centrikon TST 41.14 rotor.

TABLE 1. Pirodavir stabilization of infectivity and antigenicity[a]

Heating[b]		Amt (% of input) of residual[c]:			
		Infectivity		N antigen	
Temp (°C)	Time (days)	Without pirodavir	With pirodavir at 10 μg/ml	Without pirodavir	With pirodavir at 10 μg/ml
42	1	0.2	38	<5	94
	3	0.004	0.7	<5	90
	7	0.003	0.3	<5	69
45	1	0.006	30	<5	48
	3	<0.001	0.2	<5	35
	7	<0.001	<0.001	<5	<5

[a] ^{35}S-labeled, purified Sabin-3 virions (see Materials and Methods).
[b] Controls were held at 4°C; they showed no significant loss of infectivity or antigenicity after 7 days.
[c] See Materials and Methods for assays; results are shown for monoclonal antibody 132. Results with monoclonal antibody 875 are identical within 1 standard deviation (±5%).

After centrifugation, 400-μl fractions were collected for analysis of radioactivity. To analyze the vRNA, centrifugation was for 18 h at 4°C and 64,000 × *g* in an MSA SW 30.6 rotor.

Plaque assay. HEK cell monolayers (23) were grown in 55-mm-diameter petri dishes. For determination of the infectivity of the virus, a serial dilution of the virus suspension was prepared in PBS buffer. Of each dilution, 150 μl was added to the petri dishes. After 1 h at 4°C, the cells were washed three times with 5 ml of buffer to remove unadsorbed virus. Five milliliters of the first overlay, which consisted of modified Eagle's medium, 1.1% agar, and 10% calf serum, was added. After the petri dishes were incubated for 2 days at 37°C, 5 ml of a second overlay (with 0.006% neutral red) was added. The plaques were counted the next day.

In transfection experiments, using the vRNA, exactly the same conditions applied, except that the vRNA was serially diluted in PBS buffer containing 100 μg of DEAE-dextran per ml (25).

Monoclonal antibodies. The following monoclonal antibodies were used. (i) Antibodies 36-5h2 (site 2), 424 (site 3A), and 35-2b6 (site 3B) are serotype 1 and neutralizing (21). (ii) Antibody 39-5b4 is serotype 1, H specific, and nonneutralizing (7). (iii) Antibodies 434 (site 1) and 1037 (site 2) are serotype 2 and neutralizing (19). (iv) Antibodies 132 (site 1) and 875 (site 2) are serotype 3 and neutralizing (19). Protein A-aided immunoprecipitation was as described elsewhere (26).

RESULTS

Pirodavir stabilization of infectivity and antigenicity of Sabin-3 virus. The Sabin-3 strain was chosen for these experiments because it was the most heat sensitive of the Sabin strains (4).

^{35}S-labeled virus was heated at 42 or 45°C (controls being kept at 4°C), either in the presence of 10 μg of pirodavir per ml or in plain PBS. Samples collected after 1, 3, and 7 days were submitted to plaque titration and assayed for N antigen by immunoprecipitation. The results are shown in Table 1. Even after the shortest time (1 day at 42°C), the unprotected virus had lost 99.8% of its infectivity and all detectable N antigen. Pirodavir slowed down the loss of both infectivity and N antigen; however, the N antigenicity was better protected than the infectivity. For instance, after 3 days at 42°C in the presence of pirodavir, more than 99% of the infectivity was lost, but only 10% of the N antigen. The reason for this discrepancy was further investigated.

A similar discrepancy between the infectivity and antigenicity was observed with Sabin-1 and Sabin-2 viruses (results not shown). With those strains, the discrepancy was most marked after 1 day at 45°C.

Composition and physical characteristics of virus thermoinactivated under pirodavir protection. Unprotected heating of [^{35}S]methionine-labeled Sabin-3 virus for 3 days at 42°C yielded 80S particles (Fig. 1A). These were devoid of RNA, as shown with [^{3}H]uridine-labeled virus (Fig. 1B). When the virus was heated in the presence of pirodavir, however, it retained its original sedimentation coefficient of 160S and released little RNA. Thus, the infectivity loss of pirodavir-protected virus at 42°C was not due to a massive loss of RNA. Virions heated in the presence of pirodavir also eluted in the normal time from a high-performance size exclusion column (13, 22), suggesting that they were probably not swollen (results not shown). As shown by Boublik and Drzeniek (6), a stepwise degradation of virions may be initiated by physical tension in the capsid, resulting in its swelling (expansion). It is assumed that such

