SOME PHYSICO-CHEMICAL PROPERTIES OF RHINOVIRUSES

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VIRUSES can be isolated from people with common colds by inoculating their nasal secretions into cultures of human embryo kidney cells rolled at 33° and containing a medium of almost neutral pH. These viruses were called Salisbury strains and subsequently rhinoviruses. Certain strains are pathogenic for rhesus monkey kidney cells and are called M rhinoviruses and those which grow only in human cells are called H rhinoviruses. The cytopathic effect produced is like that of the enteroviruses and all strains are ether stable. The M rhinoviruses resemble in many ways the J.H. and 2060 strains (ECHO 28) (Tyrrell and Chanock, 1963). Other rhinovirus strains have been isolated by Hamre and Procknow (1961) using similar techniques. Johnson, Bloom, Chanock, Mufson and Knight (1962) used this method and also replaced kidney cells with human diploid cell strains ; Hamparian, Ketler and Hilleman (1961) used the latter cells exclusively. Johnson *et al.* (1962) called the viruses which they isolated from colds entero-like viruses and the latter group called theirs coryzaviruses.

Ketler, Hamparian and Hilleman (1962) have recovered infectious RNA from one H strain virus and showed that many H and M strains were not inhibited by 10^{-5} M-FUDR (5-fluoro-2′-deoxyuridine) and are therefore presumably RNA and not DNA viruses. We wished to determine whether M rhinoviruses and H rhinoviruses isolated in different laboratories were alike or different from each other and from enteroviruses in certain basic physical properties, such as size and rate of sedimentation. We also studied the effect on representative strains of inactivation by acid and by heat. Some of these results have been briefly reported earlier (Dimmock and Tyrrell, 1962) and are given here in full.

MATERIALS AND METHODS

Viruses

The following strains isolated in Salisbury were used : Sal/1/57M (H.G.P.), Sal/1/60M (B632), Sal/1/58H (F.E.B.), Sal/2/51H (D.C.), Sal/1/51H (No.) and Sal/1/59H (Th.). Dr. M. R. Hilleman supplied coryzavirus (C.V.) 11, Dr. K. M. Johnson supplied strain 1059, and Dr. K. Bögel the SD-1 strain of bovine rhinovirus. Prototype ECHO 4, the U strain of ECHO 11 and the Sal/4/60M (P.P.) strain of ECHO 28 were also used. Coxsackievirus A7 adapted to monkey kidney tissue cultures and a locally isolated strain of Coe, coxsackievirus A21 (Parsons, Bynoe, Pereira and Tyrrell, 1960), were used together with the LSc strain of poliovirus type 1.

Strains pathogenic for rhesus kidney cells were grown and assayed on secondary cultures. Others were grown first on human diploid cell strains or human embryo kidney cells and then adapted to and assayed in HeLa cell cultures. Coxsackievirus A21 and poliovirus were also grown and assayed in HeLa cells, and the bovine rhinovirus strain in calf kidney cells.

The viruses were prepared by harvesting the medium from tissue cultures showing advanced cytopathic effect and were stored at −60° or for short periods at 4°. The fluids were clarified by centrifugation at about 4000 ***g*** for 10–15 min. before use. The identity of the

virus was checked at least once by a neutralization test with homologous immune serum. Poliovirus was assayed by limit dilution titrations and the end-points calculated by the method of Reed and Muench (1938); the other viruses were assayed by microplaque assays (Parsons and Tyrrell, 1961).

Tissue cultures

Rhesus monkey cultures were prepared as described earlier (Tyrrell and Parsons, 1960). Calf kidney cultures, prepared as described earlier (Negroni and Tyrrell, 1959) were washed and maintained in 0·5 per cent lactalbumin hydrolysate and 0·1 per cent yeast extract in Hanks' saline containing 0·03 per cent sodium bicarbonate.

HeLa cells were supplied by Dr. M. S. Pereira and adapted to growth in rabbit serum. The very uniform monolayers enabled microplaques to be readily counted. Tube cultures were each made with 5×10^4 cells in 0·5 ml. of a medium containing 5 per cent rabbit serum, 0·5 per cent lactalbumin hydrolysate and 0·1 per cent yeast extract in Hanks' saline with 0·1 per cent sodium bicarbonate. After 2 or 4 days' incubation at 37° the medium was replaced with 1·5 ml. of 2 per cent calf serum and 98 per cent medium 199 and about 0·06 per cent sodium bicarbonate.

