Influenza Virus Nucleic Acid: Relationship between Biological Characteristics of the Virus Particle and Properties of the Nucleic Acid

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SUMMARY: Five A strains and three B strains of influenza virus were purified and found to contain ribonucleic acid in amounts varying from 0.75 to 1.1 %. The proportion of the purine and pyrimidine derivatives in the nucleic acid of each strain was determined. When the ratio adenine + uracil : guanine + cytosine was evaluated, the following values were obtained: A strains—PR8, 1.27 ± 0.02 ; MEL, 1.22 ± 0.01 ; WSE, 1.26 ± 0.01 ; SWINE, 1.24 ± 0.04 ; CAM, 1.28 ± 0.01 . B strains—LEE, 1.42 ± 0.04 ; MIL, 1.43 ± 0.05 ; ROB, 1.38 ± 0.01 . The nucleic acid content of PR8 virus preparations of varying degrees of incompleteness was determined both by specific absorption at 260 m μ . and by estimation of phosphorus present in the nucleic acid extract. As measured by both methods, virus preparations of low infectivity were found to have a decreased nucleic acid content. The demonstration of specific differences in the nucleic acid of A and B strains and of the relationship between the infectivity and nucleic acid content of the virus affords strong evidence that the nucleic acid is an intrinsic part of the influenza virus particle.

There can now be little doubt that the nucleic acid present in purified preparations of influenza virus is of the ribose type. Though Knight (1947) found that a sample of nucleic acid isolated from defatted virus was degraded by crystalline ribonuclease, specific chemical evidence has been obtained only recently. As judged by absorption measurements at 260 m μ , it was shown that the nucleic acid could be quantitatively extracted from defatted virus with hot 10 % sodium chloride solutions (Ada & Perry, 1954). A sensitive test for deoxypentoses, when applied to the nucleic acid extract, gave a negative reaction (Ada & Perry, 1954), and the purine-bound pentose was later identified chromatographically as ribose (Ada & Gottschalk, 1956). On treatment with alkali at 37° for 18 hr. the isolated nucleic acid was quantitatively degraded to components which remained in solution on subsequent acidification (Ada & Perry, 1954): deoxypentose nucleic acids are not appreciably degraded under these conditions (Schmidt & Thannhauser, 1945). In preliminary experiments chromatographic analysis of the purine bases and pyrimidine nucleotides, liberated by acid hydrolysis, revealed only four components, coinciding in R_f value with those from a similarly treated sample of purified yeast ribonucleic acid (Ada & Perry, 1955*a*). Liu, Blank, Spizizen & Henle (1954) reported that a hot trichloroacetic acid extract of virus contained adenine, guanine, cytosine and uracil: no thymine was found.

The small amount (0.75–1.1 %) of ribose nucleic acid associated with influenza virus preparations of high infectivity (Ada & Perry, 1954) raises the question whether the nucleic acid is a functionally significant part of the virus

particle. There are two ways in which this might be established. The first would be by the demonstration (as in the deoxyribonucleic acid of T_{even} bacteriophages) of a constituent in the nucleic acid not present in the host cell. Almost equally cogent evidence would be provided by showing that biological differences in strains or preparations of influenza viruses were correlated with changes in the amount or constitution of the virus nucleic acid. Both approaches have been used. The evidence (Ada, unpublished) indicated that the virus RNA contains only the same nitrogenous bases as are present in yeast RNA. On the other hand, the functional significance of influenza virus nucleic acid is indicated by the two sets of observations with which the present paper is concerned: (i) that 'incomplete' virus preparations have a lower content of RNA than the standard virus; (ii) that the ribose nucleic acids from influenza A and B strains show differences in constitution according to serological type. Brief accounts of these findings were reported previously (Ada & Perry, 1955*a, b*).

METHODS

Strains of influenza virus. The following strains of virus were used:

A strains: PR 8 (Francis, 1934); MEL, 'Melbourne' strain (Burnet, 1935); SWINE, Shope's strain 15 of swine influenza (Shope, 1931); WSE (Smith, Andrewes & Laidlaw, 1933); CAM (Burnet & Anderson, 1947).