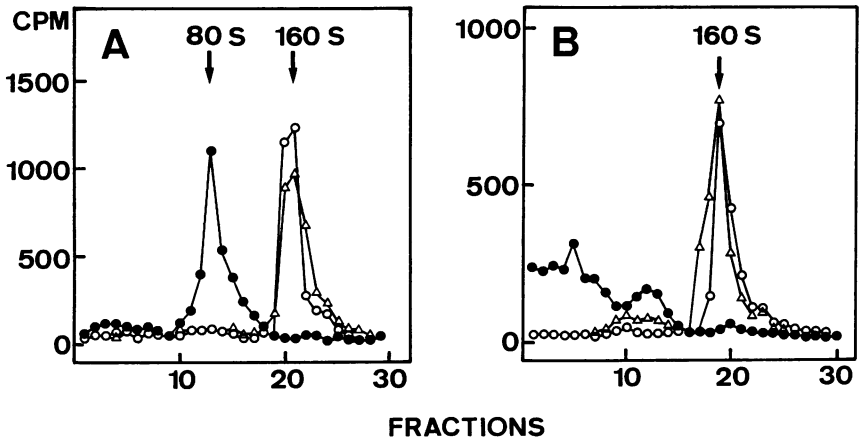

FIG. 1. Retention of RNA after thermal inactivation in the presence of pirodavir. Sabin-3 virions labeled with [^{35}S]methionine (A) or [^{3}H]uridine (B) were kept at 4°C (○) or incubated at 42°C for 3 days in the presence (△) or absence (●) of 10 μg of pirodavir per ml. After incubation, the samples were ultracentrifuged as described in Materials and Methods.

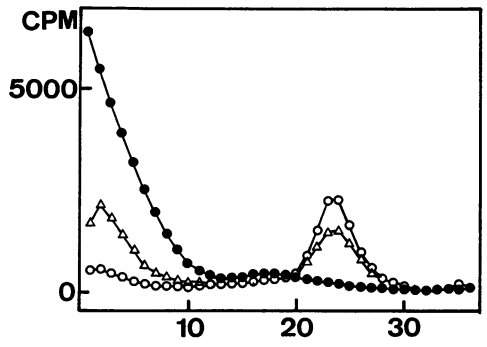

FIG. 2. Effects of incubating virions at 42°C on the sedimentation coefficient of viral RNA. Sabin-3 virions were labeled with [^{3}H]uridine. One portion of the virions was kept at 4°C (○); others were incubated at 42°C for 3 days in the presence (△) or absence (●) of 10 μg of pirodavir per ml. After incubation, the samples were cooled on ice and viral RNA was extracted by the hot phenol method (see Materials and Methods). With regard to ultracentrifugation, see Materials and Methods.

swelling would have caused a shortening of the virions' elution time, but this was not so.

The vRNA extracted from Sabin-3 virus was also examined by sucrose gradient centrifugation (Fig. 2). Roughly one-half of the vRNA from pirodavir-protected virus cosedimented with vRNA from control virus, showing that it had not undergone gross degradation. In conclusion, no observable alterations of the physicochemical characteristics of the virions or vRNA could account for the 2.1-$\log_{10}$ loss of infectivity after heating for 3 days at 42°C, in the presence of pirodavir.

Transfection with vRNA. The Sabin strains were incubated for 1 day at 45°C (types 1 and 2) or for 3 days at 42°C (type 3), controls being kept at 4°C for the duration. As in the foregoing experiments, heating was conducted in the presence and absence of 10 μg of pirodavir per ml. A sample of each virus suspension was titrated by the usual plaque assay, and another was used for the extraction of vRNA, which was titrated by transfection (see Materials and Methods). In agreement with data in the literature (for a review, see reference 17), the titer of the vRNA extracted from the 4°C controls of all serotypes was roughly 3 $\log_{10}$ lower than that of an equivalent amount of intact virus (Table 2). Unprotected heating lowered virion infectivity by ca. 4.2 $\log_{10}$, as against a 2.4- to 3.1-$\log_{10}$ reduction of the infectivity of vRNA. When the heating had been carried out in the presence of pirodavir, the infectivity loss of the virions was 2.5, 2.3, and 2.2 $\log_{10}$ for Sabin types 1, 2, and 3, respectively. When the loss was measured by transfection of vRNA, almost exactly the same figures (2.6, 2.3, and 2.1 $\log_{10}$) were found. Thus, the part of the thermal inactivation that could not be prevented by the capsid-stabilizing compound pirodavir was entirely due to reduction of the biological activity of the vRNA. The remaining 1.5-$\log_{10}$ drop of infectivity was probably due to alteration of the virions' protein moiety and was preventable with pirodavir.

