THE NUCLEIC ACID AND CARBOHYDRATE OF INFLUENZA VIRUS

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The viral diseases of plants and of animals would seem to have a common chemical background in that the causal agents which have been isolated and purified thus far appear to consist wholly or in part of nucleoprotein (7–9). The purified viruses obtained from diseased plants have been found to contain ribonucleic acid (7); an *Escherichia coli* bacteriophage has been reported to consist in part of desoxyribonucleic acid (10); and the animal viruses examined have been found to contain either ribonucleic or desoxyribonucleic acid (11).

In recent reports, the results of appropriate color tests have suggested that highly purified preparations of influenza virus contain nucleic acid, and that influenza virus contains both ribonucleic and desoxyribonucleic acids (3, 4, 6), a possibility unique in studies on viruses until now. In view of the repeatedly emphasized importance of nucleic acids in biological systems, it appeared desirable to obtain direct evidence as to the presence of nucleic acid in influenza virus by isolation, and if it is present to determine by means of appropriate tests and analyses something of its nature. In part the present report describes the isolation of, and the results of tests made on, the nucleic acid of PR8 influenza virus.[1]

Investigations on highly purified preparations of influenza virus have revealed the presence of an amount of carbohydrate apparently greater than that accountable for in the nucleic acids (4, 6). The nature of this carbohydrate had not been determined, although preliminary tests made by Taylor on the Lee strain led him to suggest the possible presence of mannose or glucose-galactose in complex form, or both (6). In the present investigation, carbohydrate-rich fractions were obtained from highly purified PR8 and Lee influenza viruses and subjected to analysis.

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[1] Throughout this paper the term influenza virus will be used to designate highly purified particulate preparations of the virus.

Methods and Findings

Preparation of Virus.—An isolation of nucleic acid was made from each of 2 lots of virus. Lot 1 consisted of a pool of virus obtained from infectious allantoic fluid by means of differential centrifugation (2) employing periods of 15 minutes at 24,000 R.P.M. in the high-speed centrifugation runs. The material was handled in 0.1 M phosphate buffer for 1 to 2 cycles and in distilled water for 2 more cycles of centrifugation. The final product was dried from the frozen state and further dried *in vacuo* over P_2O_5 at 65°C. before use. Lot 2 was obtained from infectious allantoic fluid by a combination of the methods of differential centrifugation and adsorption on and elution from chicken red cells as follows: The virus was concentrated and partially purified by means of the Sharples centrifuge (2). This concentrate was diluted with 0.1 M phosphate buffer at pH 7 until it contained about 400 to 500 standard CCA units per ml. (12). Enough red cells were then added to the virus solution at 4°C. to bring the final red cell concentration to about 4 per cent. After about an hour the agglutinated cells had settled to the bottom of the flask and most of the supernatant fluid was removed by decantation. The remaining supernatant fluid was separated from the cells by centrifuging the mixture at 3500 R.P.M. for 3 minutes and decanting. The cells were then suspended in phosphate buffer and the virus was eluted by incubating at 37° for 90 minutes with occasional swirling. The red cells were removed from the mixture by centrifuging at 5000 R.P.M. for 3 minutes. The virus in the red cell eluate was further purified by 3 cycles of differential centrifugation, one of which was made in phosphate buffer and the final 2 in distilled water. The material was then frozen and dried as described above.

Isolation of Nucleic Acid; Preparations 1 and 2.—It seemed possible that the considerable amount of lipid in influenza virus (4, 6) might interfere with the isolation of nucleic acid. Therefore, the lipid was extracted from the virus prior to the alkaline treatment customarily employed in the separation of nucleic acid from nucleoprotein (13).

Three thousand eight hundred and twenty mg. of dry virus (lot 1) in a 250 ml. centrifuge tube were mixed for about 5 minutes by means of a mechanical stirrer with 200 ml. of 3:1 alcohol-ether at room temperature. The insoluble material was separated by centrifugation and was extracted again with a fresh portion of alcohol-ether. A final extraction was made with ether alone after which the insoluble material was dried at 60°C. Two thousand nine hundred and thirty mg. of lipid-free material were obtained and 100 mg. of this were retained for analyses. The remainder was stirred at 4°C. with 40 ml. of 5 per cent sodium hydroxide. Rather than the usual 2 hour period of treatment, 4½ hours were required to get all of the material in apparent solution. The solution was made slightly acid to litmus by the addition of 4 ml. of glacial acetic acid and the precipitate obtained was removed by centrifugation. An additional small amount of precipitate was removed from the supernatant fluid after sufficient alcohol had been added to bring the concentration to 4 per cent. To the remaining opalescent solution was added concentrated hydrochloric acid until the solution was acid to Congo red and finally an equal volume of 95 per cent alcohol was added. At this point so large an amount of precipitate was obtained that it was apparent that the separation of nucleic acid from other materials had been incomplete.[2] Therefore, the precipitate was dissolved in a

[2] The failure to get at once the usual separation of nucleic acid from protein by the above procedure seems difficult to explain although the observed close association of more than 5 per cent of carbohydrate with the protein fraction and perhaps also with the nucleic acid, suggests that the carbohydrate imparts an abnormal solubility to the protein, a solubility which causes the protein to accompany the nucleic acid fraction rather than to become separated from it.

minimal amount of 5 per cent sodium hydroxide and the Johnson-Harkins fractionation procedure was repeated. This time most of the material precipitated out upon neutralization of the alkali and was left behind. Nucleic acid was obtained from the sparkling clear supernatant fluid by acidification and addition of alcohol. The nucleic acid was washed twice with 95 per cent alcohol and 3 times with ether and was dried at 60°C. The yield of preparation 1 was 21.4 mg.

