Dissociation and Reassociation of Poliovirus
I. Effect of Urea on the Virion

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Poliovirus is dissociated by urea in the presence of mercaptoethanol or dithiothreitol into RNA and protein(s). Differences exist in the sensitivity of poliovirus type I, II or III toward these agents. The decrease in infectivity titer of the virion is in the range of 10^9. After appropriate dissociation no infectious virions are found in urea-treated poliovirus concentrates. The released viral RNA is infectious. Its infectivity (1×10^6 PFU/μg RNA) is comparable to that of phenol extracted RNA. Therefore, urea dissociation is a fast and reliable method for the preparation of infectious poliovirus RNA even at the microliter scale.

The dissociation of poliovirus is dependent upon the urea concentration, temperature and pH. The decrease in virion infectivity is time dependent. It is preceded by a lag phase of different lengths for poliovirus type I, II or III. During the lag phase the virion undergoes configurational changes without losing infectivity. From the observed hyperchromic effect it is obvious that the secondary structure of the RNA inside the virion breaks down, giving rise to expanded forms of poliovirus particles. After the lag phase the RNA is released and the empty capsid dissociated into the appropriate polypetides.

Introduction

We have reconstituted infectious poliovirus particles from a mixture of RNA and protein, which was obtained by dissociating poliovirus with urea in the presence of mercaptoethanol or dithiothreitol [1, 2]. This paper deals with the dissociation process, which turned out to proceed stepwise.

Up to now different procedures have been worked out to dissect poliovirus into its components (for review, see [3]). The virus was dissociated completely by 1% sodium dodecylsulfate (SDS) and 0.5 M urea and was separated into four polypeptides by electrophoresis in polyacrylamide gels [4, 5]. This method became very useful for the polypeptide analysis not only of poliovirus but of most other types of viruses [6]. However, the polypeptides obtained were denatured and contained SDS.

For the reconstitution of poliovirus conditions had to be employed under which the virus would be completely dissociated into RNA and polypeptides without destruction of the biological activity of these components. The test procedure for an appropriate method was the ability of the dissociated sample to be subsequently reconstituted to infectious virus. Treatment of type I poliovirus with 9 M urea and 0.09 M mercaptoethanol or dithiothreitol at 25 °C for 60 min fulfilled this criteria [1, 2].

Degradation of poliovirus by urea was achieved earlier by Cooper [7] and later by Vanden Berghe and Boeye [8]. Both used 7.2 M urea at pH 7.2 for the disruption of poliovirus but at 37 °C in order to obtain information on the structure of the virion. Also Philipson *et al.* [9] degraded coxsackievirus B3 and poliovirus at 37 °C with 3 M urea at pH 9.0. From our experience ([1], Drzeniek and Bilello, unpublished results) dissociation of poliovirus by urea at 37 °C gives rise to denatured polypeptides which were not suitable for reassociation.

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Materials and Methods

Cells. HeLa S_3 cells were propagated in minimum essential medium, Joklik-modified (Grand Island Biological Co., Grand Island, N. Y., USA) (GIBCO) supplemented with 2 mM glutamine and either 5% fetal calf serum (GIBCO) or 5% calf serum (Flow Laboratories GmbH, Bonn, Germany).

Virus. The following poliovirus strains were used: Type I, strain Mahoney, type II, strain Lansing, type III, strain Saukett. Virus was propagated in HeLa

S_3 cells and purified as recently described [10]. Poliovirus RNA, *i. e.* single-stranded RNA, was obtained by the SDS-phenol procedure from poliovirus particles [11].