B strains: LEE (Francis, 1940); MIL, isolated from Melbourne epidemic (Burnet, Stone & Anderson, 1946); ROB, isolated from Melbourne epidemic (see Ledinko & Perry, 1955).

Virus fluids. Virus fluids were obtained from embryonated eggs inoculated in the allantoic cavity on the 11th day of incubation and maintained at 35°.

Standard virus. In early experiments the virus used for inoculation purposes was high titre allantoic fluid stored at -70°. Allantoic fluids were harvested 42 hr. after inoculation of 0.05 ml. of a 10^{-4} dilution of the seed virus. In later work, seed virus was passaged three times (10^{-4} dilution for 20 hr. before production of virus for the main experiment) 0.05 ml., dilution 10^{-6} ; incubation time 42 hr. In this way, inactivated virus was eliminated from the seed virus, resulting in the production of virus of higher infectivity (Horsfall, 1954).

'Incomplete' virus was grown by two or three serial passages of indiluted, infected allantoic fluid (von Magnus, 1946). Incubation times were: first (and second) passage, 16 hr.; final passage, 12 hr. (Fazekas & Graham, 1954).

Samples for haemagglutinin and infectivity estimations were taken immediately after pooling harvested fluids.

Haemagglutinin titration. Serial twofold dilutions of virus were prepared in 0.25 ml. volumes in saline (0.85 %, w/v) and 0.25 ml. of a 1 % suspension of fowl red blood cells was added to each tube. The standard + pattern of cell agglutination was taken as end-point and was read after the cells had settled for 45 min. at room temperature. The reciprocal of the end-point dilution was taken as the titre; all titrations were carried out in duplicate.

Infectivity titration. Serial tenfold dilutions of virus fluids were prepared in ice-cold saline containing 10 % normal horse serum, and 0.05 ml. of each

dilution was inoculated into the allantoic cavity into each of twelve 11-day embryonated eggs. After 3 days of incubation at 35°, the eggs were tested for the presence of virus haemagglutinin in the allantoic fluid. ID 50 end-points were calculated by the method of Reed & Muench (1938). For comparative purposes, the ratio I/A is used, where $I = \text{ID 50}$ and $A = \text{haemagglutinin titre}$.

Purification of virus. All virus preparations were purified according to the same procedure, comprising specific adsorption to and elution from human red blood cells followed by differential centrifugation. Two modifications were introduced in the method published earlier (Ada & Perry, 1954): (i) to facilitate elution of the adsorbed virus from the erythrocyte surface, approximately 20,000 units of a receptor-destroying enzyme preparation (Ada & French, 1950) were added to the eluting fluid; (ii) conditions of centrifugation in the centrifugation cycles were changed to the following: sedimentation, 38 000 g, 30 min.; clarification, 7000 g, 10 min. The yield of purified virus was expressed as the percentage recovery of haemagglutinin.

Purity of virus. The purity of the preparations was judged by examination in the ultracentrifuge and electron microscope (see the following paper, Pye, Holden & Donald, 1956) and assessed by determination of the ratio, haemagglutinin units/mg. dry weight.

Extraction of nucleic acid from virus. The purified virus was precipitated by the addition of trichloroacetic acid, dialysed against distilled water and dried from the frozen state *in vacuo*. After defatting the dried virus with chloroform + methanol (2 : 1) the nucleic acid was quantitatively extracted from the defatted virus with hot 10 % (w/v) NaCl solution (Ada & Perry, 1954). Addition of 2 vol. of ethanol to the extract precipitated the nucleic acid which was washed with 66 % ethanol, 95 % (v/v) ethanol in water, and finally with freshly distilled ethyl ether.