Preparation 2 was obtained from 2465 mg. of lot 2 dry virus. One thousand nine hundred and eighty-four mg. of lipid-free material were subjected to the alkaline treatment for separation of the nucleic acid. The lipid-free material seemed refractory to solution in the 5 per cent alkali and, therefore, was stirred with the alkali overnight. Upon neutralization with acetic acid and removal by centrifugation of the precipitate formed at this point, a supernatant fluid was obtained which was strongly opalescent. This opalescence was increased by addition of alcohol to a final concentration of 4 per cent without the formation of more precipitate. Additional material was separated from the nucleic acid by centrifugation of the opalescent solution at 24,000 R.P.M. for 15 minutes. From the supernatant fluid of the centrifugation run, crude nucleic acid was obtained by acidification and addition of alcohol as described above. The crude product was purified by solution in 1 ml. of 5 per cent sodium hydroxide at about 0° and repetition of the usual steps in the Johnson-Harkins procedure. Only 2 mg. of purified nucleic acid were obtained.

Spectroscopic Characterization of the Nucleic Acid.—The intense absorption of ultraviolet light by purines and pyrimidines (14), which are characteristic components of nucleic acid, provides a basis for the detection of nucleic acid in biological materials. The absorptions of PR8 influenza virus and of the isolated nucleic acid of preparation 1 were kindly investigated by Dr. G. I. Lavin of this Institute. No absorption characteristic of nucleic acid was observed with the intact virus[3] but the preparation of nucleic acid isolated from the virus gave a typical absorption curve with a maximum in the vicinity of 2600 Å (Fig. 1). This can be considered strong evidence for the identity of the isolated material with nucleic acid. Further tests were required, however, to establish whether ribonucleic acid, desoxyribonucleic acid, or both were present.

Composition of Nucleic Acid Preparations.—Some values obtained in analyses on the two preparations of nucleic acid are given in Table I. The analyses were made on aliquots of solutions of the nucleic acids in 0.002 N sodium hydroxide. Nitrogen was determined by nesslerization (15, 16), phosphorus by the method of King (17), carbohydrate by the method of Tillmans and Philippi (18), ribose by the procedure of McRary and Slattery (19),[4] and desoxyribose by an adaptation of the diphenylamine reaction (20).

In comparison with the theoretical values calculated for tetranucleotides, the nitrogen and phosphorus values for the nucleic acid from influenza virus are low. This and the low yields obtained are not surprising in view of the

[3] The virus particles absorb ultraviolet light so strongly that the specific absorption due to nucleic acid is completely masked.

[4] This procedure was modified to the extent that ribose was used as a standard rather than xylose. The ribose had been prepared in the laboratory of the late P. A. Levene and by analysis contained 39.87 per cent of carbon and 6.56 per cent of hydrogen. The theoretical values for ribose are 39.98 per cent of carbon and 6.65 per cent of hydrogen.

vigorous treatment required for and the difficulties attendant upon the isolation of the nucleic acid preparations. Preparation 1, but not preparation 2, contained carbohydrate manifestly in excess of that represented by ribose and desoxyribose. This is unusual, for the ordinary contaminant of nucleic acid

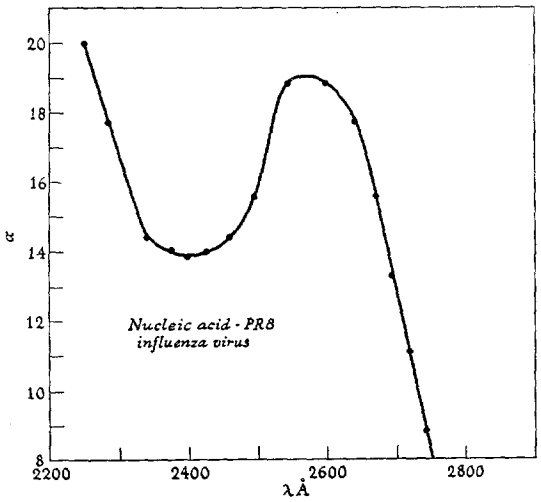

FIG. 1. Absorption in the ultraviolet of the nucleic acid isolated from PR8 influenza virus.

TABLE I

Composition of Nucleic Acid Isolated from Highly Purified PR8 Influenza Virus

Preparation	Nitrogen	Phosphorus	Carbohydrate (as glucose)	Ribonucleic acid (ribose x 3.2)	Desoxyribonucleic acid
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	10.8	4.9	37.5	44.8	19
2	9.0	6.4	33.0	47.6	50
Theoretical for desoxyribotetranucleotide ($C_{39}H_{51}N_{15}O_{25}P_4$)	16.8	9.9			
Theoretical for ribotetranucleotide ($C_{38}H_{49}N_{15}O_{26}P_4$)	16.1	9.5			

preparations is incompletely separated protein. However, preparation 1 contained very little, if any, protein as judged by its low nitrogen content, its failure to give positive ninhydrin or biuret tests, and the lack of evidence for the presence of protein in the character of its absorption of ultraviolet light.

