Properties of the Nucleic Acid of the Ryan Strain of Filamentous Influenza Virus

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SUMMARY: The amount, type and properties of nucleic acid have been estimated in the two end fractions, Ryan supernatant (Ryan Sup.) and Ryan final filaments (Ryan F.F.) of a purification procedure applied to allantoic fluid preparations of filamentary Ryan virus. Ryan Sup. consists of 80 % spherical particles and 20 % short filaments (length:diameter < 6). Analysis indicates an average nucleic acid content of 0.75 % (PR8 = 0.91 %). The value of the ratio, adenine + uracil:guanine + cytosine for the nucleic acid of A strains of influenza virus varies between 1.22 and 1.28. The value for Ryan Sup. is 1.20; for a mutant strain of Ryan virus which exists almost exclusively as spheres, the value is also 1.20.

The ratio of filaments to spherical particles in Ryan F.F. is about 50:50. On a dry-weight basis, Ryan F.F. contains about 0.25 % RNA. There may be small amounts of DNA present. On a particle basis, the residual filamentary structures contain about eight times as much RNA as do PR8 spheres. The value of the above ratio of bases however is about 0.9. Treatments of filaments with diethyl ether releases a soluble complement-fixing antigen (CFA) which on purification is found to have the same nucleic acid content per unit of CF activity as does soluble CFA isolated from either PR8 or Ryan Sup. viruses. In each case the value of the above ratio of bases is about 1.25. On a particle basis filaments in Ryan F.F. contain 3 to 4 times as much soluble CFA as does PR8 virus so that the residual RNA in Ryan F.F. must closely correspond in properties to RNA from the potential host cell which has a value for the above ratio of about 0.6. Exposure of Ryan F.F. to ribonuclease or to a procedure which degrades the filamentary form to smaller spherical units does not affect the amount or properties of the associated RNA.

A tentative scheme is proposed for the formation of virus particles of Ryan F. preparations. It is postulated that most of the spheres present in such preparations arise by fragmentation of the tip of forming filaments where there is a relative concentration of viral type RNA. The filaments which are found in the allantoic fluid thus represent only part of the original filamentary structures. This concept implies that filaments break more readily at those places where there is an enrichment of viral type nucleic acid.

Purified influenza virus preparations have been shown to contain carbohydrate, lipid, protein and nucleic acid. Some of these have been examined in detail, for example: amino acids (Knight, 1947), carbohydrate (Ada & Gottschalk, 1956; Frommhagen & Knight, 1956) and nucleic acid (Ada & Perry, 1954, 1956). The nucleic acid is the only component which has been shown to possess properties which clearly distinguish it from the corresponding component in the potential host cell. Spherical forms of influenza virus contain about 1 % RNA which features a characteristic proportion of nitrogenous bases—a pattern which is sharply distinct from that of the host cell RNA.

Evidence accumulated over the past few years indicates that the influenza

virus particle is assembled at or near the host cell wall and it is possible that components from the latter (e.g. lipid, mucoprotein) are incorporated into the virus membrane. The demonstration of a parallelism between the haemolytic power of various substances for chick erythrocytes and their capacity to damage influenza virus filaments suggests that the filament surface is less different from the host cell surface than is that of the spherical particles (Burnet, 1956). This concept implies that filaments contain an appreciable proportion of relatively unmodified host cell components. An estimate of the amount and localization within the filament of these and of specific virus components would throw light on the structure of a filament. Due to the large difference in their properties, spherical virus type RNA and host cell type RNA are suitable markers in such an investigation. The finding (Ada, Perry & Abbot, 1958) of the infectious nature of Ryan filaments enhanced the interest attached to a study of the amount, type and properties of nucleic acid present in purified preparations of Ryan filaments. The results of such an investigation are reported in this paper.

METHODS

Virus. The strains of virus used, the conditions of growth, methods of estimation of haemagglutinin and infectivity titres are described in the previous paper (Ada *et al.* 1958).

Virus fractions. The fractionation procedure applied to allantoic fluid preparations of Ryan F. virus was described previously. Two fractions were obtained, namely: Ryan Sup. which consisted of 80 % spheres and 20 % short filaments, and Ryan F.F. which was a 1:1 mixture of spheres and filaments (Ada *et al.* 1958).

Physiological saline. 0.85 g. NaCl/100 ml. distilled water.

Bicarbonate saline. Physiological saline containing 5 mg. sodium bicarbonate/100 ml. saline; pH value *c.* 7.5.