Dissociation procedure

a. *Infectivity.* 20 μl of the virus preparation (containing 1×10^{10} to 2×10^{11} PFU/ml) were incubated with 200 μl of 10 M urea and 0.1 M mercaptoethanol at 25 °C in stoppered glass tubes for the indicated length of time. To ensure complete mixing, the tubes were briefly centrifuged. All the virus accidentally spread on the side walls of the tube was dipped into the urea solution. After an appropriate length of time, 20 μl samples were withdrawn from the test tube and diluted immediately, usually in 2 ml of ice-cold PBS. The sample was further diluted into cold minimal essential medium (MEM) without serum and the infectivity was determined in the "virion assay" [12] or in the "RNA assay" [13, 14]. The infectivity of the virion was measured by an agar-cell-suspension plaque method using HeLa cells. Infectious RNA was assayed similarly except that cells were treated with DEAE-dextran and dimethyl sulfoxide before adding RNA.

b. *Optical density.* During the dissociation procedure the optical density was measured at 260 nm and at 280 nm in the Zeiss PMQ II spectrophotometer in 1 cm quartz cuvettes. Dissociation was done at room temperature (21 to 23 °C). Mercaptoethanol was omitted. For simultaneous determination of changes in optical density and infectivity, samples were withdrawn from the cuvettes, diluted 100 times into ice-cold PBS at appropriate time intervals, and assayed for virion and RNA infectivity.

c. *RNase treatment.* Treatment of the samples with ribonuclease I was performed in two ways (indicated in the Tables). Either equal volumes of the dissociated sample and RNase (0.05 mg/ml) were mixed after the dissociation had taken place and then incubated for 10 min at 25 °C, or the RNase was incorporated into the mixture of virus and urea. In the latter case, 20 μl of virus were mixed with 20 μl of RNase (0.5 to 1.0 mg/ml) and 200 μl of urea (with or without mercaptoethanol). The sample was incubated for the appropriate time, diluted 100 times with ice-cold PBS and further processed as above.

d. *Solutions.* 10 M urea (ultra pure from Schwarz/Mann, Orangeburg, N. Y., USA) or 10 M urea with 0.1 M β-mercaptoethanol (purissimum, Serva, Heidelberg, Germany) in sterile distilled water was used. The urea solutions were kept frozen at −20 °C until used. Their pH, measured after (1:10) dilution, was found usually in the range of 7 to 8.

Ribonuclease I (RNase A, EC 3.1.4.22, Serva, Heidelberg, Germany) was dissolved in water. The stock solution (1 mg/ml) was kept frozen. Isotonic phosphate buffered saline (PBS), pH 7.2, contained 0.02 M phosphate and 0.12 M NaCl. All other buffers contained 0.02 M of the buffering substance and were made isotonic with NaCl.

Results

1. *Stepwise dissociation of poliovirus by urea-mercaptoethanol*

a. *Lag phase in the dissociation process.* The first indication for a stepwise mechanism of poliovirus dissociation was obtained when examining the time dependence of the virion's infectivity during treatment with urea. In order to distinguish between infectious virus particles (virions) and infectious virus RNA, two different plaque assays were performed, namely, the "virion assay" and the "RNA assay" (see Materials and Methods). These two assays can discriminate between infectious poliovirus particles and infectious RNA [12, 15]. In the virion assay practically only infectious virus particles are measured since the infectivity of the RNA is very low (see Table V). In the RNA asasy, however, infectious virus particles have the same titer as in the virion assay. The infectivity of the RNA in the RNA assay is only 0.1 to 1% of that of infectious virus particles (Table V). Therefore, infectious RNA can only be determined in the RNA assay, if the concentration of infectious virus particles is very low or zero. In addition, infectious RNA can be distinguished from infectious virus particles by its sensitivity towards RNase (Table IV).

During the first 10 to 40 min of incubation of poliovirus with 9 M urea and 0.09 M mercaptoethanol at 25 °C the infectivity in the virion assay and in the RNA assay remains unaltered (Fig. 1 and Fig. 2). This "lag phase" in the infectivity of the virus particle is followed by a steep drop of infectivity, when the incubation is continued. The infectivity in the virion assay drops below detectability, whereas the infectivity in the RNA assay levels at values between 0.005% to 0.5% of the titer at the beginning of the urea treatment.