The results of exploratory color reactions made in this laboratory were interpreted as suggesting the presence in highly purified preparations of influenza virus of both ribonucleic and desoxyribonucleic acids (3, 4). Elsewhere, however, the results of similar tests were taken as evidence for the presence of

desoxyribonucleic acid and the absence of ribonucleic acid (1, 6, 21).[5] Owing to the chemical complexity of the intact virus, none of the positive color tests obtained with this material and attributed to nucleic acid constituents could be considered free of ambiguity. The nucleic acid preparations obtained in the present study provided the necessary material to confirm or refute the original conclusions. The conclusion that both ribonucleic and desoxyribonucleic acids were present appeared confirmed when unequivocally positive Bial and Dische (diphenylamine) tests were obtained with the isolated nucleic acid preparations. Quantitative adaptations of these tests yielded the results shown in Table I. Further confirmation of the presence of desoxyribonucleic acid was obtained when the nucleic acid preparations were found to give a positive test for desoxyribose by the Thomas reaction (24) modified as recently described (25). Additional evidence for the presence of ribonucleic acid was obtained by a study of the Bial reaction[6] and by an experiment with ribonuclease. In the case of the Bial reaction it was found, as had previously been noted (19), that the only potentially serious non-pentose interfering substances were uronic acids. For example, galacturonic acid was found to possess, on a weight basis, about 54 per cent of the chromogenic value of ribose. While it seemed unlikely from the method of preparation that uronic acid was present in the nucleic acid preparations, nevertheless tests were made to detect such material by the Tollen's naphthoresorcinol reaction (27). One mg. of nucleic acid, preparation 1, gave a negative test whereas the addition of only 0.1 mg. of galacturonic acid to such a sample yielded an intense violet-red color. From this it can be con-

[5] The conclusion that ribose was absent was based on the negation of the Bial test after hydrolysis of virus concentrates with 10 per cent sulfuric acid. The preliminary treatment of the material to be tested with 10 per cent sulfuric acid constitutes an unusual and probably unwarranted modification of the Bial test (22). Positive Bial reactions were obtained with the unaltered test in both laboratories (3, 4, 6) and it is now agreed by both groups that this can be taken as an indication that ribonucleic acid is possibly present (23).

[6] Pentose sugars yield furfural upon treatment with mineral acid. In the presence of orcinol, furfural gives a blue condensation product which appears bright green in the presence of ferric ions. It is believed that with materials of biological origin, only those substances which are converted to furfural by treatment with acid can give a color with Bial's reagent which could be qualitatively and quantitatively confused with that obtained with pentose sugars (19, 22). This conclusion was found to hold in the present study in the cases of several typical sugars and related substances. Yeast nucleic acid, thymus nucleic acid, ribose, xylose, mannose, fructose, galactose, glucose, sucrose, maltose, raffinose, starch, gum arabic, galacturonic acid, and glucosamine were tested in amounts ranging from a few hundredths of a milligram to 1 mg. Only galacturonic acid, the pentoses, ribose and xylose, and the compounds which yielded pentose under the conditions of the test, *i.e.* yeast nucleic acid and gum arabic, gave strong characteristic green colors. Some of the compounds listed above had also been tested by McRary and Slattery with essentially the same results (19). It is important to note that desoxyribonucleic acid (thymus nucleic acid) did not give a green color with the Bial reagent. This can be attributed to the fact that its sugar, 2-desoxyribose, yields levulinic acid rather than furfural upon treatment with acid (26).

cluded that the carbohydrate which is associated with the nucleic acid from PR8 influenza virus, and which is responsible for the strong Bial test given by that material, is not an uronic acid and most probably is ribose. This conclusion was confirmed by the test with crystalline ribonuclease.

The test with enzyme was based on the observation that intact ribonucleic acid gives a turbid solution around pH 1 whereas after treatment with ribonuclease this turbidity is greatly reduced or abolished.

To 2 mg. of nucleic acid of preparation 1 in 1.1 ml. of citrate buffer at pH 6.9 was added 0.5 ml. of M hydrochloric acid. This brought the pH to 0.7. A turbidity reading of 31 was obtained with this mixture in a Klett-Summerson photoelectric colorimeter using green filter 54. To another 2 mg. sample of the nucleic acid in 1 ml. of citrate buffer was added 0.1 mg. of ribonuclease in 0.1 ml. of citrate buffer. The mixture was allowed to stand for 1 hour before addition of 0.5 ml. of M hydrochloric acid. The turbidity reading for this mixture was zero.[7] In control mixtures, yeast nucleic acid behaved like the virus nucleic acid whereas the turbidity reading of thymus nucleic acid after treatment with ribonuclease was reduced only about 10 per cent.

This experiment is believed to provide direct evidence that part of the nucleic acid isolated from PR8 influenza virus is ribonucleic acid.

Examination of Fractions from Preparation 2.—A crude balance of the amounts of phosphorus and carbohydrate in the intact virus and some of the fractions arising from isolation of the nucleic acid of preparation 1 left much phosphorus and carbohydrate unaccounted for. Therefore, all of the fractions from the isolation of preparation 2 were retained and tested for nitrogen, phosphorus, and ribose. This was particularly desirable in the case of preparation 2 because of the very small amount of material appearing in the nucleic acid fraction.