Estimation of virus nucleic acid (VNA). The nucleic acid content of the 10 % sodium chloride extract was estimated in two ways. The specific absorption at 260 m μ . was determined. The formula, $\mathcal{E}_p = 30.98 E/cl$ where $\mathcal{E}_p$ = atomic extinction coefficient at 260 m μ . with respect to phosphorus, E = optical density, c = concentration of phosphorus in g./l., and l = thickness of absorbing layer (Chargaff & Zamenhof, 1948) was used to determine spectrophotometrically the nucleic acid content. The value $\mathcal{E}_p = 9200$ was experimentally determined on VNA reprecipitated as above and dissolved in water. The factors 9.7 for A strains and 9.6 for B strains, evaluated from the known proportions of bases (Ada & Perry, 1955*b*), were applied to convert the phosphorus value so obtained to amount of VNA. In this calculation of $\mathcal{E}_p$, no account is taken of possible effects of heating RNA and subsequently measuring the optical density in 10 % NaCl. To investigate this, a sample of RNA was prepared from guinea-pig liver by the method of Kay & Dounce (1953). The purified preparation contained 8.4 % phosphorus and had an $\mathcal{E}_p$ (in water) of 9300. Approximately 1.5 mg. was dissolved in 10 ml. water; one of two 1 ml. samples was added to 1 ml. water and the other to 1 ml. 20 % NaCl solution. The optical density reading in 10 % NaCl was 3 % lower than that in water (see Shack,

Jenkins & Thompsett, 1953). Both solutions, in sealed tubes, were placed in a boiling water-bath for 20 min., cooled and the optical density measured. An increase of *c.* 1.5 % occurred in both cases. Thus the depression of the optical density in 10 % NaCl is largely cancelled by the increase following heating. In view of this result, the value $\mathcal{E}_p=9200$ for VNA as determined above was used uncorrected. A direct determination of phosphorus in the sodium chloride extract and conversion of this value to nucleic acid amount using the appropriate factor given above was also made. Phosphorus determinations were carried out as previously described (Ada & Perry, 1954).

Yeast nucleic acid (YNA). A commercial preparation of yeast ribose nucleic acid was purified according to the procedure of Smith & Markham (1950).

Paper chromatography

Hydrolysis of nucleic acid. 50–250 μg . nucleic acid (purified YNA or reprecipitated VNA) was hydrolysed with 50 μl . N-HCl (100°, 60 min.) in a sealed tube. 40–50 μl . of the hydrolysate was applied to paper. Whatman no. 1 paper, washed according to the method of Hanes, Hird & Isherwood (1952), was used in ascending chromatographic analysis. The width of the paper strips varied from 0.7 to 2.0 cm. according to the amount of nucleic acid (as hydrolysate) applied. The solvent used was isopropanol + conc. HCl + water in the proportions 65 : 18 : 17 (Wyatt, 1951). The solvent front moved approximately 26 cm. in 18 hr.

Detection and estimation of the separated purine and pyrimidine derivatives

The paper was air-dried, the components located by their absorption in the ultraviolet light, and in each case a photographic record was made (Markham & Smith, 1951). The ultraviolet-absorbing spots were cut out (with corresponding areas from a blank strip) and the purine and pyrimidine derivatives eluted for 18 hr. at room temperature with 10 ml. 0.1 N-HCl (Smith & Markham, 1950). The optical density of the eluates contained in 4 cm. cuvettes was read at the wavelength of maximal absorption for each derivative, and the concentration determined by applying the appropriate extinction coefficient (Markham & Smith, 1951).

With every chromatographic analysis of a VNA hydrolysate, a YNA hydrolysate was run as a control. Unless the values obtained for the proportions of bases (expressed as the ratio adenine + uracil : guanine + cytosine) agreed within ± 2.5 % of a mean value, 0.90, obtained in a preliminary series of experiments, the result of the accompanying VNA analysis was discarded. Analytical results for the distribution of bases in incomplete virus may not have this accuracy because of the small amounts (less than 70 μg .) of nucleic acid which were examined. In several experiments, the recovery of phosphorus (evaluated from base recoveries, assuming equimolar amount of bases and phosphorus) from the chromatogram was compared with the known amount of phosphorus applied. Recoveries between 100 and 103 % were obtained with both VNA and YNA.

RESULTS

Infectivity of virus preparations. The production of standard virus (PR 8 strain) from glycerolated seed resulted in preparations with an average I/A value of 5.7 ± 0.4 . In later experiments in which the virus to be used as seed was previously passaged several times at high dilution, the average I/A value of the unpurified virus (PR 8) was 6.0 ± 0.3 . Other strains of virus, produced by a single passage from dilute inoculum, gave the following average I/A values: strains; MEL, 6.1; CAM, 6.0; WSE, 5.6; SWINE, 5.9; strains ROB, 6.5; MIL, 5.6; LEE, 6.0. The results given by preparations of incomplete virus (PR 8) were as follows: two passages of undiluted inoculum, $I/A = 4.3 \pm 0.4$; three passages of undiluted inoculum, $I/A = 3.8 \pm 0.4$.