The infectivity in the RNA assay at the plateau represents infectious RNA, since infectious virus

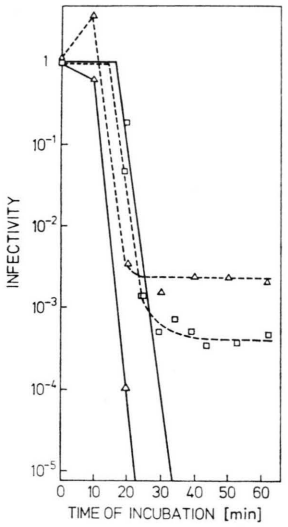

Fig. 1. Dissociation of poliovirus type I by urea. Poliovirus type I, strain Mahoney (□), or type II, strain Lansing (△) was incubated at 25 °C with 9 M urea and 0.09 M mercaptoethanol for the indicated length of time, diluted 100 times with cold isotonic phosphate buffer, pH 7.2. The infectivity was determined in the virion assay (———) and RNA assay (— — —).

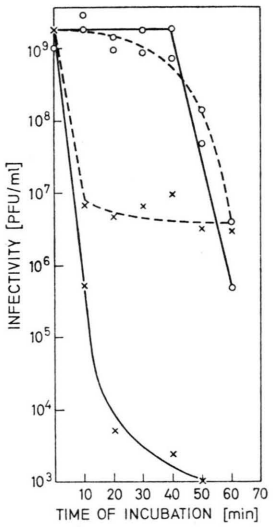

Fig. 2. Effect of temperature on poliovirus type III dissociation by urea. Poliovirus type III, strain Saukett, was incubated with 9 M urea and 0.09 M mercaptoethanol for the indicated length of time either at 25 °C (○) or at 37 °C (×). The infectivity was determined in the virion assay (———) and in the RNA assay (— — —).

particles are practically absent after 60 min of incubation (Figs 1 and 2). Under these conditions (see above) infectious RNA is measured in the RNA assay. Furthermore, the RNA obtained by incubation of poliovirus type 1 with urea for 60 min at 25 °C is indistinguishable from phenol extracted RNA by its specific infectivity, sedimentation behavior and RNase sensitivity (see section 2 b).

b. *Sensitivity of different strains.* Differences in the inactivation kinetics were found when representatives of poliovirus type II or III were used. Poliovirus type II, strain Lansing, was dissociated by urea-mercaptoethanol faster than type I virus (Fig. 1). Poliovirus type III, strain Saukett, however, was more resistant towards urea dissociation than type I virus (Fig. 2). Up to 40 min no decrease in infectivity of the virion was detected, but then the infectivity decreased in the same way as described for poliovirus type I. In contrast to type I virus, the dissociation of type III virus was not complete after 60 min at 25 °C. A rise in temperature from 25 to 37 °C resulted in complete dissociation of the virus and in the elimination of the long lag phase. A clear plateau in the RNA assay in the absence of infectivity in the virion assay indicates a complete release of infectious RNA from the virion.

It is not suggested that the differences observed in the dissociation of type I, II and III virus strains are characteristic for these serologically distinct poliovirus groups, since antigenicity and stability towards urea may be two independent features of a poliovirus particle.

c. *Effect of urea concentration.* In order to reassociate poliovirus by the stepwise dilution procedure [1, 2], it was necessary to know the stability of the virus at different urea concentrations. The results presented in Table I show that up to 2 M urea the infectivity of poliovirus type I is not significantly affected at neutral pH at 25 °C for 60 min. Higher concentrations of urea (7.2 to 9 M) destroyed completely the virion's infectivity with the concomitant release of infectious RNA. In 3.6 M urea, however, a residual infectivity remained. Its RNase resistance may be due to the presence of undissociated poliovirus particles or caused by RNase resistant substructures of the virion.

d. *Effect of pH.* In dissociation experiments performed with the aim of a subsequent reassociation of the virus, the urea employed was always dissolved in water instead of buffer. The virus used for the reassociation was dialyzed prior to the dissociation against 0.9% NaCl. Experiments in which urea was dissolved in isotonic phosphate buffer, pH 7.2, did not result in a reassociation of the virus into infec-

Table I. Effect of urea concentration on the inactivation of poliovirus particles. Poliovirus was incubated for 60 min at 25 °C with the indicated concentration of urea dissolved in water containing appropriately 0.009 to 0.09 M mercaptoethanol. RNase I was added to a portion of the incubation mixture. The pH of the samples was between 7 and 8. The incubated samples were diluted 50 times with cold PBS and the infectivity was determined in the virion and RNA assay.

