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The Component Sugars of the Influenza-Virus Particle

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The great amount of information obtained over the last 15 years about the biological activities of the influenza virus (Burnet, 1951) stimulated at an early stage detailed biophysical and biochemical investigation of this organism. Thus the particle size and shape, electrochemical behaviour (Stanley, Knight & De Merre, 1945) and enzymic properties (Gottschalk, 1954) have been intensively studied. The virus particle is known to contain four main constituents: protein, lipid, carbohydrate and nucleic acid. Taylor (1944) found for the A strain (PR8) of influenza virus a total carbohydrate content of 12.5% (orcinol method with glucose as standard), made up, on the basis of the carbazole reaction, of mannose or glucose-galactose, or both. According to Knight (1947) the total carbohydrate of the PR8 strain, as determined by orcinol with glucose as standard, is about 7%. Of this, ribose was considered to constitute approximately one-tenth, the remainder being incorporated in a polysaccharide consisting of galactose, mannose and hexosamine, as indicated by the carbazole and Elson-Morgan reactions. The identification by Taylor and Knight of the constituent hexoses is based on a technique involving, for a mixed polysaccharide, the interpretation of superimposed absorption curves. The uncertainty of such an interpretation has been previously discussed (Gottschalk & Ada, 1956). For hexosamine no attempt at identification was made. The pentose nature of the nucleic acid sugar was ascertained by

Bial's orcinol reaction, and its identity as ribose inferred from the observation that a sample of isolated nucleic acid was degraded by ribonuclease. Recently, the pentosenucleic acid content of the virus has been reinvestigated with improved techniques and a value of about 1% for highly purified and infective PR8 virus obtained; no significant amount of deoxyribosenucleic acid was found (Ada & Perry, 1954). In the preceding paper (Gottschalk & Ada, 1956), a method has been described allowing the identification and determination in mucoproteins and nucleic acids of the individual sugars after their separation from the non-carbohydrate moiety and after their chromatographic isolation. The desirability of having accurate information about the nature and amount of the sugar components of the influenza virus prompted a reinvestigation of the carbohydrate of the virus particle with the new technique.

MATERIALS AND METHODS

Influenza virus. The strain A (PR8) was used throughout; it was purified by specific adsorption to and elution from human erythrocytes, followed by two cycles of differential centrifuging in an ultracentrifuge. The purity of the virus preparation was judged by examination in the electron microscope and assessed by determination of the ratio haemagglutinin units: mg. dry wt. Details have been described previously (Ada & Perry, 1954).

Virus nucleic acid. The defatted, dried virus was extracted with hot 10% (w/v) NaCl solution and the nucleic

acid precipitated by adding 2 vol. of ethanol to the extract. In view of the small amounts available the quantity of nucleic acid used in experiments was calculated from the molecular extinction coefficient (see below).

Mucoprotein fraction from normal allantoic fluid. Normal allantoic fluid was harvested from 12-day-old embryonated hen eggs. The liquid was clarified by centrifuging (2000 g, 15 min.), and the supernatant, after dialysis against distilled water for 48 hr. at 4°, dried from the frozen state *in vacuo*. The dried material was taken up in a minimum amount of water, redialysed and freeze-dried as above. When redissolved, this material, though free of reducing sugar, gave a positive Molisch test, and it was brought out of solution by protein precipitants (sulphosalicylic acid, trichloroacetic acid) and by heating.

Ion-exchange resin. Dowex 50 (200–400 mesh) was prepared as a hydrolysing agent as described by Dixon (1955).

Determination of nucleic acid. The nucleic acid was determined spectrophotometrically by applying the formula $\epsilon_P = 30.98 E/cl$, where ϵ_P represents the atomic extinction coefficient at 260 m μ . with respect to phosphorus and found to be 9200 (Ada & Perry, in preparation), E the optical density ($\log I_0/I$), c the concentration of P in g./l. and l the thickness of the absorbing layer (Chargaff & Zamenhof, 1948). The factor 9.7, calculated from the known proportion of bases (Ada & Perry, 1955b), was applied to convert the phosphorus value into nucleic acid concentration.

Determination of total carbohydrate (non-amino sugar). As shown earlier (Gottschalk & Ada, 1956), the orcinol method, when carried out with the correct standard, is satisfactory for the determination of total carbohydrate in carbohydrate-protein complexes. In a few cases the total carbohydrate content was also estimated by the tryptophan method (Badin, Jackson & Schubert, 1953); values given by the two methods agreed within 5%. Each determination was carried out on 3–4 mg. of dried virus.

