A COMPARISON OF THE CHROMATOGRAPHIC AND THE MICROBIOLOGICAL ASSAY METHODS FOR THE DETERMINATION OF THE NUCLEIC ACIDS IN NEWCASTLE VIRUS

by

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INTRODUCTION

The chemical analysis of viral materials has revealed differences among individual viruses and groups of viruses which indicate that viruses are comparable to other biological substances. Chemical analyses have aided in the interpretation of the mechanism of virus reproduction and specificity. The study of nucleic acids in relation to their biological role has become an increasingly important phase of virus research.

It is generally assumed that the viral nucleic acids are concerned with virus reproduction and that nucleic acid is the fundamental genetic substance in viruses as well as in other organisms. These assumptions are largely based on the fact that the organized genetic material in living organisms, the chromatin, contains nucleic acid. Additional evidence which supports the genetic function of nucleic acid include: (1) mutation by agents which are known for their reaction with deoxyribonucleic acid (DNA), (2) bacterial transformation, and (3) transduction with bacteriophage. In the latter two cases, the transfer of genetic material is carried out by pure DNA or the DNA of phage.

The purpose of this study was to compare the paper chromatographic and microbiological assay methods for the analysis of the nucleic acids present in the Leavenworth strain of Newcastle disease virus (NDV). Certain properties of the virus such as stability, ease of purification and hemagglutination make it a useful organism for nucleic acid studies. Previous studies on other strains of this virus have indicated that both the deoxyribo- and the ribo- types of nucleic acid are present.

REVIEW OF THE LITERATURE

Introduction

Stanley's discovery of a high molecular weight crystalline form of tobacco mosaic virus foreshadowed the chemical analyses of viruses. He was the first investigator to draw a correlation between biological properties and physiochemical entities and thus opened a new branch of biochemical research. The literature reveals that other workers before Stanley had probably isolated the virus in a crystalline form but had failed to see the importance of their work. Subsequent to the work of Stanley many investigators, working with plant, animal and bacterial viruses, contributed to the techniques of chemical analysis.

One of the first successful attempts to determine the chemical nature of a virus was accomplished by Schlesinger in 1933 when he showed that the E. coli bacteriophage consisted mainly of protein and nucleic acid (Markham, 1953). The presence of nucleic acid in purified tobacco mosaic virus was first recognized by Pirie in 1938. He found that the nucleic acid of tobacco mosaic virus composed slightly more than 5 per cent of virus and that it was of the ribose type (Loring, 1939).

Purification of the Virus

One of the principal difficulties in performing a chemical analysis of a virus is the purification. A completely satisfactory technique has not been developed which will yield a highly purified virus and yet retain enough virus for analysis. However, techniques are available which yield a product of sufficient purity to warrant further analysis.

Ideally the virus should be completely separated from cellular materials and purified until it is free of nonviral materials.

Chemical methods for purification have been used but are not the method of choice. Many viruses are inactivated by harsh chemical treatments.

Physical methods of purification are the most widely used, the initial stage being the concentration of virus by means of the vacuum type, angle, high speed centrifuge. Knowledge of the fact that most viruses have sedimentation rates which are different from those of the starting material enables one to purify the virus by subjecting infected materials to cycles of differential centrifugation; one cycle removing the heavy impurities and the second cycle sedimenting the virus from the supernatant fluid. The cycles can be continued until a satisfactory state of purity is obtained.

Purification by differential centrifugation is valuable in that the procedure is mild and causes little change in the characteristics of the virus. It has been used to purify equine encephalomyelitis virus (Taylor et al.,1943), rabbit papilloma virus (Beard et al., 1939), influenza virus (Taylor et al., 1943; Stanley, 1944; Sharp et al., 1944), Lansing poliomyelitis virus (Loring and Schwerdt, 1946), Newcastle disease virus (Bang, 1946; Cunha et al., 1947), tobacco ring spot virus (Stanley, 1939), potato latent mosaic virus (Loring, 1939); and tobacco necrosis virus (Pirie et al., 1938).