Several significant facts emerged from the fractionation data presented in Table II. The presence of a considerable residue of phosphorus and ribose in the so called lipid-free, nucleic acid-free fraction strongly suggests that an appreciable amount of nucleic acid was not separated from the protein despite the prolonged treatment with 5 per cent alkali. A comparison of the quantities of carbohydrate and of ribose found in this same fraction demonstrates clearly that there is an excess of carbohydrate above that present as ribose. Since the bulk of the phosphorus in this fraction could be present in the amount of ribonucleic acid calculable from the ribose value, it is evident that only a small proportion of the non-ribose carbohydrate can be attributed to desoxyribose. These data lead to the conclusion reached earlier by Taylor

[7] That no residual turbidity remained due to desoxyribonucleic acid can probably be explained by the fact that the nucleic acid preparation contained less than 20 per cent of desoxyribonucleic acid and by the assumption that the native size and solubility characteristics of this small amount had been altered by the drastic treatment required to separate the nucleic acid from other virus constituents.

(6) that purified influenza virus contains carbohydrate other than that found in nucleic acid. However, the amount of extra carbohydrate indicated by the present findings is considerably less than estimated from Taylor's data since he obtained a much higher value for the total carbohydrate in the virus[8] and since he did not at that time recognize the presence of ribose, which constitutes a significant part of the total carbohydrate.

Phosphorus and ribose analyses indicated the presence of appreciable amounts of nucleic acid or nucleic acid fragments in 2 of the fractions (6 and 8)

TABLE II

Distribution of Nitrogen, Phosphorus, Ribose, and Carbohydrate in Fractions Procured during the Isolation of Nucleic Acid from 2465 Mg. of PR8 Influenza Virus

Fraction	Phosphorus		Nitrogen		Ribose		Carbohydrate (as glucose)	
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1*	20.95		240.0		17.5		150.0	
2		9.15		4.6				
3		3.57		190.0		9.5		85.5
4		0.13		0.2		0.3		
5		0.13		0.2				
6		3.16		6.9		2.8		14.6
7		0.10		0.5				
8		2.58		21.3		3.4		12.9
9		0.74		18.5				
Total....	20.95	19.56	240.0	242.2	17.5	16.0	150.0	113.0

* (1) Intact virus; (2) lipid, soluble in petroleum ether; (3) lipid-free, nucleic acid-free protein; (4) nucleic acid; (5) lipid, insoluble in petroleum ether; (6) hydrochloric acid-alcohol supernatant fluid from first precipitation of nucleic acid; (7) same as 6 after second precipitation of nucleic acid; (8) aqueous wash of lipid-free, nucleic acid-free protein; (9) sum of 5 minor fractions.

listed in Table II. These fractions were also examined in a Beckman spectrophotometer. The data obtained confirmed the presence of nucleic acid constituents, for both materials absorbed in the ultraviolet with maximum absorptions at 2600 Å. Positive color tests for desoxyribose were obtained with each fraction, but there appeared to be 9 to 10 times more ribose than desoxyribose present.

In general, the data of Table II emphasize the difficulty with which the nucleic acid of influenza virus is separated from other components of the virus by the method which has worked well in the cases of several other viruses.

[8] The data obtained in cooperative studies between the two laboratories do not yet permit a clear decision but suggest that there may actually be a real difference in the composition of the virus obtained in each laboratory, mainly with respect to the carbohydrate content.

A striking feature of the composition of the fractions containing the most nucleic acid constituents is the presence also of carbohydrate in excess of that attributable to nucleic acid.

Isolation of Carbohydrate.—The material remaining after removal of lipid and isolation of nucleic acid fom the virus was used for the attempted isolation of carbohydrate. In addition, small lots of carbohydrate were obtained from intact virus purified as described for preparation 2 of the nucleic acid separation experiments. Several procedures were tried including extraction with trichloroacetic acid (28), diethylene glycol (29), formamide (30), and tryptic digestion. Only the latter two methods yielded fractions which were substantially richer in carbohydrate than the starting material. Complications ensued from the use of the tryptic digestion method owing to the presence in the commercial trypsin employed of carbohydrate similar to that of the virus. Hence, the formamide extraction procedure was used to obtain the carbohydrate fractions which were analyzed most fully. The following represents a typical preparation.

About 1200 mg. of highly purified virus which had been dried from the frozen state were mixed with 15 ml. formamide and heated between 140 and 170°C. for 90 minutes. The resulting dark amber colored mixture was cooled and treated with 40 ml. of acid-alcohol (2 ml. of 2 N hydrochloric acid in 38 ml. of absolute ethanol). The precipitate obtained was sedimented in a low-speed centrifuge and was extracted twice with 5 ml. portions of 70 per cent alcohol. To the combined alcohol solutions was added an equal volume of acetone. The precipitate was separated by centrifugation and was dissolved in 2 ml. of water. To this solution was added 5 volumes of acetone and the resulting precipitate was removed by centrifugation. Additional precipitates were obtained by adding more acetone to the supernatant fluids so that precipitates were obtained at 5, 10, and 15 volumes of acetone. The precipitates obtained with 5 and 10 volumes of acetone were combined in a total volume of 1.5 ml. of water. The 15 volume precipitate was taken up separately in 1 ml. of water. The total yield of carbohydrate determined by the orcinol method and expressed as glucose was 25.7 mg. This represented about 30 per cent of the carbohydrate in the starting material.. Tests on the acid-alcohol precipitate indicated that considerable amounts of carbohydrate remained behind in this fraction.