Sample	Urea concentration [mol/l]	Infectivity (PFU/ml) Virion assay without RNase	Virion assay with RNase	RNA assay without RNase	RNA assay with RNase
Poliovirus type I	9	ϕ	ϕ	8×10^6	ϕ
	7.2	ϕ	ϕ	n.d.	n.d.
	3.6	1×10^5	9×10^3	7×10^5	5×10^3
	1.8	8×10^9	8×10^9	n.d.	n.d.
	0.9	1×10^{10}	1×10^{10}	n.d.	n.d.
	0	1×10^{10}	1×10^{10}	1×10^{10}	1×10^{10}

ϕ = no plaques detectable: infectivity $< 5 \times 10^2$ PFU/ml (1 : 50 dilution); n.d. = not done.

tious particles. We do not know the reason for this behavior, but in earlier work on the reconstitution of tobacco mosaic virus the necessity of using appropriate buffers was well documented [16]. On the other hand, it was important to know the effect of pH on the dissociation of poliovirus. We therefore examined the inactivation of poliovirus by urea at pH values between 5 and 9.

Using the standard dissociation procedure, *i. e.*, 9 M urea and 0.09 M mercapoethanol at 25 °C for 60 min, no measurable differences were found in the inactivation of poliovirus type I particles in the pH range of 5 to 8. Faster inactivation, however, was observed at pH 9 (not shown). In 3.6 M urea (Table II) poliovirus infectivity dropped about 100 to 1000 times during 60 min of incubation, in the pH range of 5.3 to 8.2, but as much as 10^7 at pH 9.0. In 1.8 M urea poliovirus is slightly inactivated at pH 9.0 but is resistant towards the dissociating agent(s) in the pH range of 5.3 to 8.2.

e. *Changes in optical density.* The hypothesis of a stepwise dissociation of poliovirus by urea was strengthened by measurements of the optical density of virus-urea mixtures. Earlier, Bachrach [17, 18] used optical measurements to detect structural changes in foot and mouth disease virus at elevated temperatures or after inactivation with formaldehyde.

The plateau of infectivity at the beginning of treatment suggests that the virion was not altered by urea during the lag phase. But is was very unlikely that a virus particle could withstand the attack of 9 M urea for 10 to 40 min (depending on

Table II. The effect of pH on the inactivation of poliovirus by urea. 20 μl of virus were incubated with 200 μl of 4 M urea with 0.04 M mercaptoethanol or with 2 M urea with 0.02 M mercaptoethanol in the appropriate buffer at 25 °C for 60 min. The pH value of the incubation mixture was measured with a glass-electrode. The incubated sample was diluted 100 times with cold PBS which shifted the pH to neutral conditions and was then tested for infectious particles in the virion and RNA assay.

Sample	Urea concentration [mol/l]	pH	Buffer	Infectivity [PFU/ml] Virion assay	RNA assay
Poliovirus type I	3.6	8.9	Glycine	1×10^3	2×10^3
		8.2	Tris	1×10^7	1×10^7
		7.5	Tris	3×10^7	1×10^7
		6.4	Phosphate	3×10^6	5×10^6
		5.3	Acetate	2×10^8	1×10^8
	1.8	8.9	Glycine	5×10^8	5×10^8
		8.1	Tris	1×10^{10}	n.d.
		7.3	Tris	1×10^{10}	n.d.
		6.2	Phosphate	1×10^{10}	n.d.
		5.1	Acetate	1×10^{10}	n.d.
Virus PBS (control)		7.2	Phosphate	1×10^{10}	1×10^{10}

the virus strain) without any change and then suddenly lose all of its infectivity. Of course, such an effect could be called a cooperative one, which would explain the situation but not describe the same in molecular events. In fact, measurements of the optical density of virus-urea mixtures demonstrated changes of the virion during the lag phase prior to measurable changes in the infectivity of the virion. Highly purified virus preparations were treated with 9 M urea in the same way as previously described