Yield of purified virus. Similar yields were obtained with all the A strains used, irrespective of the infectivity of the starting material. Two B strains, LEE and MIL, were recovered in lowest amounts. Few losses occurred during the purification of the B strain ROB as indicated by the high recovery of haemagglutinin. The average yields, together with the ranges, are given in Table 1.

Table 1. *The yield, haemagglutinating activity (haemagglutinin units/mg. dry weight) and nucleic acid content of some A and B strains of influenza virus*

Virus*		No. of experiments	Yield (%)	Haemagglutinin units per mg. dry weight ($\times 10^{-4}$)	Nucleic acid content† (%)
A	PR 8	9	$47 \pm 16^{\ddagger}$ (27-73)	9.2 ± 1.6 (6.9-11.2)	See Figs. 1 a and b
	PR 8§	5	47 ± 13 (40-66)	10.7 ± 1.5 (10.0-12.1)	
	PR 8	5	36 ± 14 (21-57)	7.2 ± 0.8 (6.2-8.0)	
	MEL	2	38 (30-46)	9.4 (7.2-11.6)	0.84 (0.85-0.82)
	CAM	2	33 (22-43)	8.1 (6.4-9.7)	0.88 (0.94-0.81)
	WSE	2	41 (32-50)	6.6 (6.4-6.9)	0.85 (0.81-0.89)
	SWINE	2	37 (26-48)	7.5 (7.0-8.1)	0.79 (0.75-0.83)
B	ROB	2	80 (70-90)	9.7 (8.1-11.3)	0.94 (0.87-1.01)
	MIL	3	18 (15-25)	2.9 (2.3-3.4)	0.80 (0.75-0.83)
	LEE	2	24 (20-28)	3.5 (2.8-4.3)	0.94 (0.94-0.94)

* With two exceptions, viruses grown by single passage from dilute inoculum.

† Estimated by absorption in the ultraviolet.

‡ S.D. = $\sqrt{\frac{\Sigma(\bar{x} - x)^2}{n - 1}}$.

§ Two serial passages of undiluted inoculum.

|| Three serial passages of undiluted inoculum.

Purity of virus preparations. A comparison of the ratio, haemagglutinin units/mg. dry weight, indicated that the values of all the A strains and the B strain ROB fell within the range $6-12 \times 10^4$. The two B strains, LEE and MIL, consistently gave values of a lower order ($2.3-4.3 \times 10^4$) (Table 1).

Nucleic acid content. In earlier work the nucleic acid content of the A strain PR8 (standard preparations) was found to be c. 0.8% as estimated by ultra-violet absorption (Ada & Perry, 1954). It is clear from Table 1 that all of the other strains of virus tested contain similar amounts of nucleic acid.

Relationship between the infectivity and nucleic acid content of virus. The nucleic acid content of PR8 virus preparations, whose I/A values ranged from 6.5 to 3.2, was determined both by specific absorption at 260 m μ . and by estimation of phosphorus present in the nucleic acid extract. The values obtained are plotted in Fig. 1*a*, *b*, the nucleic acid content being expressed as %/mg. dry weight of virus. It is evident that, as measured by both methods, the virus preparations of low infectivity had a decreased nucleic acid content.

Figs. 1*a* and *b*. Relationship between the infectivity/haemagglutinin ratio and the nucleic acid content of influenza virus. $\times$ = single passage (0.05 ml., ID 50 = 10^{-4} – 10^{-6} , 42 hr.); $\circ$ = double passage (each 1.0 ml. undiluted fluid); $\bullet$ = triple passage (each 1.0 ml. undiluted fluid). Fig. 1*a*. Nucleic acid content estimated by absorption at 260 m μ . of nucleic acid extract. Fig. 1*b*. Nucleic acid content estimated from phosphorus content of nucleic acid extract.