Determination of component non-amino sugars. The component sugars of 20–25 mg. of the dried virus were released by hydrolysis (0.25N-H₂SO₄, 18 hr., 100°), unhydrolysed protein and liberated amino acids removed respectively by precipitation with ethanol and treatment with ion-exchange resin, and the purified sugars were isolated and quantitatively estimated by chromatography (Gottschalk & Ada, 1956). An average recovery of 80% (the orcinol value of the original viral material being taken as 100%) of the component sugars was obtained by this technique. This recovery must be regarded as very satisfactory, considering that about 5% of the orcinol-reacting material was left behind after hydrolysis in an insoluble fraction of the virus preparation.

The purine-bound sugar present in virus nucleic acid was liberated by hydrolysis with N-H₂SO₄ at 100° for 1 hr. and then processed as described previously (Gottschalk & Ada, 1956).

Determination of amino sugars. Hydrolysis of the virus preparation (3–4 mg.) and purification and estimation of the liberated amino sugars were carried out as reported elsewhere (Gottschalk & Ada, 1956).

Identification of non-amino sugars. The component sugars were identified by comparison with a set of standard sugars, run on paper in four suitable solvents (Gottschalk, 1955). Since the sugars found to be present in influenza

virus were those most frequently occurring in carbohydrate-protein complexes, no sugar derivatives for identification by melting point were prepared.

Identification of amino sugars. The amino sugars were identified by their conversion into the corresponding pentoses, followed by chromatographic analysis (Gardell, Heljenskjöld & Rochnovlund, 1950). This identification procedure was checked by chromatographic separation of the purified hexosamines, a technique which also allowed an estimate of the approximate proportion of glucosamine and galactosamine (Gottschalk & Ada, 1956). About 5 mg. of dried virus was used for this estimation.

RESULTS

The sugar component of virus nucleic acid. The purine-bound sugar present in virus nucleic acid, after liberation and purification, was chromatographed together with arabinose, xylose, lyxose and ribose in four different solvents (Gottschalk, 1955), all affording satisfactory separation, and a single spot obtained coinciding in each case with the ribose standard in both R_F value and colour. Quantitative determination of the isolated ribose by the chromatographic method gave in a total of three experiments a mean value amounting to $92 \pm 9\%$ of the theoretical value calculated from the purine content of the virus nucleic acid. In a representative experiment on two portions (0.20 mg.) of nucleic acid the following values were obtained. In one portion 35 μ g. of ribose was found; in the other, 20 μ g. of adenine and 17 μ g. of guanine, equivalent to 39 μ g. of ribose (expressed as free pentose) were present. This result compares with that previously found for yeast nucleic acid (Gottschalk & Ada, 1956).

Since the quantity of virus nucleic acid available did not allow identification of the pyrimidine-bound pentose by its conversion into the corresponding acid (Levene & La Forge, 1912), an attempt was made to hydrolyse the *N*-glycosidic linkages with the hydrogen form of a sulphonated ion-exchange resin, conditions which would ensure the survival of the liberated sugar for an extended period (see Dixon, 1955). Virus nucleic acid (0.12 mg. in 0.20 ml. of water) was heated for 100 hr. at 100° in the presence of 25 mg. of resin. After separation of the resin the solution was concentrated to dryness at about pH 6 and the residue analysed by chromatography. The amount of sugar recovered and identified as ribose (as above) was 10 μ g., accounting for no more than 25% of the theoretical calculated from the purine and pyrimidine base content (see above). This low figure would suggest that not only, as in mild acid hydrolysis, are the cytosine- and uracil-*N*-ribosides unaffected by the resin, but that even part of the purine-*N*-ribosides escapes hydrolysis with the latter as catalytic agent.

Non-amino sugars (other than ribose) of the virus particle. The total carbohydrate content of the virus particle, as estimated by the orcinol method, was found to vary between 3.0 and 5.6%, the mean value of the results from experiments with nine different virus batches being 4.6 (see Table 1). Of this amount, 6% is accounted for by ribose; the remainder is distributed between galactose, mannose and fucose in the proportion 10:6.4:5.3.

Amino sugars of the virus particle. The total amino sugar content of six different batches of influenza virus varied between 2.2 and 3.3%, with a mean value of 2.6%. Glucosamine was the only amino sugar (see Table 1).

In the six batches of virus in which both the non-amino sugar content and the amino sugar content were determined the value of the non-amino sugar: amino sugar ratio was calculated for each individual batch and found to vary between 2.0 and 1.1, with a mean value of 1.6.

The carbohydrate moiety of the mucoprotein fraction of normal allantoic fluid. The mucoprotein fraction prepared from normal allantoic fluid has been analysed, with the results tabulated.

DISCUSSION

The evidence presented leaves little doubt that the pentose isolated from the purine nucleotides of the virus nucleic acid is ribose. The hope that the pentose linked to the pyrimidines might be detached by a cation-exchange resin was not substantiated. However, from the ribose nature of the purine-bound pentose of virus nucleic acid, from the ribose identity of the purine- and pyrimidine-bound pentose in yeast nucleic acid (Levene & La Forge, 1912) and from the identity of the bases in yeast ribose and virus pentose nucleic acid (Ada & Perry, 1955*a*), it seems most likely that the pyrimidine bases of virus nucleic acid are also linked to ribose.