Stanley (1944), in an evaluation of methods of purification and concentration of influenza virus, found that high speed centrifugation of infected allantoic fluids resulted in an almost quantitative removal

of activity from the supernatant fluids, whereas a treatment with red blood cells effected only a removal of 50 to 90 per cent of the activity.

A method of purification utilizing adsorption and elution from red blood cells has been used quite extensively in conjunction with differential centrifugation to purify the viruses of influenza (Knight, 1946; Sheffield et al., 1954; Ada and Perry, 1954, 1955, 1956; Miller, 1956) and Newcastle disease (Franklin et al., 1957).

Many of the workers listed above have found it advantageous to initially purify the virus by adsorption on red blood cells. Even though the red cells adsorb the virus preferentially they still adsorb other materials. Also, all viruses that adsorb to red blood cells do not elute equally well from them. This gives rise to considerable difficulty, particularly when any loss of the volume of virus is a major factor. The use of this method has proven successful for workers using influenza virus, but it has been suggested that it is not an indispensable step in obtaining influenza virus in a state of maximum purity.

Another method of purification which has met with some success is that of adsorption of the virus on aluminum phosphate columns. Miller and Schlesinger (1955) studied five strains of influenza virus each of which was found to have a characteristic adsorption pattern which could be used for rapid and efficient differentiation with no significant inactivation of the virus.

The nonviral particles found in tissues in which animal viruses are cultivated range in size from 10 to 300 millimicrons. Since viruses fall in this same size range, it is difficult to separate them. Another factor which complicates virus purification is the

presence of low molecular weight particles dissolving in or being adsorbed on the viral particles (Knight, 1949). Beard (1948) has shown that cholesterol is present in the lipid extracts of most animal viruses.

At present, the ultra-centrifuge remains the most effective tool for animal virus purification. It is possible to make the centrifuge selective by an appropriate choice of high and low speed cycles, but this is seldom done in the purification of animal viruses due to the great loss of virus particles.

Criteria of Purity. Stanley (1939) stated that it was practically impossible to establish that a substance was 100 per cent virus. Several methods are now available which can be used with a fair degree of accuracy. Lauffer and Stanley (1939) listed several tests which may be used for establishing the purity of a purified virus: (1) constancy of properties in successive preparations and in preparations from different sources, (2) homogeneity in sedimentation, (3) electrochemical homogeneity, and (4) serological purity. More recently, additional methods such as the electron microscopy and the determination of the ratio of hemagglutinin units to milligram of dry weight have been used.

Methods for Analyses of Viruses

Chromatography and Ultraviolet Spectrophotometry. Recently the techniques of chromatography and ultraviolet spectrophotometry have been developed by Hotchkiss (1948) and by Vischer and Chargaff (1948). These techniques have provided a quantitative and qualitative method for the determination of the constituent purine and pyrimidine bases of nucleic acids. Once the nucleic acids have been separated they

can readily be detected directly by spectrophotometry. According to Wyatt (1955) as little as 0.5 milligrams of nucleic acid can be detected by this method.

The R_F value[1] of each nucleic acid base is influenced by: (1) the composition of the vapor phase in the chromatography vessel, (2) the temperature, (3) the direction (ascending or descending) and length of run, and (4) the paper used. R_F values can be reproduced accurately if careful attention is given to the above factors (Wyatt, 1955).

Various methods have been used to determine the positions of nucleic acid derivatives on filter paper. Hotchkiss (1948) cut out the spots and eluted them in acid for measurement of the ultraviolet extinction in the spectrophotometer. Vischer and Chargaff (1948) treated the paper with salts of mercury, the mercury fixed by the purine and pyrimidine bases was made visible by conversion to black mercuric sulfide. Fries et al. (1951) described a microbiological method for detecting purine and pyrimidine nucleosides on paper chromatograms with the aid of nutritionally deficient strains of Ophiontoma. The most useful techniques appear to be those making use of the absorption of ultraviolet light by the nucleic acid bases.