Proportions of nitrogenous bases of the nucleic acid isolated from virus preparations (PR8) of varying infectivity. It was thought possible that some indication of the specificity of the missing components in incomplete virus might be obtained by a comparison of the base proportion in the nucleic acid isolated from incomplete virus with the values of the nucleic acid from standard virus. In each case, the nucleic acid was hydrolysed, chromatographed and the amount of purine and pyrimidine derivatives estimated. The results (Table 2) indicate that in two preparations of low infectivity ($I/A = 4.5$, 3.9), the values were within experimental error of those found in preparations of high infectivity.

Specific differences in the nucleic acids from A and B strains of virus. Five A strain and three B strain viruses were analysed for their base proportions as described above (Table 3). It can be seen that, in general, the guanine content

of the B strains is lower than that of the A strains; the uracil content of the A strains is lower than that of the B strains; and both strains have similar amounts of cytosine. Comparison of the values of the ratio adenine + uracil : guanine + cytosine indicates that an A strain virus can be sharply differentiated from a B strain virus. The difference between the means of any pair of A and B viruses is significant (*t* test, $P < 0.05$). Differences of the mean values within each category are not significant ($P > 0.1$).

Table 2. *Proportions of bases in the nucleic acid of preparations of influenza virus (PR8) having varying infectivity/haemagglutinin ratios*

Virus PR 8 (A)	<i>I/A</i>	No. of experi- ments	Ratio of bases referred to adenine				Ratio: adenine + uracil guanine + cytosine
			Adenine	Guanine	Cytosine	Uracil	
	6.5-5.6	5	10	8.7 ± 0.2	10.4 ± 0.5	14.2 ± 0.13	1.27 ± 0.02
	4.5	1	10	8.5	10.2	13.7	1.27
	3.9	1	10	8.8	10.6	14.0	1.25

Table 3. *Proportions of bases in the nucleic acids of A and B strains of influenza virus*

Virus strain		No. of experi- ments	Ratio of bases referred to adenine				Ratio: adenine + uracil guanine + cytosine
			Adenine	Guanine	Cytosine	Uracil	
A	PR 8	5	10	8.7 ± 0.2	10.4 ± 0.5	14.2 ± 0.13	1.27 ± 0.02
	MEL	2	10	8.3, 8.8	10.7, 11.3	13.3, 14.5	1.22 ± 0.01
	WSE	2	10	8.9, 8.9	10.8, 10.5	15.0, 14.3	1.26 ± 0.01
	SWINE	2	10	9.1, 8.8	10.9, 10.6	14.1, 14.5	1.24 ± 0.04
	CAM	2	10	8.5, 8.5	10.9, 10.7	14.7, 14.8	1.28 ± 0.01
B	LEE	4	10	8.0 ± 0.5	10.0 ± 0.3	15.5 ± 0.5	1.42 ± 0.04
	MIL	3	10	7.7 ± 0.2	10.4 ± 0.3	15.8 ± 0.2	1.43 ± 0.05
	ROB	2	10	8.4, 8.2	10.4, 10.4	15.7, 15.9	1.38 ± 0.01

DISCUSSION

A comparison of the results of chemical analyses carried out on different virus preparations will be valid only if each substrain or type can be obtained in a 'pure' state. In the present investigation, the degree of purification of each preparation was estimated by determination of the ratio haemagglutinin units/mg. dried weight for each purified preparation and by examination of the purified virus in the ultracentrifuge and less frequently by the electron microscope (see the following paper, Pye, Holden & Donald, 1956). By the first criterion, the figures in Table 1 suggest that all the A strain viruses had been purified to an equal extent. The ultracentrifuge patterns of the individual virus preparation generally revealed a single boundary; in some cases, a small amount of a more slowly sedimenting component was present. As the surface characteristics of incomplete virus are indistinguishable from those of standard virus (von Magnus, 1954), the similar haemagglutinating activity of the purified preparations of incomplete and standard virus in the present investigation strongly suggests that a comparable degree of purification had been achieved

in each case. Comparison of the ultracentrifugal diagrams in this series might not furnish a clear indication of purity because of the more complex pattern frequently given by incomplete virus. While the recently isolated B strain ROB had a haemagglutinating activity similar to those of A strains, the low values of the well-established B viruses, MIL and LEE may cast some doubt on the purity of these preparations. However, the consistently low values of MIL and LEE, when considered together with the ultracentrifugal evidence of a single sedimenting boundary, favour the concept that the low haemagglutinating activity is an intrinsic property of the virus particle. Preparations of virus examined by the electron microscope showed no appreciable contamination with obviously non-viral material. The available evidence suggests therefore that the purified virus preparations which were chemically analysed did not contain significant amounts of extraneous material.