Tests on Carbohydrate Fraction.—Different preparations of PR8 carbohydrate differed quantitatively in their compositions but were very similar in general respects.

All of the preparations contained nitrogen in addition to that later identified with part of the carbohydrate, and hence contained protein residues, presumably attached to the carbohydrate. These probably consisted of degraded protein, for the biuret test was weakly positive and no precipitate was obtained with one of the preparations by treatment with ammonium sulfate or with mixtures of ammonium sulfate and acetic acid. The particles of the carbohydrate complex were apparently of colloidal dimensions as judged by their failure to pass through a collodion membrane. By subtracting the nitrogen found to be a part of the carbohydrate and multiplying the remaining nitrogen by the factor 6.25, it was calculated that the carbohydrate preparations described above contained 50 to 75 per cent protein residues. Very

little nucleic acid was present. This was shown by the lack of phosphorus and by the negligible amounts of ribose and desoxyribose present as indicated by Bial and diphenylamine tests, respectively. The weak Bial test also eliminated the possibility of the presence of appreciable amounts of any ordinary pentose or uronic acid. The iodine test for starch and glycogen was negative but the orcinol test for carbohydrate was strongly positive. Tests for reducing sugars with Benedict's qualitative reagent were negative but became strongly positive after hydrolysis with mineral acid. Likewise, tests for glucosamine were negative before hydrolysis but were strongly positive afterwards. Seliwanoff's test for fructose (or ketoses) was negative when some of the hydrolysate which gave a strong test for reducing sugar was used. The result of color tests with heroin in sulfuric acid (31) was compatible with the presence of mannose. Upon treatment of some hydrolysate of PR8 carbohydrate with phenylhydrazine hydrochloride and sodium acetate, an osazone was formed which microscopically appeared identical with glucosazone. The carbohydrate preparations described above appeared to possess serological activity for they gave precipitates with antisera to intact PR8 influenza virus and to the sedimentable protein of normal allantoic fluid but not with normal rabbit sera.

Thus the carbohydrate fraction isolated from PR8 virus by the formamide extraction method appeared to consist of a polysaccharide attached to protein residues. The polysaccharide contained glucosamine and possibly mannose. Its composition was further elucidated by the following experiments.

Carbazole Color Reaction.—It appeared unlikely that sufficient quantities of virus carbohydrate of adequate purity could be readily obtained in order to identify the component sugar or sugars by the classical approach of isolation and preparation of characteristic derivatives. However, the results obtained by Gurin and Hood (32, 33) in a study of the colors developed by various sugars in the presence of carbazole and sulfuric acid seemed to offer promise, for this method requires only 0.1 mg. or less of carbohydrate and the carbohydrate need not be pure. The use of the carbazole reaction to identify sugars is based on the fact that the colors produced by the various sugars are significantly different, varying from pink to brown. These differences were brought out by Gurin and Hood by determining the relative transmissions of light of the colored solutions when they were examined in an Evelyn photoelectric filter photometer using filters which transmitted maximally at 420, 500, 540, and 660 mμ. This technique was considerably refined by Seibert and Atno in the substitution of a Beckman spectrophotometer for the Evelyn instrument (34).

In the present study the following conditions were used:—

The carbazole employed was purified by twice washing with cold sulfuric acid and recrystallization from toluene and 70 per cent ethyl alcohol (32). The colors were developed as described by Gurin and Hood (32) and were then analyzed in the Beckman spectrophotometer at wave lengths of light ranging from 380 to 600 mμ with intervals of 20 mμ. The results reported here were obtained using 2 blanks as described by Seibert and Atno (34). Results obtained earlier using only a carbazole blank as a reference solution were very similar to the later ones with the exception of those obtained with complex mixtures of substances such as intact influenza viruses.

Carbazole colors were developed with 0.1 mg. samples of glucose, fructose, galactose, mannose, and ribose and the absorption curves obtained are plotted in Fig. 2. The shapes of the curves closely resemble those obtained by Seibert and Atno for the sugars which were studied in common. It is clear that the individual sugars give distinctive and characteristic curves. From the spectrophotometer data, ratios of the optical densities of the colored solutions at two selected wave lengths can be calculated (32, 34). These ratios, though they may vary by as much as 10 per cent between laboratories (*cf.* 34) or 5 per cent in the same laboratory, are never-

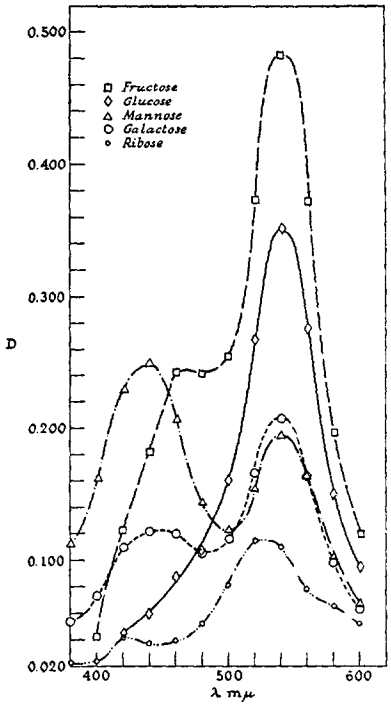

FIG. 2. Light absorption in the Beckman spectrophotometer of colored solutions obtained by reaction of carbazole in sulfuric acid with 0.1 mg. portions of some sugars.

theless distinctive for each sugar. It is apparent that if experimental conditions could be exactly reproduced each time, the ratio for any given sugar would be constant. An individual sugar can thus be characterized by the shape of its absorption curve as in Fig. 2 and by the value of the ratio of absorptions at two wave lengths (see 32, 34, and Table III). Moreover, since a mixture of two sugars gives a curve and an absorption ratio which are averages of what is obtained with the two alone, one can identify a number of mixtures of two sugars. More complex mixtures are increasingly difficult to identify with any degree of assurance although limited applications can be made even in the case of these (34).