Sucrose density gradient

Sucrose solutions of 0 to 18 per cent w/v in 3 per cent concentration steps were mixed with an equal volume of tissue culture fluid. The seven sucrose solutions were passed consecutively to the bottom of the centrifuge tube through a finely drawn out glass tube starting with the 0 per cent solution. A concentration gradient was thus formed grading to the highest concentration at the bottom of the tube. No appreciable mixing of the sucrose layers was shown to take place during this procedure when a crystal violet solution replaced the tissue culture fluid in alternate layers.

RESULTS

Ultrafiltration

We attempted to estimate the size of the viruses by ultrafiltration using carefully standardised Gradocol membranes (supplied by Dr. F. Himmelweit of the Wright–Fleming Institute) and calibrating these with poliovirus type 1, a similar virus the size of which is accurately known.

The filters were all "satisfied" with 10 ml. of 5 per cent horse serum before use and filtrations were performed with a positive pressure of 10 lb. per sq. in. The culture fluids were all passed through a membrane of average pore diameter (APD) 570 mμ and then divided into several portions of about 5 ml., each of which was passed through another membrane. The filtrates passing the 570 mμ membrane and the other membranes were all titrated. When poliovirus was mixed with a rhinovirus the titrations were done at 37° in stationary cultures to reveal the presence of poliovirus; poliovirus antiserum was added and the mixture titrated at 33° in rolled cultures to reveal the presence of rhinoviruses. (Preliminary experiments showed no difference in the titre of the poliovirus when antiserum against the rhinovirus was added.)

The results of filtering mixtures of poliovirus and H.G.P. or B632 are shown in Fig. 1. This shows that the titre of all three viruses was reduced to approximately the same extent by passage through various membranes. Some rather low titre pools of D.C. and F.E.B. were also filtered in this way and similar reductions were observed on passage through membranes of 63 mμ APD or greater. These results suggest that poliovirus and both M and H rhinoviruses are about the same size. Poliovirus was completely held back by a 39 mμ membrane and H.G.P. and B632 were held back by a 45 mμ membrane The difference could have been

due to the higher titre of the former virus and clearly this " limiting " size is an unreliable measure of the diameter of virus except when using high titre virus preparations. Nevertheless, using Black's factor (Black, 1958) the probable diameter of H.G.P. and B632 calculated from these data is 31 mμ and of polio-