Under ultraviolet light the spots of nucleic acid appear as dark regions against the background of the filter paper. Markham (1951) reports the use of low-pressure mercury resonance lamps to detect nucleic acids. These lamps have a sensitivity such that 0.2 micrograms of adenine spread over a circle 1.5 cm in diameter can be detected.

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[1] R_F value = $\frac{\text{distance moved by the nucleic acid spot}}{\text{distance moved by the solvent}}$

Mineralite ultraviolet lamps have been used with success but their sensitivity is less. The use of ultraviolet absorption is particularly valuable since the absorption of nucleic acids at 260 millimicrons is very intense and specific. Nucleic acids can be separated from all other cellular constituents in this way. There are no significant differences in the absorption of ribonucleic acid and deoxyribonucleic acid.

Hotchkiss (1948) listed several advantages of the use of chromatography for separation of nucleic acid bases: (1) The five bases, cytosine, thymine, uracil, adenine, and guanine, can be completely separated from one another in microgram quantities. (2) The separated substances are detected by ultraviolet spectrophotometric examinations of solutions prepared by soaking exised portions of the paper in water. (3) An organic solvent is used which does not interfere with ultraviolet spectrophotometry. (4) The recovery of individual constituents is essentially quantitative, with an error of 10 per cent or less. (5) Spectrophotometric standards of purity are provided which make it possible to identify each component and to determine with considerable accuracy the composition of binary mixtures which may result from a migration not carried far enough to give altogether complete separations. (6) One milligram or less of nucleic acid can be examined for the rate of liberation of its nitrogenous bases during partial enzymatic or acid hydrolysis. (7) Some simple derivatives such as nucleosides can be purified and identified in this procedure. (8) Less soluble substances, or dilute solutions, are used by evaporation of moderately large volumes of solutions upon a small spot in the paper.

Hydrolysis Procedures. It is essential that a good method of hydrolysis be employed. There are several hydrolytic procedures used for breaking the nucleic acids into their purine bases and pyrimidine nucleotides, but all methods cannot be applied to both types of nucleic acid. It is more probable that a separate procedure must be used for the detection of DNA and RNA respectively. Markham and Smith (1949) recommend the use of 1N hydrochloric acid at 100° for 1 hour. This procedure breaks down RNA to purine bases and pyrimidine nucleotides that may be separated readily by paper chromatography. Marshak and Vogel (1951) recommend the use of 98 per cent formic acid at 175° for 30 minutes for the breakdown of DNA to its constituent bases. The use of 12 N perchloric acid at 100° C for 1 hour is recommended by Wyatt (1951) for the breakdown of DNA.

Attempts have been made to separate the mononucleotides obtained from RNA by the use of alkaline hydrolysis, but there are no solvent systems presently available which will achieve this rapidly. A method of separation of nucleotides is that of ionophoresis (Markham and Smith, 1952) which makes use of the mobility of the nucleotides in an electric field. In this procedure the spots are detected, as with chromatograms of hydrolyzed material, after they have been treated with a citrate buffer and the spots have been allowed to separate.

The free purine bases, adenine and guanine, are readily found during acid hydrolysis of both types of nucleic acids, whereas the pyrimidine bases remain for the most part as mononucleotides in the case of RNA or as nucleoside diphosphates in the case of DNA. 1N hydrochloric acid hydrolysis is not applicable to DNA, since nucleotides or nucleosides cannot be obtained quantitatively by chemical hydrolysis,

because of the lability of the deoxy-sugar.

Wyatt (1951) in the analysis of deoxypentose nucleic acids from eight animal sources and one plant source found that the ratios of the purines and pyrimidines vary with the species source of the nucleic acid, but not with different tissues from one species.