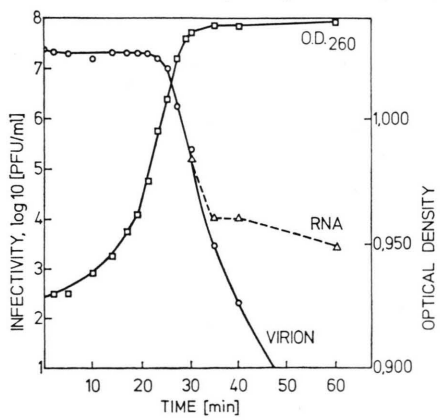

Fig. 3. Changes of optical density and infectivity of poliovirus treated with urea. Poliovirus type I was incubated at room temperature (21 to 23 °C) with 9 M urea in a cuvette of the spectrophotometer. The optical density (□—□) was measured at 260 nm ($O.D._{260}$); infectivity was determined in the virion assay (○—○) and RNA assay (△— — —△) at the time indicated on the abscissa.

except that dissociation took place in a cuvette in a spectrophotometer and in the absence of mercaptoethanol. In the time course of treatment the optical density at 260 nm increased as outlined in Fig. 3. Most of the increase occurred during the lag phase in which the infectivity of the preparation remained unaltered. At the end of this phase the infectivity of the virion dropped rapidly more than 10^6 times. Infectious RNA was detected at that very moment at which the optical density reached its highest value.

Mercaptoethanol was omitted from the incubation mixture since its slow oxidation gave rise to an increase in optical density which could be mistaken for the optical density increase of the virion itself solely due to the action of urea. It was noted previously ([2] and unpublished results) that mercaptoethanol had no decisive effect on the dissociation, being important for the reconstitution process.

Incorporation of RNase into the virus-urea mixture gave a higher increase in optical density than treatment in the absence of this enzyme (Fig. 4). The onset of the additional increase of optical density clearly began after the first phase of optical density increase in the absence of RNase.

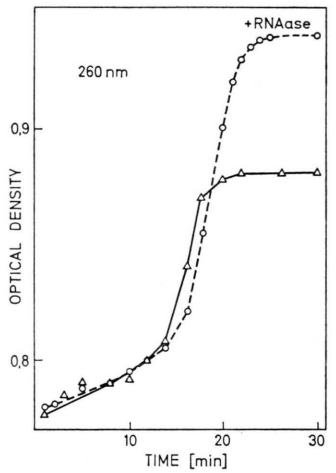

Fig. 4. Effect of RNase on the optical density of poliovirus treated with urea. Poliovirus was incubated with urea as described in Fig. 3 and the optical density (△—△) determined at different lengths of time at 260 nm. In addition, a parallel sample was run in the presence of 50 μg/ml of RNase (○- - -○).

The maximum of optical density was at the wave length of 260 nm for native poliovirus and after dissociation of the virion with urea. The ratio of optical density at 260 nm to that at 280 nm remained constant during urea treatment. From this and from the observed increase in optical density of urea-treated poliovirus it is concluded that the changes in optical density were due to the hyperchromic effect of poliovirus RNA. This hyperchromicity demonstrates that poliovirus RNA has a secondary structure inside of the virus particle which is lost when the virion is treated with urea under the employed experimental conditions. Following release of the RNA from the virus particle, *i. e.*, at the end of the lag phase, the RNA becomes accessible to the action of the added RNase, giving rise to the additional hyperchromic effect of a single-stranded RNA depolymerized to its subunits.

2. *Absence of infectious virions in urea-dissociated samples*

A complete absence of infectious poliovirus particles after their treatment with urea-mercaptoethanol is crucial for the demonstration of virus reconstitution. In the foregoing publication [2] the absence of infectious virus particles was demonstrated only down to a level of 1×10^3 PFU/ml of the dissociated sample. This limitation was brought about by the (1:100) dilution of the dissociated sample. A high dilution was chosen to avoid any interfering effect of urea or mercaptoethanol in the virion assay and to prevent a spontaneous reconstitution. In order to determine lower infectivity titers, it was first necessary to examine the effect of urea and mercaptoethanol on the virion assay.

a. *Effect of urea on the plaque assay.* Poliovirus type I was diluted in decreasing concentrations of urea and mercaptoethanol. The diluted virus was applied directly to HeLa cells and its plaque-forming capacity was measured in the virion assay. A slight decrease in the number of plaques was ob-

Table III. Effect of urea on the plaque assay of poliovirus. Poliovirus in isotonic phosphate buffered saline (PBS) was diluted with urea, mercaptoethanol, urea and mercaptoethanol or PBS. 100 μl of each sample containing 150 PFU of virus were assayed for their ability to produce plaques in the virion assay.