Characteristic curves were obtained with the carbohydrate fractions obtained by the formamide extraction procedure from 3 different samples of PR8 influenza virus and from one lot of Lee virus. One curve for each of preparations

TABLE III

Light Absorption in Beckman Spectrophotometer of Colored Solutions Obtained by Reaction of Carbazole in Sulfuric Acid with Sugars, with PR8 and Lee Influenza Viruses, and with Carbohydrate Fractions of PR8 and Lee Influenza Viruses

Substance	D440*	D540	$\frac{D540}{D440}$
0.1 mg. glucose	0.066	0.349	5.30
0.1 mg. fructose	0.183	0.483	2.64
0.1 mg. galactose	0.122	0.208	1.70
0.1 mg. mannose	0.250	0.195	0.78
0.1 mg. ribose	0.038	0.110	2.90
0.025 mg. galactose, 0.075 mg. mannose	0.209	0.211	1.01
0.05 mg. each of galactose and mannose	0.186	0.210	1.13
2 mg. PR8 influenza virus	0.167	0.252	1.51
2 mg. Lee influenza virus	0.169	0.257	1.52
PR8 carbohydrate	0.178	0.195	1.10
Lee carbohydrate	0.311	0.322	1.04

* Optical density at 440 mμ corrected for carbazole blank color and for color of substance treated with all reagents except carbazole.

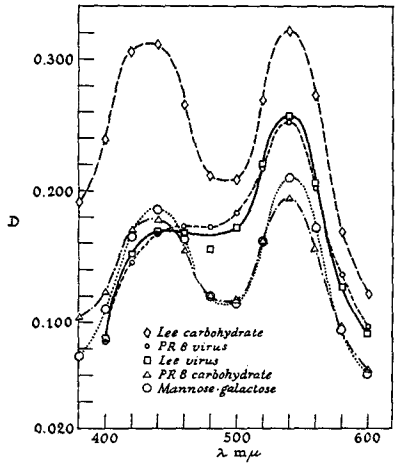

FIG. 3. Light absorption in the Beckman spectrophotometer of colored solutions obtained by reaction of carbazole in sulfuric acid with 2 mg. samples of PR8 and Lee influenza viruses, 0.1 ml. of their carbohydrate fractions, and an equimolar mixture (0.05 mg. of each) of mannose and galactose.

procured under identical conditions is given in Fig. 3 and the ratios D540:D440 are presented in Table III. The nature of these curves and the ratios D540:D440 immediately suggested a mixture of mannose and galactose. This

was true for each preparation tested. In the cases given in Fig. 3 and Table III, the composition of the carbohydrates isolated from PR8 and Lee viruses closely resembled in behavior an equimolar mixture of mannose and galactose. With other preparations of PR8 carbohydrate the results suggested a composition of about 3 parts of mannose and 1 of galactose. The proportion of these two hexoses in the isolated carbohydrate is probably dependent upon the conditions of isolation although unequivocal proof for this is lacking. The curves and data obtained with the intact viruses are, as would be expected from the chemical complexity of these substances, difficult to interpret except in a general sense. They are included mainly to illustrate the remarkable identity of behavior of the two strains of virus in the carbazole reaction. This indicates that there is no pronounced qualitative difference in the carbohydrate components of the two strains of influenza virus.

Glucosamine Analyses.—It seemed possible that part of the nitrogen of the isolated virus carbohydrate might be represented by a nitrogenous sugar derivative, since tests for protein with some preparations were weak. This proved to be the case, for strong color tests for glucosamine were obtained with hydrolysates of the carbohydrate fractions, and later with hydrolysates of the virus itself.

Quantitative analyses for glucosamine were made essentially as described by Sørensen (35). To 4 mg. of virus in 1 ml. of 0.1 M phosphate buffer, or to an appropriate volume of virus carbohydrate solution in water, was added enough 38 per cent hydrochloric acid to bring the final concentration of acid to about 5 N. Such a mixture in a sealed pyrex test tube was heated for 3 hours in a boiling water bath. Heating for 6, 12, or 24 hours was found to result in no increase in glucosamine; hence a 3 hour period of hydrolysis was regularly employed. After cooling, the tube was opened, immersed in ice water, and the acid was neutralized with sodium hydroxide using an amount of 8 N alkali just insufficient to neutralize all of the acid. Neutralization was continued with 0.2 N sodium hydroxide until the solution was faintly alkaline to alkacid test paper. The neutralized hydrolysate was then filtered into a graduated test tube, and the tube and filter were washed 3 times with distilled water. An appropriate amount of the combined filtrate and washings was introduced into a 30 ml. glass stoppered cylinder and enough distilled water was added to bring the volume to 2 ml. Standard solutions of glucosamine hydrochloride and a reagent blank were made to the same volume. To each cylinder was added 2 ml. of fresh acetylacetone reagent and, after mixing, the cylinders were placed in a boiling water bath for 20 minutes. After cooling, 20 ml. of 95 per cent alcohol and 2 ml. Ehrlich's reagent were added and the colors were allowed to develop for 30 minutes. The colors were read in a Klett-Summerson photoelectric colorimeter using the green filter 54 and setting the instrument to zero with the reagent blank. Since it was found that Beer's law was obeyed in the range 0.03 mg. to 0.10 mg. of glucosamine, the size of the sample to be analyzed was chosen so that its glucosamine content would fall within this range.