Calcium magnesium saline (Ca Mg saline). Prepared according to Mayer, Osler, Bier & Heidelberger (1946) as follows: NaCl, 9.0 g.; 5:5-diethyl barbituric acid 0.575 g.; Na-5:5-diethyl barbiturate, 0.375 g.; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.168 g.; CaCl_2 , 0.028 g.; made up to 1 l. with distilled water. After autoclaving, the solution had a pH value of 7.2.

Complement-fixation titration. The micro-technique used was first described by Donnelley (1951). Serial twofold dilutions of antigen were prepared in volumes of 0.04 ml. in Ca Mg saline, 0.04 ml. of complement diluted to 3 HD 50 was added to each tube, and then 0.04 ml. of 1/5 or 1/10 rabbit antiserum. The tubes were incubated for 1 hr. at 37°, and then 0.04 ml. of 3 % sensitized sheep red cells were added to each tube. The test was read after a further 30 min. incubation. The titre is expressed as the reciprocal of the antigen dilution showing 50 % haemolysis.

Nucleic acids. Purified yeast nucleic acid (YNA) and deoxyribonucleic acid (DNA) from calf thymus were purified as described previously (Ada & Perry, 1954, 1956).

Extraction and estimation of nucleic acid from virus. The nucleic acid was extracted from the dried virus using a hot 10 % (w/v) NaCl solution. The

amount present in the salt extract was determined by using the formula $Ep = 30.98 E/cl$ (Chargaff & Zamenhof, 1948), where Ep = atomic extinction coefficient at 260 $m\mu$. with respect to phosphorus, E = optical density, c = concentration of phosphorus in g./l., and l = thickness of absorbing layer. Details have been given previously (Ada & Perry, 1954, 1956).

Paper chromatography. The nucleic acid samples were hydrolysed and the purine and pyrimidine derivatives separated by paper chromatography. The separated components were located by their absorption in ultraviolet light, eluted with 0.1 N-HCl and the optical density of the eluate read at the wavelength of maximal absorption for each derivative (Ada & Perry, 1956). The precaution was taken of checking the absorption ratio of each derivative as isolated from the chromatographic paper. In order of increasing R_f values guanine was read at 248 and 262 $m\mu$, adenine at 248 and 262 $m\mu$, cytidylic acid at 262 and 278 $m\mu$ and uridylic acid at 262 and 278 $m\mu$. Low values for the ratios of the virus derivatives compared with the values given by the derivatives of a sample of YNA treated in a similar fashion, indicated either faulty separation or contamination with other u.v. absorbing substances.

In one instance a departure was made from the technique outlined above. Samples of the soluble complement-fixing antigen derived by ether treatment from Ryan Sup. and Ryan F.F. were obtained in small amounts. To avoid losses involved in precipitating the nucleic acid present in low concentration in the 10 % salt extract, the whole complex was digested with N-HCl and run on paper. As a control, an equal weight of bovine serum albumin was digested and run on paper. The absorption ratios of the isolated purine and pyrimidine derivatives, when adjusted by using the values of the protein control, agreed well with the YNA values in the case of guanine and adenine but were 12 and 17 % lower than the YNA values in the case of the cytidylic acid and uridylic acid derivatives, respectively. Estimates of the amounts of these two components were therefore too high, but this effect was largely abolished when calculating the value of the ratio adenine+uracil:guanine+cytosine, and was allowed for when determining the total amount of nucleic acid associated with the soluble complement-fixing antigen.

RESULTS

Chemical properties of purified virus

Lipid content. Prior to the determination of the nucleic acid content, the dried virus was defatted with a chloroform + methanol mixture (Ada & Perry, 1954). The difference in weight before and after such treatment gives an approximate value for the lipid content of the preparation. In two experiments, the following values were obtained: Ryan Sup. 48 and 51 %; Ryan F.F., 54 and 59 %. These values are higher than the mean value of 44 % quoted earlier for PR8 (Ada & Perry, 1954).

Nucleic acid content. The nucleic acid content of the defatted virus preparation was determined as described in Methods. Extraction with a 10 % salt solution was found to give a less accurate estimate in the case of filament

preparations than with spherical viruses. With the latter it was shown (1) that the u.v. spectrum of the salt extract agreed well with that of a purified sample of yeast nucleic acid (YNA) and (2) that material which absorbed with a maximum at 260 $m\mu$ was quantitatively extracted by the hot salt treatment. Thus, measurement of the optical density at 260 $m\mu$ of the salt extract gave a reliable estimate of the nucleic acid content (Ada & Perry, 1954, 1956).