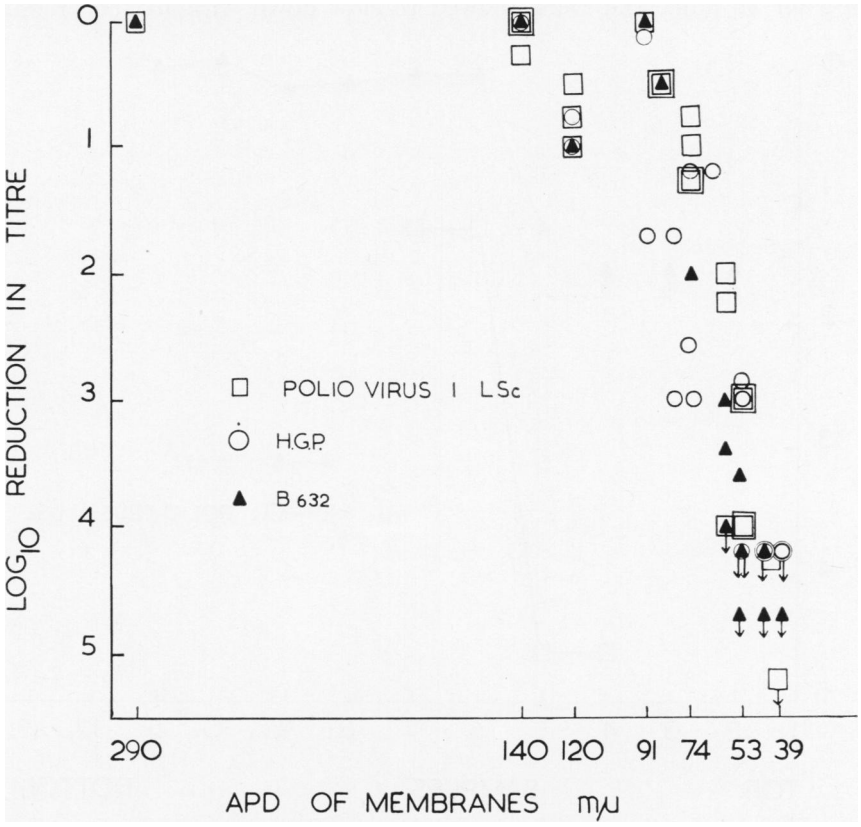

FIG. 1.—Filtration of viruses through Gradocol membranes. All virus suspensions were initially filtered through a 570 mμ APD membrane. The points with arrows represent experiments where no virus was detected in the filtrate.

virus type 1 is 29 mμ ; those for D.C. and F.E.B. are less than 47 mμ and less than 40 mμ respectively.

It was concluded that by ultrafiltration M strains and probably H strains are about the same size as poliovirus type 1.

Ultracentrifugation

In these experiments we again used poliovirus as a reference preparation since the sedimentation rate of thoroughly purified virus has been determined by optical methods (Schwerdt, 1957) and we expected that it would behave rather like the viruses we were studying. We prepared a sucrose density gradient of either 10 ml. in nitrocellulose tubes for the S40·2 angle rotor of a Spinco model L

centrifuge or of 3·5 ml. for the polypropylene tubes of the horizontal rotor of an M.S.E. Superspeed 40 centrifuge. The nitrocellulose tubes were pretreated with 5 per cent calf serum overnight, which improved the recovery of virus and the sharpness of the boundary (Buckland and Tyrrell, unpublished).

The centrifuges were run at room temperature, at a speed to produce about 75,000 *g* for 30 min., and were allowed to slow down without the brake. One

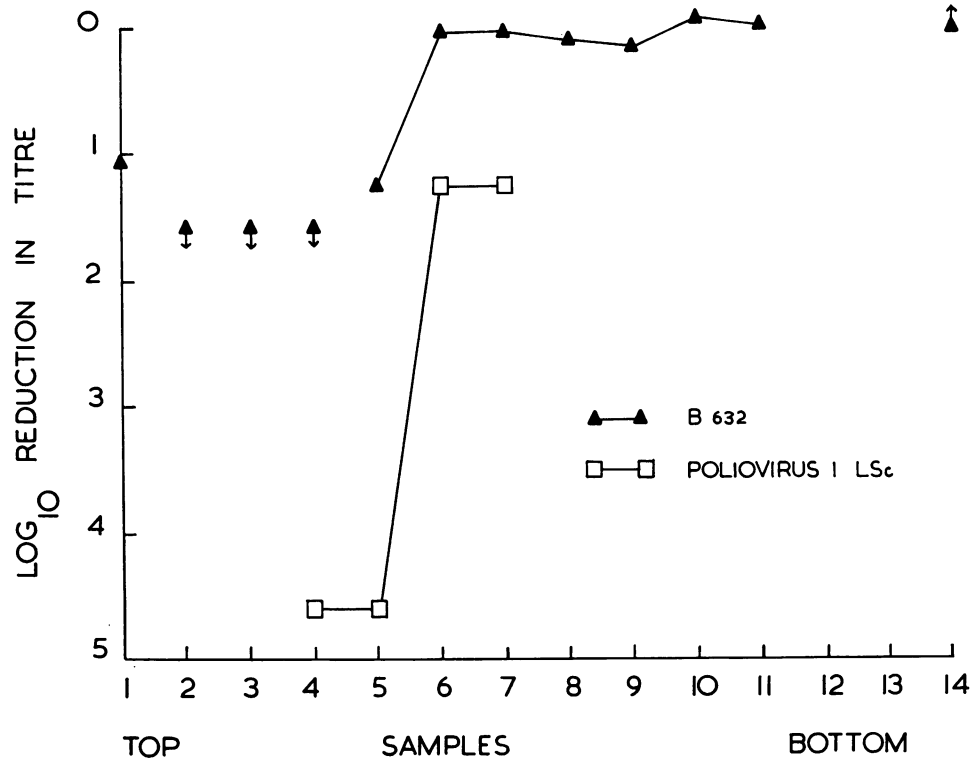

FIG. 2.—Ultracentrifugation of a mixture of B632 and poliovirus type 1 LSc using a Spinco S40.2 rotor and a Model L preparative centrifuge. Titres are compared with those of uncentrifuged material. Virus was not found in sample 1 in subsequent experiments.

third of the total acceleration and deceleration times was added to the time at full speed when calculating the results (Pardee and Schwerdt, 1952). About 16 consecutive samples were removed from the top of the tubes using a calibrated Pasteur pipette for the small tubes, and a tuberculin syringe with a flat-ended needle for the large tubes. The fractions were titrated and compared with the titre of uncentrifuged material; the procedure mentioned above for virus mixtures was followed.