Final concentration of		PFU/plate of sample diluted with			
Urea [mol/l]	Mercaptoethanol [mol/l]	Urea	Mercaptoethanol (ME)	Urea +ME	Buffer (PBS)
1	10^{-2}	102	109	102	
10^{-1}	10^{-3}	141	132	172	
10^{-2}	10^{-4}	162	110	146	
10^{-3}	10^{-5}	163	147	161	
10^{-4}	10^{-6}	149	127	—	
0	0	—	—	—	145

served in 1 M urea and 0.01 M mercaptoethanol (Table III). Lower concentrations of urea and mercaptoethanol had no effect on the virion assay. Therefore, the infectivity of urea-treated virus was never measured at higher concentrations of urea than 1 M of final concentration in the plaque assay.

b. *Residual infectivity in the virion assay.* When poliovirus containing 2×10^{10} PFU/ml was dissociated with 9 M urea and 0.09 M mercaptoethanol and then diluted 10 times before the test in the virion assay, 1 to 5 plaques per plate were seen. Since each plate was infected with 0.1 ml, this corresponds to an infectivity of 10 to 50 PFU/ml of the dissociated sample. The titer, calculated to the original virus, was then 1×10^2 to 5×10^2 PFU/ml because of the 10 times dilution of the input virus by the urea-mercaptoethanol mixture (Table IV). In a number of experiments no plaques were observed. Here the titer was less than 1×10^2 PFU/ml of input virus.

Table IV. Sensitivity of infectious material obtained by the urea dissociation procedure towards RNase. Poliovirus type I was kept at 25 °C in 9 M urea and 0.09 M mercaptoethanol for the indicated time. Thereafter, half of each sample was treated with ribonuclease I for 10 min at 25 °C. All samples were then chilled and the infectivity was determined in the virion and RNA assay. Untreated virus was kept in phosphate buffered saline (PBS) instead of urea-mercaptoethanol.

Sample	Time of incubation [min]	RNase [μg/ml]	Infectivity [PFU/ml]	
			Virion assay	RNA assay
Virus in PBS	60–180	0	2×10^{10}	2×10^{10}
	60	25.0	2×10^{10}	1×10^{10}
Virus in urea and mercaptoethanol	60	0	1×10^2	2×10^6
	60	12.5	ϕ	ϕ
	120	0	3×10^2	1×10^7
	120	12.5	ϕ	ϕ
	180	0	5×10^2	3×10^7
	180	12.5	ϕ	ϕ

ϕ = no plaques detectable.

The detection of the very low amount of plaques of urea dissociated poliovirus in the virion assay raised the question as to the nature of this infectivity. Since up to now either virus particles or isolated poliovirus RNA have been the only infectious entities known, it had to be determined which of them was the reason for this low infectivity in the virion assay. Poliovirus type I was therefore dissociated at 25 °C with urea and mercaptoethanol for 60, 120 and 180 min. The infectivity of the dissociated sample was determined in the virion and in the RNA assay. Half of the sample was incubated with ribonuclease after the dissociation step. The results presented in Table IV demonstrate that the infectivity in the virion assay was destroyed by ribonuclease. Also the infectivity in the RNA assay was completely abolished by ribonuclease action.

Untreated poliovirus did not show any decrease in the infectivity titer after ribonuclease treatment both in the virion and in the RNA assay.

These experiments demonstrate the destruction by RNase of the infectious entity found in the virion assay obtained by dissociating poliovirus properly by urea-mercaptoethanol. They prove the absence of any detectable infectious virions after the dissociation procedure. The residual infectivity observed is due to the fact that poliovirus RNA is also infectious in the virion assay [19] as summarized in Table V.