In the present investigation, similar conditions applied to preparations of Ryan spheres and, to a lesser extent, to Ryan Sup. In the case of final Ryan filaments, however, the u.v. absorption curve was much flatter (260 $m\mu$ /280 $m\mu$ = 1.4–1.5) than that of the control YNA (260 $m\mu$ /280 $m\mu$ = 2.08), indicating the presence in the extract of other u.v. absorbing substances. Secondly, extraction with hot perchloric acid (Ogur & Rosen, 1950; Martin & Morton, 1956) of the salt extracted virus residue indicated that small amounts of nucleic acid (or material with a maximum absorption at 260 $m\mu$) was not extracted by salt solution. At most this amounted to 12 % of that present in the salt extract and because of the small amount involved (max. 12 μ g.) was not further examined. An alternative method for estimating the amount of nucleic acid present in filaments was to determine the recovery of the nitrogenous bases following chromatography of the acid-digested nucleic acid. This gives a minimum value owing to incomplete precipitation of the nucleic acid by ethanol. In the case of two preparations of Ryan Sph. virus the recovery amounted to over 90 % of that estimated to be present from the u.v. spectrum of the salt extract. Values of 60–70 % were similarly found for two preparations of Ryan F.F. In Table 4 where the values for the nucleic acid content of the virus preparations are given, a maximum and a minimum value are given for the Ryan F.F., representing the estimates from the salt extract and from the recovery of bases respectively. In later calculations, the mean of these two values is used.

The sodium chloride extracts from these batches of Ryan F.F. were tested for the presence of DNA by the method of Keck (1956). The extract from one batch gave no peak at 490 $m\mu$. The extracts from the other two preparations gave definite peaks at 490 $m\mu$, indicating the presence of deoxyribose. Based on these figures, the amount of DNA calculated to be present in the salt extracts of the two batches amounted to 11 and 16 % of the total nucleic acid present. These figures are considerably too high, however, as the absorption curves were found to be higher at 450 and 520 $m\mu$ and yet lower at 490 $m\mu$ than curves given by the appropriate control DNA solution. There may therefore be a small amount of DNA associated with filaments.

The values for the nucleic acid content of the virus preparations expressed on a percentage dry weight basis are given in Table 1. In the case of Ryan Sup. and Ryan F.F., the mean nucleic acid contents expressed on a virus particle basis are also presented, using the values for particle weights found earlier (Ada *et al.* 1958). For comparative purposes, a value is included for the nucleic acid content/PR8 particle. Though this figure is obtained from the nucleic acid content of a purified preparation of virus and the particle counts of virus present in allantoic fluid preparations, it is unlikely that any significant

Table 1. *Nucleic acid content of PR8 virus and of spherical and filamentary forms of Ryan virus*

Virus preparation	Nucleic acid content (expressed on a dry-weight basis)			Particle weight of virus* ($\times 10^{10}$ μ g.)	Mean nucleic acid content (expressed on a virus particle basis) ($\times 10^{13}$ μ g.)
	Mean (%)	No. of experiments	Range of values		
PR8†	0.91	11	0.79–1.12	6	5.5
Ryan spheres	0.87	3	0.75–1.0	—	—
Ryan supernatant	0.75	6	0.60–0.93	5.8	4.4
Ryan final filaments	$\left\{ \begin{array}{l} \text{max.} = 0.80\ddagger \\ \text{min.} = 0.20\ddagger \\ \text{mean} = 0.25\ddagger \end{array} \right\}$	8	max. 0.23–0.35	175	44

* Ada *et al.* (1958).

† Ada & Perry (1956).

‡ See text.

Table 2. *Proportion of bases in nucleic acid of PR8 and Ryan viruses*

Virus	No. of experiments	Nucleotide composition (as mole/100 mole nucleotide)				Adenine + uracil
		Adenine	Guanine	Cytosine	Uracil	Guanine + cytosine
PR8*	5	23.1 $\pm$ 0.2	20.2 $\pm$ 0.5	24.0 $\pm$ 0.7	32.9 $\pm$ 0.5	1.27 $\pm$ 0.02
Ryan spheres	2	21.8	20.2	25.1	32.9	1.21
		22.3	20.5	24.9	32.3	1.20
Ryan supernatant	5	22.7 $\pm$ 1.4	21.7 $\pm$ 1.6	23.7 $\pm$ 1.1	31.9 $\pm$ 1.5	1.20 $\pm$ 0.03
Ryan final filaments	6	21.2 $\pm$ 1.3	25.3 $\pm$ 1.4	26.2 $\pm$ 2.1	27.3 $\pm$ 1.5	0.94 $\pm$ 0.05
Host cell RNA†	1	19.6	32.6	27.5	20.3	0.66

* Ada & Perry (1956).