The two species of influenza virus A and B used in this study are differentiated primarily by their serological behaviour. The clearest difference is in terms of the soluble complement-fixing antigen (c.f.a.) which is group specific, all A strains having a c.f.a. sharply distinct from that produced in the course of infection with a B strain. The antigens of the virus surface are more complex and the A strains in particular show very wide serological differences. In general, however, there is some cross-reaction between all A strains, while the antigenic diversity in the B strains is considerably less. There are other reasons too for thinking that the difference between the A and B species is a real one, notably the failure to obtain any evidence of recombination (Perry & Burnet, unpublished). The results of the chemical analyses are in broad agreement with the biological findings. There was a consistent and reproducible difference between the values of the ratio adenine + uracil : guanine + cytosine of the five A and three B strains tested. On the other hand, in no case were the differences between mean values within either the A or the B strains significant. If fine differences between the values of this ratio do exist within each species, it appears unlikely that the present technique, when applied to very small amounts of nucleic acid, would detect them.

With both plant and bacterial viruses examples have been found where infection is associated with the formation of particles which, though closely related (either serologically, chemically or morphologically) to the corresponding virus particle, are non-infectious. In each of the instances which have been adequately studied, the non-infectious component(s) contains little or none of the nucleic acid associated with the infectious virus particle (Markham, 1951; Jeener & Lemoine, 1953; Rich, Dunitz & Newmark, 1955; see Epstein, 1953).

A more complex situation is found with the influenza virus. The conditions giving rise to 'incomplete' virus, i.e. virus in which infectivity titrations give lower values than would be expected from haemagglutinin titres or particle counts by electron microscopy, have been discussed by numerous workers (von Magnus, 1954; Fazekas & Graham, 1954; Finter, Liu & Henle, 1955; Burnet, 1955). Here it need only be noted that exposure at 35° will also lower the infectivity titre without changing haemagglutinating activity. For this

reason precautions were taken to ensure that the virus did not remain long in the egg after its production. It can be assumed that the virus preparations used contained only a small proportion of thermally degraded virus. Even so there was considerable variation in the infectivity of virus preparations of similar nucleic acid content and in the nucleic acid content of virus preparations of similar infectivity. Though the same trend is shown independently of the method of nucleic acid determination, the estimates given by phosphorus analyses are higher than those evaluated from ultraviolet absorption measurements, due probably to the presence in the salt extract of small amounts of other organic phosphates. Clearly there is no simple proportionality between infectivity and nucleic acid content; a ninety-nine-fold decrease in infectivity was accompanied by approximately a twofold decrease in nucleic acid content. It should be mentioned that in a more limited series of experiments, it was found (Donald & Ada, unpublished) that essentially the same relationship was present when the I/A ratio was plotted against the nucleic acid content calculated on a virus particle basis. It may well be that only virus particles with a nucleic acid content above a certain value can induce continuing infection. With decrease in the amount of nucleic acid in the parent particle there will be a progressively greater decrease in the proportion of infectious progeny produced and at a certain value of nucleic acid amount, only non-infectious haemagglutinin will be produced. Such an interpretation is consistent with the concept of intermediate grades of incompleteness (Burnet, Lind & Stevens, 1955), based on the observation that incomplete virus (WS strain) produced a much higher yield of haemagglutinin than would be expected from its infectivity titre, as determined by limit dilution in the embryonated egg. Until much more is known of the process of virus replication, it would be premature to attempt a more detailed interpretation of these relationships. The fact that the proportion of bases is not significantly different in standard and incomplete virus perhaps suggests that the decrease in nucleic acid content is determined by some random process but by no means excludes the possibility that a specific moiety of the nucleic acid is concerned in determining infectivity.

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