The quantity and character of the carbohydrate in the viral carbohydrate preparations were estimated from the quantitative carbazole reaction data and the glucosamine determinations. From such data, it was calculated that the one preparation of Lee carbohydrate which was obtained contained a poly-

saccharide consisting of 2 molecules of glucosamine and 1 molecule each of mannose and galactose. The PR8 carbohydrate preparation obtained simultaneously with the Lee preparation just described appeared to contain a polysaccharide composed of equal amounts of glucosamine, mannose, and galactose.

TABLE IV

Glucosamine Content of Preparations of Influenza Viruses and of Normal Allantoic Protein at Various Stages of Purification

Material	Preparation	Sharples concentrate Glucosamine	First cycle vacuum centrifugate Glucosamine	Purified red cell eluate* Glucosamine
		per cent	*per cent*	*per cent*
Lee inflenza virus	1			3.7‡
	2		4.1	3.7
	3		4.3	3.7
	4		4.5	4.1
	5		4.4	3.7
	Average....		4.3	3.8
PR8 influenza virus	1			3.0‡
	2		3.2	3.1
	3		3.2	2.7
	4	1.5	2.9	2.7
	5	1.9	3.5	3.0
	6	1.7	3.2	2.9
	Average....	1.7	3.2	2.9
Normal allantoic protein	1		5.0‡	
	2		4.9‡	
	3		4.8	
	4		5.0	
	Average....		4.9	

* See text for description of fractions.

‡ Dry samples; all others were solutions in which the concentration of material had been determined by nitrogen analyses and use of an appropriate factor.

The polysaccharide was attached to an amount of protein residue which in this instance was calculated to be about 3 times that of the carbohydrate.

Glucosamine analyses were made on influenza viruses and their carbohydrate components and on the sedimentable protein of normal allantoic fluid as described above. Preparations of the allantoic protein were obtained from normal allantoic fluid by 2 to 3 cycles of centrifugation (4). Since the glucosamine content of the virus preparations proved dependent upon the stage of purification, the various steps in the purification procedure will be described in order to provide a basis for interpretation of the results presented in Table IV and elsewhere.

The virus in 3 to 7 liters of infectious allantoic fluid was concentrated in the Sharples supercentrifuge (2). The virus concentrate obtained by suspending the bowl lining in 0.1 M phosphate buffer was clarified by spinning at 5000 R.P.M. for 5 minutes in an angle centrifuge. The supernatant fluid was centrifuged at 24,000 R.P.M. for 15 minutes. The supernatant fluid from this run was reserved for analyses; the pellets were suspended in phosphate buffer and, after clarification in the angle centrifuge, the virus was adsorbed on and eluted from red cells as described under the preparation of lot 2 virus for the nucleic acid isolation experiment. The final product, designated as purified red cell eluate in Table IV, consisted of the material obtained from the red cell eluate after 2 or more cycles of differential centrifugation.

After the purification procedure the preparations consist of particles which are highly active biologically and which are uniform in size, in electrochemical behavior, and in serological reactions (5). In short, the material at this point appears to be highly purified influenza virus. Tests, which had not previously been made, also showed that the material gives no precipitin reaction with antiserum to chicken red cells, whereas when first eluted the virus preparation does so.

From Table IV it can be seen that the sedimentable protein of normal allantoic fluid contained the most glucosamine of any of the fractions tested. Since this substance constitutes a separable impurity in partially purified preparations of virus, it was expected that in a series like that shown for PR8 virus, the Sharples concentrate would be richest in glucosamine and the succeeding fractions progressively poorer. However, the Sharples concentrate was found to contain much less glucosamine than the other fractions. This apparent anomaly led to the discovery that all of the allantoic fluid was not being removed from the Sharples bowl by the final wash with phosphate buffer (2). As a result, the Sharples concentrate contained, in addition to the comparatively large virus particles and those of the well characterized "sedimentable protein of normal allantoic fluid" (4), components of allantoic fluid which were much too small to have been included in the virus concentrate by virtue of their sedimentation characteristics. The small material just described remained in the supernatant fluid when the Sharples concentrate was run in the vacuum centrifuge (2) at 24,000 R.P.M. for 15 minutes. Upon examination, it was found to possess virtually no red cell agglutinating (CCA) activity, a glucosamine content of 0.7 per cent or less, and when treated with antiserum to the sedimentable protein of normal allantoic fluid, it gave a precipitate containing less than one-tenth the amount of nitrogen given in the homologous reaction with an equal quantity of material. Removal of this material, which constituted 40 to 50 per cent of the nitrogen of the Sharples concentrate in the case of PR8 virus and 50 to 60 per cent for Lee, was reflected by sharp and proportionate increases in CCA activity and in glucosamine content. Further purification of the virus by adsorption on and elution from chicken red cells followed by 2 cycles of differential centrifugation resulted in a decrease in glucosamine content and an increase in CCA activity as would be expected by removal of ex-

traneous normal allantoic protein. The following example with PR8 virus will serve to illustrate these findings.