† Ada & Perry (1955).

error is involved. Expressed on a dry-weight basis, PR8 and Ryan Sph. viruses yield comparable values while that for Ryan Sup. is only slightly less. The mean value for Ryan F.F. is less than one third as much. On a particle basis however, Ryan F.F. has a much higher nucleic acid content than Ryan Sup. Although Ryan F.F. contains about equal amounts of spherical particles and of filaments, the filaments account for over 95 % of the weight so that the nucleic acid content of a filament in Ryan F.F. will not be significantly different from 44×10^{-12} μ g. This figure will therefore be used in later calculations. On a particle basis the nucleic acid content of Ryan Sup. is 80 % of the value for PR8.

Proportion of bases in the virus nucleic acid. As well as yielding information concerning the identity of the bases present in the nucleic acid this analysis indicates the degree of contamination of the virus particle by the host cell type nucleic acid. Results are given in Table 2.

In every case only four spots, agreeing in R_f values with the spots from a control yeast nucleic acid digest, were present. For purposes of comparison, the values for each virus are conveniently expressed by the ratio, adenine +

uracil:guanine + cytosine. The value of this ratio given by Ryan Sph. virus and by Ryan Sup., 1.20, is close to the range previously found for A strains of virus, 1.22–1.28. Ryan F.F., however, has a proportion of bases which yields a value for the ratio which is intermediate between that of spherical viruses and the value given by the RNA of a crude extract of chorio-allantoic membrane (Ada & Perry, 1955).

Action of ribonuclease on Ryan virus. Short exposure of influenza virus (PR8) to pancreatic ribonuclease has previously been shown not to affect the nucleic acid content of the virus particle (Ada & Perry, 1954). It was of interest to see whether the exposure of filamentary forms of virus to ribonuclease affected the content or base proportions of the associated nucleic acid.

In a typical experiment, 80 ml. of an eluate of Ryan F. virus containing 200×10^4 AD was divided into two parts. To one part was added 1 ml. of saline containing 380 μ g. ribonuclease and both parts were then incubated at 37° for 2 hr. The virus in each sample was obtained by the usual procedure of differential centrifugation as described in the preceding paper (Ada *et al.*, 1958). In two cases, preparations of Ryan Sph. virus were also treated with ribonuclease, the enzyme being added at the eluate stage and the virus subsequently purified by differential centrifugation (Ada & Perry, 1956). The nucleic acid content of each virus preparation was estimated and the proportion of bases determined.

The results of several experiments are presented in Table 3.

Table 3. *Action of ribonuclease on the infectivity and nucleic acid content of Ryan virus*

Virus	No. of experiment	Amount of ribonuclease added/mg. virus (μ g.)	Length of incubation at 37° (hr.)	EID 50: AD		Nucleic acid content (%)	Ratio Adenine + uracil Guanine + cytosine	Ratio Purines Pyrimidines
				Allantoic fluid	Purified virus			
Ryan spheres	1	15	2	6.2	6.3	0.87	1.26	0.72
	2	Nil	2½	6.2	{5.9	0.87	1.20	0.74
		25	2½		{5.9	0.85	1.21	0.73
Ryan final filament	1	14	1	6.8	—	0.23	0.89	0.85
	2	Nil	2½	5.5	{5.4	0.30	0.99	0.80
		12.5	2½		{5.3	0.33	1.03	0.83
	3	Nil	2½	6.1	{5.8	0.26	0.94	0.88
		13	2½		{5.1	0.27	0.94	0.86

Exposure to ribonuclease was found to leave unchanged the following properties of Ryan F.F. preparations: (1) Yield of virus and the value of the ratio, AD : dry weight of virus. (2) Amount of nucleic acid. (3) Value of the ratios, adenine + uracil:guanine + cytosine and purine:pyrimidine. As ribonuclease is unable to split the link between purine nucleotides the value of the latter ratio is a sensitive test for enzymic degradation of the nucleic acid. It can be concluded therefore that the nucleic acid has not been affected by the enzyme although the amount of the latter added corresponded in some experiments to three times the estimated amount of nucleic acid present. (4) Value of the ratio EID 50:AD. The one exception in the last experiment quoted appears to be anomalous in view of the nucleic acid results.

