COMPARISON OF DIFFERENT PREPARATIONS OF INFLUENZA VIRUS RIBONUCLEIC ACID

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It was shown previously that influenza virus RNA can be isolated by the extraction with cold phenol of the protein from purified preparations of the virus which had been treated briefly with ether (Portocala et al., 1959; Sokol and Szurman, 1959). However, the yield of viral RNA was variable and usually low (Table I). Difficulties were also encountered in quantitative removal of proteins from the aqueous phase. When pretreatment with ether was omitted, extraction with phenol at 4° C liberated only a small proportion of RNA (Table I) derived probably from damaged virus particles. Since representative samples of viral RNA were necessary for biological and physical studies, attempts were made to find a method which would reproducibly ensure high yields. Extraction with hot phenol seemed to be promising, but RNA exposed to relatively high temperatures is not suitable for the study of its secondary structure. Recently Laver (1961) reported that influenza B virus can be disrupted by treatment with sodium deoxycholate (SDC). We tried therefore to isolate viral RNA by phenol extraction also from SDC-treated virus. The virus was purified and the RNA isolated as follows.

The inhibitor resistant line of A2/Singapore/57 influenza virus strain was used in all experiments. Virus grown in the allantoic cavity of embryonated eggs was adsorbed onto and eluted from chicken erythrocytes

and sedimented twice at 40,000 X g for 40 minutes. The final virus pellet was resuspended in phosphate buffered saline (0.13 M NaCl, 0.007 M phosphate, pH 7.2). In addition to this procedure two of the preparations were also treated with ribonuclease and deoxyribonuclease and purified by chromatography on DEAE-cellulose columns (Sokol and Schramek, 1963). The bulk of the virus was eluted from the column with 0.2 M NaCl buffered to pH 7.2. All preparations of purified virus showed a single, asymmetric peak ($s_{20,w}$ = 610 ± 12 S) when sedimented in the Phywe analytical ultracentrifuge or analyzed in the Antweiler type electrophoretic apparatus. The number of hemagglutination units per mg virus in these preparations was about 5×10^4 and the ratio of infectivity (estimated by titration in embryonated eggs) to hemagglutinating activity about 10^6. Ten ml amounts of intact or SDC-pretreated virus preparations containing 1-3 mg virus per ml were extracted three times for 8 minutes with equal volumes of 80% phenol at 4, 25, and 60° C, respectively. For treatment with SDC varying amounts of solid detergent were added to the virus suspensions and allowed to dissolve under gentle shaking. The turbidity of the suspension decreased markedly. After about 1 minute phenol was added and the mixture was shaken vigorously. After each extraction the water phase was separated by centrifugation in the cold. The aqueous phase was freed of dissolved phenol by repeated extraction with ether and the RNA precipitated at -5° C by 2.5 volumes of ethanol.

In Table I the recovery rates of precipitable RNA isolated by different methods are shown, assuming that influenza virus contains 0.9% RNA (Miller, 1956) and that the optical density of a solution containing 0.1 mg RNA per ml (1 cm path) at 258 mμ is 2.5. Increasing the temperature of extraction from 4 to 25° C resulted in an appreciable increase of the recovery rate of RNA. A further increase in temperature to 60° C did not improve the yield of viral RNA. Almost quantitative yields were obtained both at 4 and 25° C when SDC-treated virus was extracted. No attempts were made to re-extract the phenol phase with

buffer for recovery of RNA adhering to the denatured protein. The RNA liberated could not be derived from host cell nucleic acids adsorbed onto the virus particles, since virus preparations treated with nucleases gave the same yields as untreated preparations.

TABLE I

Yields of RNA isolated from purified influenza virus preparations by different methods

Mode of isolation	Conc. of virus (mg/ml)	Weight ratio SDC/virus	Recovery of RNA %
Phenol			
4° C	1.97	-	9.8
25° C	1.97	-	44.3
60° C	2.64	-	41.1
Ether + phenol			
4° C	1.34	-	57.3
4° C	1.00	-	31.9
25° C	1.37-2.11	-	29.7 ± 12.9*
SDC + phenol			
25° C	2.44-3.32	1.8	86.0 ± 3.3*
25° C	1.14	4.4	105.5
25° C	1.04	4.8	106.6**
4° C	2.76	1.8	79.7**

*Mean and standard deviation from five independent experiments.

**After the first high speed centrifugation the resuspended virus was treated with pancreatic nucleases (10 μg per ml, 25° C, 45 minutes) and purified by chromatography on DEAE-cellulose column. The virus-containing fractions were then centrifuged and the pelleted virus resuspended in the buffered saline solution.

Attempts of several investigators to demonstrate the infectivity of RNA's isolated from purified preparations of influenza (Sokol and