The results of an experiment on a mixture of viruses are shown in Fig. 2. This shows that an unequivocal boundary is formed by the sedimenting virus and that the boundaries of the poliovirus and the rhinovirus lie at the same level in the tube. Numerous experiments of this type were performed with only one virus in the tube; the results are listed in Table I. The sedimentation coeffi-

cient of the particles forming the boundary has been calculated and corrected for the effects of temperature, sucrose concentration and viscosity on the rate of sedimentation. It can be seen that the coefficients calculated from the experiments in the angle rotor are very similar to that found for purified poliovirus (Schwerdt, 1957), namely 160*S*. When the experiments were repeated with the horizontal rotor, poliovirus and all the rhinoviruses again sedimented at exactly the same rate, but the calculated coefficient was smaller. When the tube returns to a vertical position during deceleration a small amount of stirring may be set up and cause the boundary to move sufficiently to account for this difference in coefficient. The mean coefficient of the H rhinoviruses (130*S*) was not significantly different from that of the M rhinoviruses and ECHO 28 (133*S*).

TABLE I.—*Ultracentrifugation of Viruses*

Virus	Rotor	Sedimentation coefficients (S_{20})			Mean S_{20}
Poliovirus 1 (LSc)	Horizontal	130	142	137	136
H.G.P.	,,	133	136	—	135
B.632	,,	145	135	—	140
ECHO 28 (P.P.)	,,	120	129	—	125
F.E.B.	,,	128	142	—	135
No.	,,	123	130	—	127
Th.	,,	123	121	—	122
D.C.	,,	136	135	—	136
1059	,,	138	123	—	130
C.V.11	,,	134	125	—	130
Poliovirus 1 (LSc)	Angle	—	165	—	—
H.G.P.	,,	—	155	—	—
B.632	,,	—	165	—	—

Acid stability

It was shown (Tyrrell, Bynoe, Hitchcock, Pereira and Andrewes, 1960; Hitchcock and Tyrrell, 1960) that the ability of H.G.P. virus to cause colds, and to produce interference or a cytopathic effect in tissue cultures was abolished by treatment at pH 2 overnight. As poliovirus is not inactivated at a low pH it was thought that this property might distinguish rhinoviruses from enteroviruses. Further experiments were therefore prepared.

Tissue culture fluid was mixed with an equal volume of 0·1 M buffer. Citric acid-sodium citrate buffer was used from pH 2·5 to 4·5, acetic acid-sodium acetate or sodium phosphate buffers at pH 5 and the latter buffer from pH 6 to 8, and glycine-sodium hydroxide buffers from pH 9 to 11·5. The pH was checked for every experiment with a dummy mixture containing non-infectious culture medium. The mixtures were held at 37° for 60 min. in screw-capped bottles and during this period no significant changes in pH occurred. A parallel mixture at pH 7 was kept at 4° for this duration. The pH of the mixtures was then adjusted to neutrality by adding an equal volume of 1 M-phosphate buffer—pH 7·2 for mixtures of pH 4·5 or less and pH 6·9 for mixtures of higher pH. The neutralised mixtures were titrated, starting with a 1 : 10 dilution to avoid the toxic effect of the buffers on the cells. The results of this test on H.G.P., B632 and ECHO 4 are shown in Fig. 3 and expressed as the $\log_{10}$ reduction of the non-incubated control. Further viruses were tested at pH values of 4·3 to 4·5, 5·0 to 5·4, 6·8 to 7·2, and 9·1 to 9·6 and the results are summarised in Table II.