Degradation of filaments

Treatment with ethyl ether. It has been shown by numerous workers that under suitable conditions, exposure of influenza virus to ethyl ether results in the structural breakdown of the virus particle with a concomitant loss of infectivity and the release of a soluble haemagglutinin and a soluble complement-fixing antigen (CFA). The soluble haemagglutinin is a mucoprotein, whereas the CFA is a nucleoprotein (Frisch-Niggemeyer & Hoyle, 1956; Schafer, 1957). The latter probably contains all the nucleic acid in the virus particle and, at least in the case of PR8, the proportion of bases in the CFA nucleic acid is the same as that in the parent virus particle (Ada, 1957). It was of interest to know (1) whether filaments contain any CFA; (2) the properties of the nucleic acid (if any) associated with the CFA.

Ether treatment was carried out following a slight modification of the technique described by Schafer & Zillig (1954). Purified virus (5–10 ml.; $1-4 \times 10^6$ AD) was shaken 16 hr. at room temperature with two volumes of peroxide-free ether. After centrifugation (5000 g, 15 min.) the sedimented and interfacial precipitates were pooled, suspended in saline and this and the aqueous layer reshaken with ether as above. This procedure was again repeated. The aqueous layers obtained by centrifugation were pooled, the ether removed by brief exposure to vacuum and intact virus particles removed by centrifugation (20,000 g, 45 min.). The supernatant contained the CFA and soluble haemagglutinin. The latter was removed by exhaustive extraction with freshly washed, packed red cells. The CFA was sedimented from the extracted solution by centrifugation (98,000 g, 120 min.), resuspended in saline and tested for biological activity and the presence of nucleic acid.

Table 4. *Amount and properties of soluble complement-fixing antigen (CFA) obtained by ether treatment of PR8 and Ryan viruses*

Virus	Units of CFA released/AD of virus ($\times 10^{-3}$)	AD/mg. dry weight* ($\times 10^{-4}$)	Units of CFA released/ mg. dry weight virus	Units of CF activity/ mg. dry weight of CFA ($\times 10^{-4}$)	Nucleic acid content (%)	Ratio Adenine + uracil Guanine + cytosine
PR8	10.3, 9.1, 14.3	9	1020	1.7	4.6	1.23
Ryan supernatant	8.7	4.6	400	1.5	3.6	1.25
Ryan final filaments	12.5, 13.3, 8.6	1.1	124	0.7	2.0	1.21

* Ada *et al.* (1958).

The amount and properties of the CFA obtained in this way from PR8 and Ryan viruses is shown in Table 4. The amounts of CFA released/AD of intact virus (calculated from the CF activity of the pooled aqueous layers before removal of intact virus and haemagglutinin) are comparable in the cases studied—PR8, Ryan Sup. and Ryan F.F. When these values are converted to units of CFA /mg. dry weight virus, a big difference is immediately apparent.

The figure for PR8 is almost eight times larger than that for Ryan F.F. with Ryan Sup. in between these two. In the next column, the units of CF activity/mg. CFA are given. PR8 virus and Ryan Sup. have similar values, that for Ryan F.F. being about half as great. The values for nucleic acid content in the following column show the same trend. This has two consequences: (1) due to the fact that both the nucleic acid content and the CF activity are low, the preparation of CFA from Ryan F.F. is probably impure and not intrinsically less active than antigens from other virus preparations; (2) the close agreement between the CF activity and amount of nucleic acid associated with the antigens from the three virus preparations suggests again that the presence of nucleic acid in the antigen preparations is not fortuitous. Finally in the last column are given the values of the ratio, adenine + uracil : guanine + cytosine, which characterize the CFA nucleic acids. For reasons given in Methods, these analyses are subject to a slight error. Nevertheless, the main conclusion to be drawn is quite clear. Whereas with PR8 and Ryan Sup. antigens the values agree well with those obtained with the nucleic acid extracted from the intact virus, the value given by the Ryan F.F. antigen clearly differs from that of the intact virus nucleic acid and agrees well with that of spherical virus preparations. This not only indicates that the nucleic acid found in Ryan F.F. is heterogeneous but also that it contains a fraction with the 'viral type' proportion of bases.

It has recently been found that treatment of virus with deoxycholate also results in the release of a soluble haemagglutinin and CFA. The optimum conditions for isolation of these components have not yet been determined but the results indicated that filaments contain a soluble CFA in approximately the same proportions/AD as given in Table 4. Details of the procedure will be published later.

Freezing of filaments. Donald & Isaacs (1954) have shown that following exposure to ultrasonic vibrations, filaments break up into smaller units which are approximately spherical. This results in an increase in haemagglutinin titre, although the infectivity is not affected. It is possible that such a process might liberate any nucleic acid or nucleoprotein either adsorbed to the filaments or in a situation where, following fragmentation of the filaments, diffusion into the medium could take place.