DISCUSSION

A moderate heating (42 to 45°C) of the Sabin strains of poliovirus in the presence of the capsid-binding compound pirodavir yields paradoxical results: the virions and their RNA remain physicochemically and antigenically intact, but the virions' infectivity is reduced by 2 $\log_{10}$. Looking for an explanation of this phenomenon, we used transfection to determine the infectivity of the vRNA extracted from the heated virions. It turned out that the virions heated in the presence of pirodavir and the vRNA extracted from them suffered almost exactly the same degrees of inactivation (Table 2). Thus, the part of the inactivation that could not be prevented by pirodavir was due to RNA inactivation.

There was little difference (0.2 to 0.5 $\log_{10}$) in the infectivity of the vRNA, whether the heating had taken place in the presence or absence of pirodavir. This was surprising, since heating of unprotected virus caused the RNA to be expelled from the virions (Fig. 1B) and extensively degraded (Fig. 2), whereas in the presence of pirodavir the RNA remained associated with the virions and roughly one-half of the molecules kept their sedimentation coefficient of 35S (Fig. 2). Presumably the vRNA undergoes cleavages which fail to lower the RNA's sedimentation coefficient, as secondary structure holds the molecule together. These cleavages might be caused by a virion-associated ribonuclease. Such an enzyme exists in purified foot-and-mouth disease virus, in which it was shown to degrade the genomic RNA in situ upon prolonged incubation at relatively high temperatures (10, 11). The biological activity of the vRNA might also be affected by other changes in the RNA, such as the removal of the protein VPg from the vRNA or alterations in the secondary structure of the RNA.

The 4-$\log_{10}$ loss of infectivity suffered by unprotected virions is reduced to 2 $\log_{10}$ by pirodavir. As we just noted, this protection cannot be accounted for at the vRNA level and must be sought at the level of the capsid. This interpretation is fully compatible with the observations (retention of the 160S sedimentation value and of the native epitopes); it is also in agreement with data in the literature, as pirodavir probably stabilizes the capsid by entering the pocket in the floor of the canyon (8, 24). In this, pirodavir resembles $MgCl_2$, which also prevents protein, rather than RNA, damage, presumably by bonding adjacent protein components in the capsid (12).

Our conclusion that the loss of infectivity suffered by the Sabin strains after moderate heating (42 to 45°C) has both an RNA component and a protein component is in keeping with views expressed by Dimmock (12), who postulated the existence of these two mechanisms. Inactivation at the protein level was said to become dominant from 47°C up.

TABLE 2. Pirodavir stabilization of virion and vRNA infectivities

Virus strain	Heating		Concn of pirodavir (μg/ml)	Infectivity ($\log_{10}$ PFU/ml)[a] of:			
				Virions		vRNA	
	Temp (°C)	Time (days)		Titer	Loss due to heating[b]	Titer	Loss due to heating[b]
Sabin-1	4	1	0	7.6		4.8	
	45	1	0	3.4	4.2	1.7	3.1
	45	1	10	5.1	2.5	2.2	2.6
Sabin-2	4	1	0	7.7		4.8	
	45	1	0	3.4	4.3	2.3	2.5
	45	1	10	5.4	2.3	2.5	2.3
Sabin-3	4	3	0	6.6		3.9	
	42	3	0	2.5	4.1	1.5	2.4
	42	3	10	4.4	2.2	1.8	2.1

[a] See Materials and Methods for infectivity assay; the observed infectious titer was recalculated to the original volume of the virus.
[b] Compared with 4°C control.

This similarity of the protective mechanism and the inability to prevent RNA damage explain why the actual protection by capsid-binding compounds was not better than that effected by $MgCl_2$ (4). The findings also suggest that the search for more efficient stabilizers should focus on the stabilization of the vRNA.

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