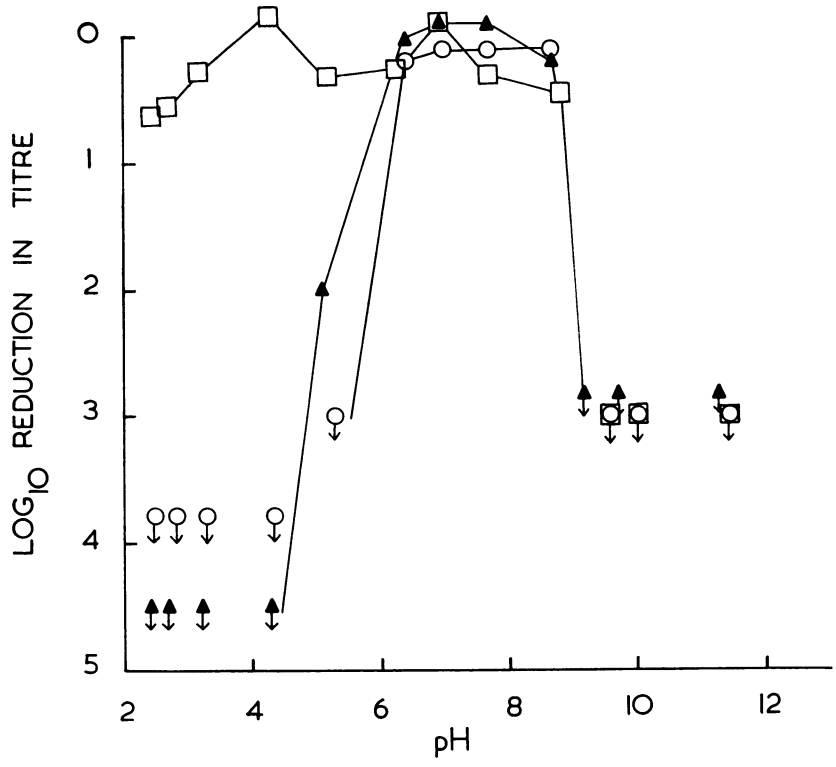

FIG. 3.—Incubation of viruses in buffer solutions for 60 min. at 37°. ○——○ H.G.P.; ▲——▲ B632; □——□ ECHO 4. Titres are compared with those of parallel virus-buffer mixtures at pH 7 kept at 4°.

TABLE II.—*Loss of Infectivity after Incubation in Buffer Solutions*

Group	Strain	Reduction of titre (Log_{10})† after incubation at pH: 4·3–4·5	5·0–5·4	6·8–7·2	9·1–9·6
Enterovirus	Coxsackie A7	N.D.	0*	0·1	0·6
	Coxsackie A21	0·1	0·2	0·1	>3·1
	ECHO 4	0	0	0·2	>3·0
	ECHO 11	0	N.D.	0·1	>3·6
	ECHO 28	>3·4	>3·4	−0·1	>3·4
Rhinovirus	H.G.P.	>3·8	3·0	0·1	>3·0
	B.632	>4·5	2·0	0	>2·9
	F.E.B.	N.D.	>1·9	−0·1	1·4
	No.	N.D.	>2·3	0·3	1·9
	D.C.	>2·6	1·3	0	>2·6
	1059	N.D.	>2·9	−0·1	>2·5
	C.V. 11	>2·1	1·3	0·1	2·2

* pH 4·7.
† Each value is the mean of the results of at least two experiments.
N.D. = not done.

All the enteroviruses tested, with the exception of ECHO 28 virus, were acid stable and all the rhinoviruses were acid labile. Apart from Coxsackie A7 all the viruses of both groups were considerably inactivated in alkaline solutions.

Heating at 50° in the presence of magnesium chloride

Poliovirus types 1, 2 and 3 are rapidly inactivated on heating at 50°, but this inactivation is completely prevented for 1–2 hr. by the addition of 1 M-magnesium chloride (Wallis and Melnick, 1961). The following experiments were performed to see if the same phenomenon occurred among rhinoviruses.