As a suitable ultrasonic generator was not available, purified virus was subjected to numerous cycles of freezing and thawing in a bath of ethanol and solid CO₂. While the appearance of Ryan Sup. preparations was unchanged, the marked streaming shown by Ryan F.F. decreased until between the 6 and 10th cycle, it was no longer apparent. Following this treatment, the virus preparations were re-adsorbed on to red cells and after elution (1½ hr. 37°), the eluate was centrifuged (35,000g, 30 min.). The biological properties of the deposited virus were tested and the nucleic acid investigated using the procedures described earlier.

The results are given in Table 5. In columns 3*a* and 3*b* the amount of haemagglutinin was estimated from the haemagglutinin titre of the solution immediately before and after the freezing and thawing process. In both

Table 5. *The effect of repeated cycles of freezing and thawing on some biological and chemical properties of Ryan filaments*

No. of experiments	No. of cycles of freezing and thawing	Virus	Recovery of haemagglutinin		(log 10) EID 50 purified virus	AD/mg. dry weight		Nucleic acid content of virus		Ratio				
			Amount of haemagglutinin	after freezing and thawing		Before* ($\times 10^{-4}$)	After ($\times 10^{-4}$)	Before (%)	After (%)	Adenine + uracil				
										Before	After	Guanine + cytosine	Before	After
1	6	Ryan supernatant	320	280	—	—	—	—	—	—	—			
	6	Ryan final filaments	160	340	—	—	—	—	0.30†	0.33	—			
2	10	Ryan supernatant	38	22	52	9.6	9.0	—	—	—	—			
	10	Ryan final filaments	19	45	150	8.8	8.6	—	—	—	—			
3	10	Ryan supernatant	115	112	56	—	—	4.6 (2.2-7.7)	6.7	0.75† (0.6-0.93)	0.8			
	10	Ryan final filaments	60	180	136	—	—	1.1 (0.8-1.7)	4.7	0.30† (0.23-0.35)	0.3			
										0.94†	1.04			

* Mean value and range from Table 8. (Ada, Perry & Abbot, 1958).

† Mean value and range from Table 1.

‡ Mean value from Table 2.

preparations of Ryan Sup., there was no increase in titre. In the case of Ryan F.F., however, the titre rose two to threefold and in the second experiment, this is reflected in the yield of haemagglutinin as estimated from the haemagglutinin titre of the final sedimented virus. As filaments in Ryan F.F. are about 6 times more efficient as haemagglutinin than are the spheres (Ada *et al.*, 1958), it is readily seen that this treatment breaks down the filaments into particles which are equivalent (as haemagglutinin) to about 15 (i.e. $2\frac{1}{2} \times 6$) Ryan F. spheres. Ryan Sup. showed a slight drop in EID₅₀ during freezing and thawing but the value for Ryan F.F. remained unchanged. Donald & Isaacs (1954) found the infectivity of filament preparations to remain unchanged after fragmentation with ultrasonic vibrations. In the second experiment, the ratio AD/mg. dry weight was determined. The value of Ryan Sup. is within the range previously found for untreated preparations but the Ryan F.F. preparation shows an increase (three to fourfold) compared with the mean value and range of untreated preparations. The nucleic acid content of both Ryan Sup. and Ryan F.F. remained unchanged following freezing and thawing indicating that there was no preferential loss of nucleic acid or protein from the fragmented filaments. The value of the ratio, adenine+uracil:guanine+cytosine was not very different from the mean value found previously for untreated preparations.

Plate 1, fig. 6, in the preceding paper (Ada *et al.* 1958) shows an electron micrograph of a preparation of Ryan F.F. after ten cycles of freezing and thawing. No filaments remained and the picture is rather similar to that found by Donald & Isaacs (1954) after exposure of filaments to ultrasonic vibrations.

In summary then, the process of freezing and thawing, though drastically changing the morphology of filaments had not appreciably changed the content or properties of the associated nucleic acid. To this extent, these results support those from the experiment with ribonuclease in suggesting that all the nucleic acid found associated with filaments is incorporated into the structure of the virus particle.

DISCUSSION

The original aim of this investigation was to study the properties, particularly the nucleic acid content, of filamentous forms of virus. In confirmation of the experiences of previous workers, it soon became clear that filaments were fragile structures which readily fragment, e.g. during purification procedures. The smaller of these breakdown products are isolated in the fraction, Ryan Sup., which contains the bulk of the spherical particles originally present in the infected allantoic fluid. While the other end fraction, Ryan F.F., which contains a high concentration of filaments (50 %), would seem to be the more important for study, the properties of both these fractions must be taken into account when attempting to define the mode of formation and structure of a Ryan filament.