Solvent Systems. There are many different solvent systems available which will give good results with various preparations depending upon the type of separation desired. However, no solvent system is absolutely free from criticism. Wyatt (1955) lists several factors which should be considered in preparing solvents: (1) Water content. By using a miscible solvent the water content can be varied over a wide range. (2) pH. The relative rates of movement of ionizable substances can be controlled by regulation of pH, having regard to their dissociation constants. (3) Salt content. The local presence of salts may cause distorted spots, and the addition of salts slows the movement of solutes.

In the preparation of solvent systems it is advisable to avoid substances which absorb in the ultraviolet range. Some of the solvent systems which are used with success are: (1) aqueous n-butanol with or without added ammonia, (2) butanol saturated with water, (3) isopropanol-hydrochloric acid and water, and (4) n-butanol with formic acid.

Microbiological Techniques for Estimating Nucleic Acid Content. A microbiological assay method for the determination of deoxyribosides and deoxyribonucleic acid was described by Hoff-Jorgensen (1952) with the organism Thermobacterium acidophilus R-26. The method was specific and allowed the determination of a few micrograms of deoxyribonucleic

acid with an error of about 10 per cent at the lowest concentration. The method is based on the finding that deoxyribonucleosides are essential growth factors for Thermobacterium acidophilus R-26.

Both ribonucleic acid and deoxyribonucleic acid have been found in viruses and a tremendous range of quantity has been found among different viruses. Variations range from less than one per cent in viruses of influenza and Newcastle to about 40 per cent in tobacco mosaic virus.

All plant viruses contain only ribonucleic acid but both types of nucleic acid have been found in animal viruses. However, both types are apparently not present in all animal viruses. Some investigators working with the same virus have reported conflicting results as to which nucleic acid is present or if both types are present. Taylor et al. (1942) has shown that Shope papilloma contains only DNA and Hoagland (1943) has shown that vaccinia contains only DNA. Influenza (Graham, 1950) and Newcastle disease (Cunha et al., 1947), viruses have been found to contain both DNA and RNA.

At present viruses in general seem to be composed of various combinations and amounts of nucleic acid, protein, and frequently lipid, with polysaccharide, vitamins, and other materials appearing as yet only in special cases. Nucleic acid and protein can be singled out from the other constituents because these substances, combined to form specific nucleoproteins, appear to comprise the minimum chemical requirement for virus activity, and nucleic acid and protein constitute the only components common to all viruses. The potential importance of nucleic acids in virus reproduction is evidenced by the results obtained with the turnip yellow mosaic virus (Knight, 1949). Data indicated that

only the nucleoprotein possesses virus activity.

Chemical Analyses of Viruses

Taylor et al. (1942) found the nucleic acid content of rabbit papilloma virus to be 6.8 per cent of the whole virus protein. In a comparison of two strains of influenza virus, viruses A and B, by Taylor (1944) it was discovered that the materials isolated were of similar complexes of protein, lipid, and carbohydrate and contained small amounts of deoxyribonucleic acid.

In studying the California strain of Newcastle disease virus (Cunha et al. 1947) it was found that the virus was composed of about 67 per cent complex protein, about 27 per cent lipid, and a small amount of nucleic acid.

Wyatt and Cohen (1953) reported a new pyrimidine base from bacteriophage nucleic acids. The new base was identified as 5-hydroxymethyl cytosine. They concluded after careful study that the new base was a unique virus constituent.

Working with influenza virus, Knight (1947) found both DNA and RNA in the purified particles. Ada and Perry (1954) analyzed for the nucleic acid content of influenza virus by extracting the nucleic acid from the virus particle with 10 per cent hot sodium chloride. They reported that a negligible amount of DNA was present in the virus and that the RNA content of the virus was 0.78 per cent to 0.98 per cent. In 1955 Ada and Perry extracted nucleic acid from the two strains of influenza virus (A and B) and compared the two strains. They found the ratios of adenine plus uracil to guanine plus cytosine were significantly different in the two strains.