The spectrum of the remainder of the virus carbohydrate, consisting of galactose, mannose, fucose and glucosamine, closely resembles that of neutral mucoproteins (Gottschalk & Ada, 1956). Indeed, no heteropolysaccharide of such a composition is known to exist in nature unless bound to protein. Though the proportion of the individual non-amino sugars is remarkably constant, the total amount of non-amino sugar and of amino sugar varies appreciably and independently from batch to batch of virus. The variation in the ratio non-amino sugar:amino sugar may reflect the variation from virus to virus in the carbohydrate composition of a single mucoprotein or the presence in the virus particle of two or more different mucoproteins.

Table 1. *Analysis of the carbohydrate of the influenza-virus particle and of the mucoprotein fraction of the normal allantoic fluid*

Material (1)	Total carbohydrate content* (non-amino sugar) (%) (2)	Ribose content† (%) (3)	Proportion of non-amino sugars			Total hexosamine content (%) (7)	Proportion of amino sugars		Non-amino sugar* Amino sugar (10) (6) 1.6±0.3 1.4
			Galactose (4)	Mannose† (5)	Fucose† (6)		Glucos- amine (8)	Galactos- amine (9)	
Influenza-virus particle	4.6±1.0§ (9)	0.28±0.04 (3)	10.0 (6)	6.4±0.6 (6)	5.3±0.5 (6)	2.6±0.4 (6)	10 (3)	0 (3)	1.6±0.3 (6)
Mucoprotein fraction of allantoic fluid	3.8	0	10	15	8	2.8	10	1	1.4

* Orcinol value.

† Evaluated from the ribose recovered from the purine nucleotides and assuming the ribose nature of the pyrimidine-bound pentose.

‡ Referred to galactose.

§ Standard deviation = $\sqrt{\frac{\sum(\bar{x} - x)^2}{n - 1}}$.

|| Figures in brackets refer to the number of experiments.

The percentage values given are expressed as anhydro sugars. The virus figure in the last column is not computed from the means as given in columns 2 and 7, but is calculated from the figures of each individual batch of virus.

As may be seen from Table 1, the mucoprotein fraction of the allantoic fluid, which derives from potential host cells of the virus, contains the same sugars as are present in the virus particle. This observation, taken together with the variation of the carbohydrate content of the virus, raises the question whether or not the mucoprotein present in the virus preparation is a contaminant or an integral part of the particle. The nature of the virus purification process, including specific adsorption to and elution from red cells, eliminates the possibility of a contaminant unassociated with the virus particle. A contaminant associated with the virus by adsorption to its surface seems to be excluded on quantitative grounds; even with a low protein content (e.g. 50%) such a contaminant would entirely cover the particle surface, a situation incompatible with the known enzymic and antigenic properties of the virus. The mucoprotein is therefore considered to be an integral part of the virus particle. It remains to be determined, however, whether the virus exerts a genetic control over the pattern and amount of the mucoprotein or whether its presence in the virus is merely a necessary concomitant of the process by which new virus particles are produced. Whether gene-controlled or not, the general resemblance of the virus mucoprotein to that produced by the non-infected cell would suggest that the same basic mechanism operates in both cases.

SUMMARY

1. The purine-bound pentose of influenza virus nucleic acid has been identified chromatographically as ribose.

2. The remainder of the virus carbohydrate, consisting of galactose, mannose, fucose and glucosamine, is built into a polysaccharide considered to be the carbohydrate moiety of a mucoprotein.

3. Though the proportion of the component non-amino sugars is fairly constant, the total amounts of non-amino sugar and of amino sugar vary appreciably and independently.

4. The potential host cells of the virus were found to produce mucoprotein with a carbohydrate moiety of similar composition to that of the virus.

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Liver Glycerylphosphorylcholine Diesterase

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Recent isotopic experiments have led to the conclusion that free glycerylphosphorylcholine (GPC) and glycerylphosphorylethanolamine (GPE) occur in liver tissue as intermediates in the catabolism of phosphatidylcholine and phosphatidylethanolamine respectively (Dawson, 1955). This being so, it appeared likely that an enzyme would be present in the tissue which was capable of further hydrolysing these phosphodiester. The present paper describes some of the properties and distribution of such an enzyme, which catalyses the hydro-

lysis of the diesters into glycerophosphoric acid and the free base. Some of the preliminary observations on the enzyme were made in collaboration with Mr A. Bowyer in 1954.

Contardi & Ercoli (1933) reported that the product obtained by the action of lecithinase B on lecithin was acted on by a further enzyme present in kidney, intestine and pancreas, which liberated choline. The enzyme concerned, however, appears to differ from the present enzyme in having a pH optimum of 4 (cf. 7.5) and in being inhibited by