On a dry weight or a particle basis, the particles present in Ryan Sup. contain about 80 % as much nucleic acid as do PR 8 spheres (Table 1). Further-

more, the proportion of bases in the nucleic acid from the particles in Ryan Sup. is the same as that found in the nucleic acid from the mutant strain, Ryan Sph. virus, that is, adenine + uracil:guanine + cytosine = 1.20. This value is only slightly lower than the range found previously for A strain viruses, 1.22–1.28. This may indicate that both Ryan preparations contain host cell type RNA but, if so, the amount involved must be very small unless it is assumed that all A strains are also contaminated. Marked contamination is unlikely in view of the observed specific difference in the nucleic acid base proportions of A and B types of influenza virus (Ada & Perry, 1955). On the other hand, Ryan F.F. preparations have less nucleic acid than PR8 preparations when expressed on a dry-weight basis (0.25%:0.9%) but about eight times more when the figures are compared on a virus particle basis. The value

Table 6. *Comparison of the base ratio of filamentous virus nucleic acid with that of an artificial mixture of equal amounts of spherical virus nucleic acid and potential host cell nucleic acid*

Source of nucleic acid	Nucleotide composition (as mole/100 mole nucleotide)				Adenine + uracil Guanine + cytosine
	Adenine	Guanine	Cytosine	Uracil	
Ryan filaments	21.2	25.3	26.2	27.3	0.94
1 part Ryan spheres + 1 part host cell	20.8	26.5	26.3	26.4	0.90

of the ratio for nucleic acid bases in Ryan F.F. preparations is quite different (0.9) from that found in Ryan Sup., being about half way between the latter value and that given by the RNA isolated from the uninfected host cell. A theoretical mixture of equal amounts of spherical virus type RNA and host cell type RNA yields values for the proportion of the individual bases which are very close to the value found in the RNA of Ryan F.F. (see Table 6). Support for such a concept is provided by two experimental results. First there is the finding that the nucleic acid associated with the soluble complement fixing antigen (CFA) isolated from Ryan F.F. gives a value for the proportion of nucleic acid bases which lies within the range given by the nucleic acid bases of A strain spherical viruses. Secondly, it was shown in Table 4 that the amounts of nucleic acid per unit of soluble CFA from PR8 and from Ryan F.F. are similar. (Nearly all the soluble CFA from Ryan F.F. will derive from filaments, the contribution from spherical particles being negligible.) There is also good agreement for the amount of soluble CFA released per AD of virus. As filaments in Ryan F.F. are 3 to 4 times more efficient as haemagglutinin than are PR8 spheres, it follows that these filaments have 3 to 4 times as much of the same type of nucleic acid as is present in PR8 spheres. Now Ryan F.F. preparations on a particle basis have 8 times as much nucleic acid as PR8 spheres. Nearly half of this is the 'spherical virus type'. By difference the remainder of the nucleic acid must have a composition close to that of unmodified host cell RNA. Is the host cell type RNA incorporated into the

structure of the filament? It would be very difficult to prove this but the evidence suggests that this is indeed the case. Treatment with high concentrations of ribonuclease did not alter the composition of the nucleic acid. Even after repurification, the breakdown products of filaments degraded by freezing and thawing contained the same amount of nucleic acid, with relatively unchanged properties, as the original Ryan F.F. preparation.

There are four further points which must be considered in any hypothesis concerning the formation of Ryan virus. (1) The spherical particles present in Ryan F. fluids are less efficient as haemagglutinin than are those particles present in preparations of PR8 or the mutant strain, Ryan Sph. viruses. (2) The particle weight of Ryan F. spheres is less than those of spheres in preparations of PR8 or Ryan Sph. (Ada *et al.* 1958). (3) The bulk of these

Fig. 1. A scheme illustrating the mode of formation of spherical and filamentary particles present in preparations of Ryan F. Spherical virus type RNA, ---●; host cell type RNA ■.