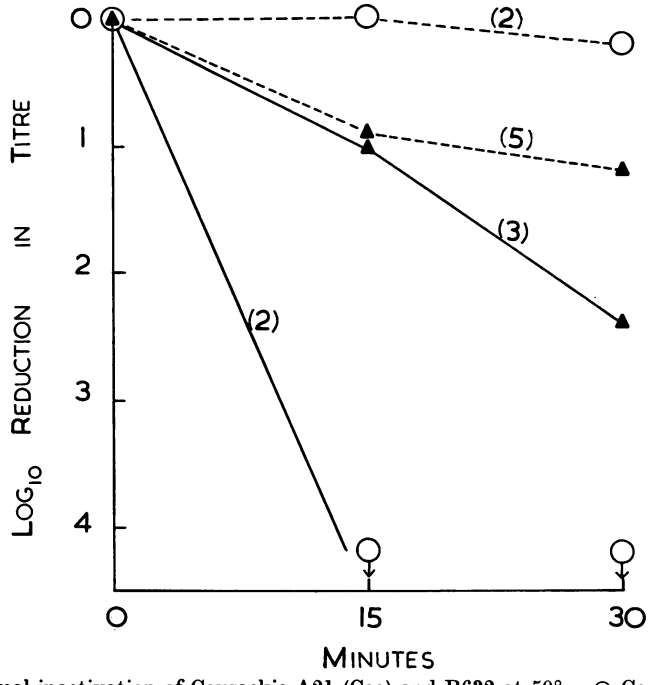

FIG. 4.—Thermal inactivation of Coxsackie A21 (Coe) and B632 at 50°. ○ Coe; ▲ B632; ——— = control; - - - - = 1 M-$MgCl_2$. The points are the mean of the number of experiments marked on each curve. Titres are compared with those of unheated virus mixtures.

Tissue culture fluid was mixed with an equal volume of either distilled water or 2 M-magnesium chloride and the mixtures were held at room temperature for at least 10 min. before being added in volumes of 0·8 ml. to screw-capped bottles preheated at 50°. Similar mixtures were held at room temperature. Bottles were transferred at intervals from the 50° waterbath to an icebath. Serial dilutions, commencing at a 1 : 10 dilution of the mixture were inoculated into tissue cultures. The loss of infectivity at various times compared with that of unheated virus is shown in Fig. 4, which indicates that Coxsackie A21, an enterovirus which nevertheless grows freely in the upper respiratory tract, was rapidly inactivated at 50°, but completely stabilized by magnesium ion while B632 was more slowly inactivated at 50° and incompletely stabilized by $MgCl_2$. It was thought that the $MgCl_2$ might lower the pH of the mixture sufficiently to in-

activate the virus, but the pH was found to lie between 6·2 and 6·9 which is outside the range that caused inactivation (see Fig. 3). Further when a mixture of HGP. and magnesium chloride was adjusted to neutrality with tris (hydroxymethyl) amino methane the rate of inactivation at 50° was identical with a similar mixture without the extra buffer at pH6·2.

The results of further experiments with other viruses are included in Table III. This shows that in general rhinoviruses were more slowly inactivated at 50° than were the enteroviruses tested and that the rhinoviruses were stabilized by magnesium chloride to a varying extent. ECHO 28 virus was completely stabilized by $MgCl_2$. Although there is a clear difference between the two groups it would be difficult to choose a criterion or a combination of criteria which would satisfactorily distinguish all rhinoviruses from all enteroviruses.

TABLE III.—*Loss of Infectivity after Incubation at* 50°

Group	Strain	Reduction of titre (Log_{10})†	
		Control	+1M-Mg^{++}
Enterovirus	*Coxsackie A21	>4·2	0·2
	*Poliovirus 1 (LSc)	>6·0	0·2
	*ECHO 4	>3·0	0·3
	ECHO 11	3·7	0·2
	*ECHO 28	>3·5	0·1
Rhinovirus	H.G.P.	2·8	1·6
	*B.632	2·4	1·2
	F.E.B.	2·5	1·2
	No.	3·6	1·1
	D.C.	>3·3	0·6
	1059	2·7	0·6
	C.V. 11	2·7	1·4
	S.D. 1	>3·9	1·5

* Incubated for 30 min.; the others for 60 min.
† Each value is the mean of the results of at least two experiments.

DISCUSSION

When we started this work it seemed possible that the viruses under study might prove to be a heterogeneous group. It was known that some were pathogenic for rhesus monkey kidney cells and others were not and although all were ether stable and produced similar cytopathic effects in human cells it was possible that their size or other physical properties might vary significantly like the biological properties—the pathogenicity for monkey kidney cells seems to be a graded property and some strains (*e.g.* 30/60, Hobson and Schild, 1960; Tyrrell, unpublished observation) are only feebly pathogenic.