Table V. Comparison of the infectivity of poliovirus virion and RNA.

Preparation	Amount of substance		Infectious particles [PFU]		Physical particles / Infectious particles	
	Mass	Physical particles	Virion assay	RNA assay	Virion assay	RNA assay
Poliovirus particles (virions)	3 μg virion [a]	2×10^{11}	2×10^9	2×10^9	10^2	10^2
Poliovirus RNA	1 μg RNA [b]	2×10^{11}	–	1×10^6	–	10^5
Urea dissociated virion	2 μg protein + 1 μg RNA	2×10^{11} [c]	10–50	2×10^6	10^{10} [c]	10^5 [c]
Poliovirus RNA [d] (ref. [22, 11])	1 μg RNA	2×10^{11}	10–20	2×10^6	10^{10}	10^5

[a] Calculated from the O.D.$_{260}$ using $E^{1\%}_{260,\,1\,cm} = 81.6$ [20] and mol wt $= 6.8 \times 10^6$ Dalton [21].
[b] Calculated on the basis $E^{1\%}_{260,\,1\,cm} = 240$ [3].
[c] RNA molecules.
[d] Data of Breindl and Koch (1972), Ref. [22], and Koch (1973), Ref. [11].

3. *Characterization of the RNA obtained by the urea procedure*

Sufficient concentration of infectious RNA in the dissociated sample is a necessary prerequisite for the reconstitution process. In the course of many reconstitution experiments it was observed that one of the reasons for unsuccessfulness was the presence of ribonuclease in virus preparations. The action of ribonuclease was visualized by a decrease of the titer in the RNA assay after the dissociation had taken place (for example as in Fig. 3). In RNase-free samples the infectivity in the RNA assay remained unchanged for hours after the dissociation as demonstrated in Table IV.

The sedimentation velocity in sucrose gradients of poliovirus RNA obtained by urea dissociation of the virus was indistinguishable from RNA derived by the SDS phenol procedure. Both RNA preparations sedimented at about 35S [2].

Furthermore, the specific infectivity of RNA obtained by dissociating poliovirus particles with urea is the same as for phenol extracted RNA, both in the virion and in the RNA assay (Table V). Our data are in excellent agreement with results published for single-stranded RNA obtained by phenol-extraction of poliovirus particles [22, 11].

Data compiled in Table V demonstrate the infectivity of poliovirus RNA in the virion assay as mentioned in the foregoing section. From the amount of physical particles obtained from optical density data, the ratio of physical particles (PP) to infectious particles (IP) was also calculated. Roughly, one plaque was formed in the virion assay by 100 physical virus particles but by 10^{10} RNA molecules. In the RNA assay, however, only 10^5 RNA molecules were necessary to give one plaque.

From physical and biological data it is thus evident that RNA released from the virion by the urea procedure is indistinguishable from RNA obtained by the SDS phenol procedure.

Discussion

Reconstitution of infectious poliovirus particles from its components demonstrates that the process of poliovirus dissociation is in principle a reversible phenomenon. This holds true for the dissociation of poliovirus by urea at appropriate conditions estimated in the previous papers [1, 2]. The dissociation process is therefore of primary importance for all reconstitution experiments. Since high concentrations of poliovirus particles were used for the reconstitution experiments, the dissociation procedure had to be very efficient to destroy all infectious particles. We started with 1×10^{11} PFU/ml of original virus or with 1×10^{10} PFU/ml of virus in the "incubation mixture" containing the 9 M urea and 0.1 M mercaptoethanol. When the incubation mixture was tested for residual infectivity after 60 min at 25 °C, 50 to 250 PFU/ml were detected in the virion assay and about 1×10^7 PFU/ml in the RNA assay (Table V). The fact that the infectivity in the RNA assay is much higher indicates that almost all infectious entities obtained after dissociation of poliovirus particles by urea were RNA molecules. The infectivity in the virion assay was not due to infectious virus particles but caused by poliovirus RNA, as demonstrated by its sensitivity towards RNase, by co-sedimentation [2] and by the same specific infectivity as of phenol extracted RNA. It is well established [11] that poliovirus RNA can be detected in the virion assay when employed at high concentrations. Thus, treatment of poliovirus particles by 9 M urea results in the complete dissociation of all poliovirus particles into RNA and protein.