Szurman, 1959; Ada et al., 1960; Schäfer, 1959) or Newcastle disease (Benedict et al., 1960) and Sendai (F. Sokol, unpublished) viruses were unsuccessful. This fact together with the finding that the helical internal nucleoprotein component of myxoviruses, containing all the viral RNA, is composed of two strands (Horne et al., 1960; Hoyle et al., 1961) suggested that the structure of myxovirus RNA differs from that of infectious RNA isolated from small viruses. Therefore some physical properties of RNA isolated from SDC-treated influenza virus were investigated. Our RNA preparations gave negative colorimetric tests for DNA and proteins with diphenylamine and Folin-biuret reagents, respectively. Hyperchromic effects up to 30% were obtained upon exposure to pancreatic ribonuclease, but not to deoxyribonuclease. The character of the ultraviolet absorption spectrum suggested the absence of absorbing impurities. The temperature dependence of the optical density of RNA solution is shown in Fig. 1. The absorption increased gradually over a relatively broad temperature range, the midpoint of this rise being at 51° C. In Fig. 2 are shown the time-dependence of the reaction of viral RNA with formaldehyde at 27 and 45° C, and the increase of absorbancy after heating at 100° C for 10 minutes in the presence of formaldehyde and cooling to room temperature. It is evident that a fraction of amino groups of the purine bases and of cytosine was accessible for the reaction with formaldehyde at 25° C, while others became available only at higher temperatures. The reactivity with formaldehyde of viral RNA heated at 100° C for 10 minutes and then cooled to 25° C was the same as that of unheated RNA. The character of the melting curve and that of the reaction with formaldehyde suggested that influenza virus RNA is single stranded and that it contains helical regions in which the amino groups are hydrogen-bonded (Fresco et al., 1960). The base composition of the isolated A2 influenza virus RNA (20.7, 26.4, 22.4 and 30.5 mol% of guanine, adenine, cytosine and uracil, respectively) offers additional evidence about its single stranded nature (Sokol and Schramek, 1962).

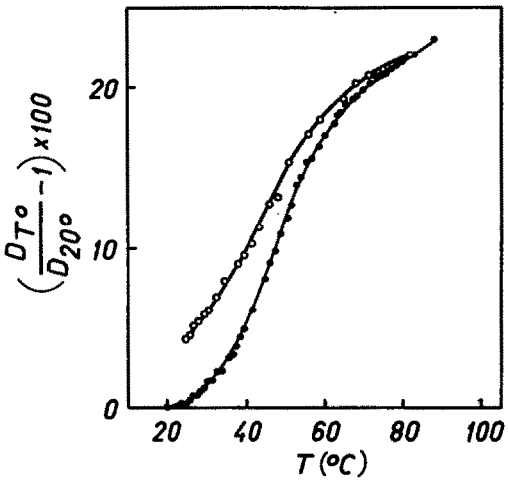

Fig. 1. The temperature-dependence of the optical density of influenza virus RNA in the buffered saline solution. Ordinate: increase of optical density at 260 mμ in %. Solid circles: heating; open circles: cooling.

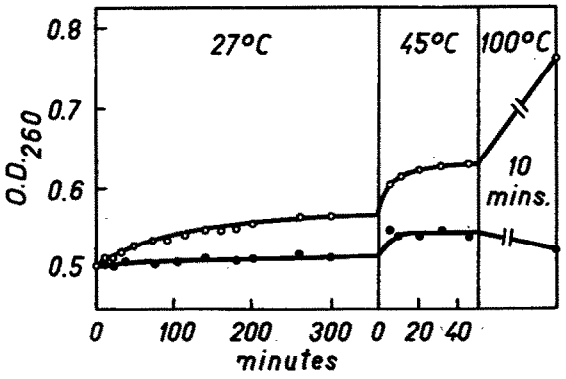

Fig. 2. The reactivity of influenza virus RNA with formaldehyde. The optical density at 260 mμ is shown as a function of time. RNA in 0.1 M phosphate buffer pH 6.9 reacted with formaldehyde (1.0%) at 27° C. After 300 minutes the temperature was changed to 45° C for 40 minutes. Then the RNA was heated at 100° C for 10 minutes and cooled to room temperature (open circles). Increase of absorbancy after heating at 100° C and cooling: 51%. Solid circles: control without formaldehyde.

A solution of viral RNA (64 μg/ml) was centrifuged at 59,780 rev/min in a Spinco model E centrifuge and pictures were taken at intervals using the ultraviolet absorption optical system. A single peak with a sedimentation

coefficient of 11.0 S was observed. However, chromatography on Ecteola-cellulose columns revealed that in addition to the 11 S component, which can be eluted only with 0.1 M NaOH, some RNA preparations contained up to 30% of another component eluted by 0.2 M NaCl. It will be necessary to compare the molecular weights of RNA's isolated by different methods to decide whether fully infectious influenza virus particles contain several RNA molecules or whether the 11 S component is a split product of the labile viral RNA.

REFERENCES

1. Ada, G. L., Lind, P. E., Larkin, L., and Burnet, F. M., Nature 184, 360 (1959).
2. Benedict, A. A., Lee, D., and West, B., Nature 188, 98 (1960).
3. Fresco, J. R., Alberts, B. M., and Doty, P., Nature 188, 98 (1960).
4. Horne, R. W., Waterson, A. P., Wildy, P., and Farnham, A. E., Virology 11, 79 (1960).
5. Hoyle, L., Horne, R. W., and Waterson, A. P., Virology 13, 448 (1961).
6. Laver, W. G., Virology 14, 499 (1961).
7. Miller, H. K., Virology 2, 312 (1956).
8. Portocala, R., Boeru, V., and Samuel, I., Acta virol. 3, 172 (1959).
9. Schäfer, W., In "Perspectives in Virology", 20, John Wiley and Sons, Inc., New York, 1959.
10. Sokol, F., and Schramek, Š., Acta virol. 6, 373 (1962).
11. Sokol, F., and Schramek, Š., Acta virol. 7, in press (1963).
12. Sokol, F., and Szurman, J., Acta virol. 3, 175 (1959).