The CCA activities (12) were 14,500, 36,000, 41,000, and 40 per mg. of nitrogen for the Sharples concentrate, the first cycle vacuum centrifuge concentrate, the purified red cell eluate, and the supernatant fluid from the first cycle vacuum centrifuge run, respectively. Glucosamine values were 1.5, 2.9, 2.7, and 0.7 per cent, respectively. Milligrams of nitrogen in the precipitates obtained with antiserum to the sedimentable protein of normal allantoic fluid using 0.2 mg. of antigen were 0.05, 0.09, 0.07, and 0.018, respectively, compared with 0.195 obtained in the homologous reaction.

Similar results were obtained in the case of Lee virus but the glucosamine values for the purified virus fractions were always significantly higher and the CCA activities considerably lower than those obtained with PR8 virus. It is particularly interesting that the ratio of average glucosamine values for the two strains of virus (Lee/PR8 = 3.8/2.9) is almost identical with the inverse ratio of the average CCA activities per milligram of nitrogen obtained on 16 preparations of highly purified virus (PR8/Lee = 40,490/31,500).

DISCUSSION

On the basis of the analyses made during the isolation of nucleic acids, it is tempting to postulate the existence in influenza virus of nucleocarbohydrate, that is, nucleic acid combined with carbohydrate. However, at present the data do not exclude the fortuitous association of carbohydrate with the isolated nucleic acid of preparation 1 nor its incidental inclusion in nucleic acid-rich fractions of preparation 2. Probably of greater importance so far as the composition of the virus is concerned is the evidence which can be taken to support Taylor's conclusion (6) that the particles of highly purified preparations of influenza virus contain carbohydrate in addition to that of the nucleic acids. Owing to the lack of a reliable value for the amount of desoxyribose in the intact virus, it is difficult at present to calculate precisely the quantities of nucleic acid and non-nucleic acid carbohydrate present. On the assumption that the phosphorus of the virus is present only in phospholipid and nucleic acid components, it can be calculated from the data of Table II that the PR8 virus employed in these studies contains approximately 5 per cent of nucleic acid. Further, it can be estimated from the ribose value that the virus contains about 2.3 per cent ribonucleic acid. Taylor reported a value for desoxyribonucleic acid of 2.1 per cent (6). The sum of these last two values gives a total nucleic acid content which approaches that calculated above from the phosphorus analyses. On the basis of a nucleic acid content of 5 per cent, the purified viral particles obtained in this laboratory contain about 4 to 5 per cent of non-nucleic acid carbohydrate.

Bacteria have been shown to contain both ribonucleic and desoxyribonucleic acids (36), but the data of the present report appear to represent the first direct

demonstration of the presence of both types of nucleic acid in a virus. Even vaccinia virus, which has been found to resemble bacteria in many respects, contains only one type of nucleic acid (8).

The methods tried thus far for separating polysaccharide from the virus have necessitated such drastic treatment or have yielded such impure preparations, or both, that it is not possible to state the exact composition of the carbohydrate as it exists in nature. However, it seems reasonable to conclude on the basis of the evidence reported here that the particles representing the PR8 and Lee influenza viruses contain a polysaccharide composed of mannose, galactose, and glucosamine units. One is reminded of the polysaccharides associated with egg albumin (37), serum proteins (34), certain bacteria, and blood group A specific substance (38). It seems possible that polysaccharides of this general type may play a rôle of hitherto unsuspected importance in certain biochemical systems.

Recent serological data have indicated that the highly purified particles representing the influenza virus contain an antigen characteristic of the host (5). In the case of virus isolated from infectious allantoic fluid, it was estimated from quantitative precipitin data that a minimum of 20 and 30 per cent, respectively, of the antigen characteristic of the sedimentable protein of normal allantoic fluid is present in the particles representing PR8 and Lee viruses. In the course of the present study the sedimentable protein has been found to contain an appreciable amount of glucosamine. It has seemed probable, therefore, that glucosamine analyses might be used to follow the separation of the normal component during purification of the virus. The data reported here support this idea. Furthermore, it appeared that the amount of normal antigen present in the most highly purified virus-containing particles could be ascertained from the glucosamine content. Such calculations made from the data in Table IV indicate that the particles of Lee and of PR8 virus contain about 78 and 59 per cent, respectively, of antigen characteristic of the host, values which are about 2.5 times as great as those disclosed by the serological method. These values, however, rest on the assumption that the glucosamine analysis is a direct measure of the amount of normal allantoic protein and particularly that that portion of the virus particle unrelated to the host is devoid of glucosamine. At present this has not been proved and hence the calculation just mentioned must be regarded as speculative.

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SUMMARY

Both ribonucleic and desoxyribonucleic acids have been obtained from purified particles of PR8 influenza virus. These particles were also found by

extraction with formamide to contain carbohydrate in addition to that of the nucleic acids. Carbohydrate-rich fractions, essentially devoid of nucleic acid, were obtained not only from the particles representing PR8 virus but from those of Lee influenza virus as well. The carbohydrate in each case appeared to be a polysaccharide composed of mannose, galactose, and glucosamine units.

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