We have reported that after dissociation of poliovirus by urea its protein components sedimented at about 5 to 20S in sucrose density gradients containing 5 M urea and 0.05 M mercaptoethanol [2]. A closer examination revealed that the S_{20} value of the dissociated protein is about 2S under these conditions (U. Yamaguchi-Koll and R. Drzeniek, unpublished results). This suggests a complete dissociation of the virus capsid into the appropriate polypeptides. The addition of urea to sucrose gradients was necessary in order to prevent reaggregation of the released polypeptides. Reaggregation was a serious problem in our work. In other studies concerned with the dissociation of picornaviruses [23, 9, 8] the high tendency of poliovirus protein(s) to aggregate was also observed. At the moment there remains some doubt as to which reported substructures of picornaviruses are resulting directly from dissociation and which are the product of aggregation. Therefore, our data about poliovirus protein obtained by dissociation of the virion by urea will be presented in a subsequent paper dealing with the substructures of

poliovirus. These results will be set into relation to the recent finding of other investigators [9, 3, 8].

The dissociation of poliovirus by urea at 25 °C revealed a lag phase in the time course of incubation. This lag phase was also observed by others but it was much shorter due to the higher temperature of incubation (37 °C)[7, 8]. Since no interpretation of this lag phase has been available so far, the following explanation is proposed.

During the first 10 to 40 min (depending on the strain) of incubation of poliovirus in 9 M urea and 0.1 M mercaptoethanol the infectivity in the virion assay remains unaltered. After this lag phase the infectivity of the virion drops very fast (Figs 1 and 2) to level below detectability (<10 PFU/ml). However, after the disappearance of the infectivity of the virion the infectivity of the RNA becomes prominent. Thus, two phases of poliovirus dissociation can be distinguished. In the first phase the infectivity of the virus particle remains unchanged (lag phase) but drops sharply in the second phase in which infectious RNA is demonstrated.

From infectivity measurements it could be concluded that nothing happens to the virion in the lag phase. But this is unlikely to occur. The optical density at 260 nm of poliovirus treated with 9 M urea increases in the lag phase, indicating changes of the poliovirus particle without effect on its infectivity. The increase in optical density seems to be due to structural changes of the virus RNA. Inside the virus particle the RNA is highly ordered as demonstrated by its hyperchromicity. A secondary structure of RNA inside of foot and mouth disease virus was well documented [17]. Since urea breaks down hydrogen bonds, it either dissociates the double-stranded regions of the virion's RNA or it dissociates non-covalent bonds existing between the RNA and the polypeptides in virus particles. The result is a loosening of the virion's structure to a point at which the virus falls apart with the concomitant release of RNA.

In earlier publications it was suggested [23, 24, 9, 8] that the degradation of picornaviruses is a stepwise mechanism in which the polypeptides of the virion were released in an ordered sequence beginning with VP4 [25]. This has to be proven for our dissociation procedure. We anticipate that in the lag phase no release of polypeptides from the virion occurs. If polypeptides would dissociate from the virion in the lag phase, this process should become irreversible when a high dilution of the incubation mixture is employed. But we never observed a decrease in infectivity of the virion in the lag phase, although very high dilutions were used prior to the plaque assay. Furthermore, one would expect that the dissociation of a polypeptide from the virion would make the virus RNA accessible to RNase, but this was never observed in the lag phase. Therefore, it is suggested that in the lag phase the virion expands without loss of polypeptides, as it was observed for phage MS2 when treated in low ionic strength buffers [26]. Kaper [27] described experiments in which formamide, which is chemically related to urea, kept the protein shell of turnip yellow mosaic virus (TYMV) in the expanded state so long that this could easily be demonstrated. Extended forms of picornaviruses were observed after heat treatment [28] or after fixation by glutaraldehyde [29].

Experiments are under way to characterize and isolate the different substructures of poliovirus and then to establish the sequence of events during which poliovirus particles are dissociated by urea into RNA and polypeptides.

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