Ryan F. spheres are liberated into the allantoic fluid before the long filaments and hence may be formed before the latter. (4) Groups of linearly arranged spheres are often seen (unshadowed micrograph) in Ryan F. preparations. These findings suggest that many of the spheres in Ryan F. fluids are not formed as distinct particles as is known to be the case in a strain such as PR8 virus (Morgan, Rose & Moore, 1956), i.e. the virus particles form at the surface of the membrane and separate cleanly as spherical bodies (Fig. 1*f*). Formation of Ryan F. spheres in this way would not readily explain their low efficiency as haemagglutinin or the smaller particle weight compared with spheres from Ryan Sph. preparations. A more attractive scheme is depicted in Fig. 1*a*–*e*. It is suggested that to a large extent the viral nucleoprotein is channelled into a filamentous structure (Fig. 1*a*); as the latter increases in length, the leading portion breaks up into particles (Fig. 1*b*) which, except for their smaller weight and a probable deficiency in surface components (including haemagglutinin), are similar to those in Ryan Sph. fluids. In some cases the filamentous particle continues to lengthen (Fig. 1*b*, *c*) and incorporation of spherical virus type RNA tapers off. Whether this decreased incorporation is gradual or sporadic, and whether inclusion of host cell type RNA is an attempt to remedy this deficiency, are unknown. There may be sectors which contain a

relative enrichment of spherical virus type RNA as suggested in Fig. 1*d*; breakage of such structures here would yield short filaments or spheres (Fig. 1*e*), many of which would be recovered in Ryan Sup. This interpretation implies that the filament breaks preferentially at those locations where there is an enrichment of viral type RNA. This is not unreasonable as there may be a constriction or weakening of the wall at these places, representing an abortive attempt by the infected cell to form spherical particles.

Does this scheme fit in with current ideas of the structure of filaments? If, for the purpose of discussion, it be assumed that filaments found in the allantoic fluid infected with different strains of virus are basically similar, the more important observations for which we must account are: (1) Filaments may have, particularly at one end, a large round structure called the Archetti body (Archetti, 1954) which may contain the virus nucleoprotein. (2) Sections of infected (Persia F. virus) allantois which were actively producing filaments show in the electron microscope no evidence of internal structure in filaments or structures corresponding to Archetti bodies; in comparable sections of membranes infected with a non-filamentary strain of virus, the virus spheres show an electron-dense core (Morgan, *et al.* 1956). On this basis it was suggested that the spherical form of the virus is the elementary infectious unit and that the filamentary form is largely or completely non-infective. (3) Filaments when treated with acid develop along their length rows of spheres which can be completely digested with trypsin. Spheres treated similarly reveal trypsin-resistant polygonal rings which have been identified as ribonucleoprotein (Valentine & Isaacs, 1957). (4) When subjected to procedures which break up the filament into approximately spherical particles, there is an increase in the haemagglutinin titre but no change in the infectivity titre (Donald & Isaacs, 1954; Burnet & Lind, 1957; this investigation).

These observations may be discussed in order. Classical Archetti bodies are rarely seen in Ryan F. preparations (Ada *et al.* 1958). The presence or absence of electron-dense material in virus structures has restricted meaning until it is correlated with the presence or absence of some chemical fraction. Valentine & Isaacs (1957) have carried out this further step by relating the appearance of specific structures visible in the electron microscope with their susceptibility to different reagents, including ribonuclease. Their evidence does not eliminate the presence of ribonucleoprotein along the length of the filament, but does show (i) that it is not present in as great a concentration as in spheres or (ii) that it is not in the same configuration which, when exposed to acid, causes it to be rearranged into a similar structure to that given by spheres. The final requirement, retention of infectivity following breaking up of the filament into smaller units, is not necessarily at variance with our scheme. It must be stressed that there is little information to indicate why an influenza virus particle may or may not infect the host cell. In the case of spheres it was found previously that 'incomplete' virus had a decreased nucleic acid content compared with the amount found in standard virus (Ada & Perry, 1955; Morzycki *et al.* 1956). On this basis the enhanced nucleic acid content of a filament may

be a contributing factor to the high infectivity. It is not inconceivable that a filament might break down into a number of smaller units containing varying amounts of nucleic acid but perhaps only one or two (presumably those containing sufficient RNA) are capable of inducing continuing infection (Ada, 1957) at the appropriate dilution. It is probable that in this case factors other than nucleic acid are involved.

The suggested mode of formation of spherical particles in cells infected with Ryan F. virus is different from that known for Persia F. virus where many spheres form as discrete particles (Morgan *et al.* 1956) and are as efficient as haemagglutinin as are spheres present in preparations of PR8 or Ryan Sph. viruses (Ada *et al.* 1958). That is, Persia F. virus is a strain where both types of virus production (filamentous and as discrete spheres) occur. It is unknown whether virus particles in the human host are produced as discrete spheres or by a filamentous process, similar to that postulated for Ryan F. virus. Attempts to examine in the electron microscope throat washings from humans suffering from influenza have so far been unsuccessful. The filamentous mode of virus formation may merely represent a transition stage in the adaptation from human host to egg host or it may characterize the production of virus in the human host. The apparent difference lies in the ability or otherwise to seal off the particle immediately after incorporation of the genetic material.

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