Ultrafiltration, though a cumbersome and not very precise method, showed that the size of all these viruses was not very different from that of poliovirus, although a coryzavirus had been reported to have a diameter of 17–18 mμ (Hamparian *et al.*, 1961). Ultracentrifugation experiments showed that the sedimentation coefficient was very similar to that of poliovirus, and the margin of experimental error in this comparison was probably less than that of filtration. Of course, the size of this coefficient is affected by size, density and shape of the virus particle, but it would be an odd coincidence if the different strains varied a great deal in any of these three parameters in such a way that the sedimentation coefficient was the same in all cases.

Infectious RNA has been isolated from a number of enteroviruses and recently from one H type coryzavirus by Ketler *et al.* (1962) who also showed that other coryzavirus strains multiplied freely in the presence of the DNA inhibitor FUDR. Since all rhinoviruses are probably the same size and density as polioviruses and all apparently contain RNA there is good experimental support for the decision of an International Committee to group the viruses under the title of PICORNA-viruses since they all appear to be small RNA viruses (International Enterovirus Study Group, 1963).

Our results also indicate that ECHO 28 and all rhinoviruses isolated from colds may be distinguished from enteroviruses because they are inactivated by acid. Similar observations were also reported by Ketler *et al.* (1962). It has already been suggested that the fact that a virus is inactivated at pH 3 or 4 should be the cardinal test for distinguishing a rhinovirus from an enterovirus (Tyrrell and Chanock, 1963) and on this ground, as well as others, ECHO 28 virus should be regarded as a rhinovirus. Ketler *et al.* (1962) suggest grouping the viruses together under the name ERC (ECHO 28, rhinoviruses and coryzaviruses), but Andrewes, Burnet, Enders, Gard, Hirst, Kaplan and Zhdanov (1961) have called them all rhinoviruses. This name emphasizes that they can be readily isolated from the nose and throat rather than from the faeces. This fact may be reflected in the laboratory test used to distinguish the two groups; acid labile viruses would be readily inactivated in the gastric juice and therefore would neither be passed passively into the faeces nor be able to establish an infection in the intestine. Infection of the intestine would also be difficult for rhinoviruses because most of them multiply less well at 36° than at 33°.

As in so many instances these results suggest further experiments. It would be desirable to show experimentally that more of the rhinoviruses, particularly M strains, yield infectious RNA, and whether this can be distinguished from enterovirus RNA. It is also desirable to obtain good electron micrographs of purified virus particles. We suspect that rhinoviruses are acid labile because the surface protein is more readily denatured than that of enteroviruses, and the protective effect of magnesium ions against heat inactivation of infectivity may also be due to protection of the virus protein. It should be possible to confirm or refute these suggestions experimentally and further results will be reported later.

We have considered only the behaviour of the virus particle in isolation, so to speak, but it is clear that there may well be differences and similarities between the way in which the viruses multiply. The cytopathic effect of M and H strains closely resembles that of enteroviruses, but the latent period of rhinoviruses is longer than that of poliovirus (Tyrrell, 1963). Also most rhinoviruses are inhibited by α-hydroxybenzyl-benzimidazole (H.B.B.) (Tamm and Eggers, 1962) while enteroviruses are not. Further experiments will be needed to determine what differences or similarities there may be in the way in which enteroviruses and rhinoviruses multiply.

SUMMARY

The properties of certain viruses isolated from common colds and called rhinoviruses, entero-like viruses or coryzaviruses, have been compared with those of representative enteroviruses.

Two M rhinoviruses (strains H.G.P. and B632) appeared by ultrafiltration to be very close in size to poliovirus type I.

The rates of sedimentation in sucrose gradient of 2 M and 4 H rhinoviruses were similar to those of poliovirus and ECHO 28.

The rhinoviruses and ECHO 28 were all rapidly inactivated at pH 5 and below, while enteroviruses were not, and it is thought that ECHO 28 virus should be classified as a rhinovirus.

Rhinoviruses on the whole were more slowly inactivated than enteroviruses when heated at 50°, and this inactivation of rhinoviruses was less effectively prevented by molar $MgCl_2$ than was the inactivation of enteroviruses.

One strain of coryzavirus (C.V.11) and of an entero-like virus (1059) behaved in all tests like the H rhinoviruses and it is thought that they should be classified as members of that group.

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