In a continuation of studies with the two strains of influenza virus by Ada and Perry (1956) it was reported that there were specific differences in the nucleic acids of the two strains. The nucleic acid isolated from the incomplete virus showed on analysis the same proportion of bases as that from standard virus, but the content of nucleic acid was decreased. These experiments gave strong evidence for the functional significance of influenza virus nucleic acid.

A study of influenza virus by Frisch-Niggemeyer and Hoyle (1954) revealed that the content of ribonucleic acid was 0.7 to 0.74 per cent. This figure is in excellent agreement with that found by Ada and Perry (1954).

Function of Nucleic Acids in Viruses

It is generally assumed that the nucleic acid components of viruses are somehow concerned with the ability of these agents to reproduce. Knight (1954) interpreted the work of Markham and Smith, with turnip yellow mosaic virus, and Hershey and Chase, with bacteriophages labeled in the protein and nucleic acid constituents, to mean that virus activity can be resolved into two phases: (1) the infectious process, in which protein is essential, and (2) the reproductive process, in which nucleic acid alone is sufficient.

It has been a common practice to think of nucleic acid as the fundamental genetic substance in viruses and in other organisms. This assumption is made largely on the basis that the organized genetic material in living organisms, the chromatin, contains nucleic acid, and that the wave lengths of ultraviolet light, which cause mutation in certain organisms, parallel the absorption spectrum of nucleic acids

(Knight, 1954). Evidence also suggests that viral nucleic acids may function in holding the protein in a specific configuration in which its biological properties are manifest. This later hypothesis is supported by the proposed DNA structure (Watson and Crick, 1953) consisting of two helical polynucleotide chains coiled around the same axis and held together by bonds between the purine and pyrimidine bases. This structure would make possible the reduplication of specific genetic properties embodied in DNA organization. Additional evidence which supports DNA as the carrier of genetic properties includes: (1) mutation by agents which are known for their reaction with DNA, (2) bacterial transformation, and (3) transduction in bacteriophage.

Knight (1954) believed that a single nucleic acid should be sufficient for each virus since many viruses contain only one nucleic acid. Another hypothesis is that the more complex the virus the more nucleic acid it requires or the more types of nucleic acid it requires. Influenza virus and Newcastle disease virus have been reported to contain both RNA and DNA. These viruses are more complex than plant viruses. This hypothesis does not stand since vaccinia virus, which is large and complex, contains only DNA. The possibility remains that those viruses which have been reported to contain two types of nucleic acid were not pure when the chemical analysis was made.

Summary

Since Stanley's purification and subsequent chemical analysis of tobacco mosaic virus many of the plant viruses and a few of the animal viruses have been purified and analysed for their chemical constituents. The purification of the animal viruses has been difficult and it has

been impossible to isolate them in a crystalline form. This is due to the similarity of the virus particle size to the size of many materials in the tissues in which they must be grown. The purity of any except the largest of the animal viruses is questionable.

Techniques are now available by which microgram quantities of nucleic acid extracted from viral substances may be detected. With the advancement of finer methods the importance of purification is also accentuated.

Analyses of viruses thus far have revealed that plant viruses contain only RNA, animal viruses contain either RNA or DNA or both, and bacterial viruses contain DNA. The molar quantities of the nucleic acid reportedly present in viruses vary over a wide range.

MATERIALS AND METHODS

Virus Stock

The virus used in this study was the Leavenworth Kansas-1948 strain of Newcastle Disease virus (NDV) obtained from the stock collection of the Department of Bacteriology, Kansas State College. Leavenworth Kansas-1948 virus was isolated in June 1948 by L. D. Bushnell at Kansas State College from infected chickens obtained in Leavenworth, Kansas. The virus is still capable of hemagglutination after treatment at 56° C for 240 minutes and the same infectivity remaining after 180 minutes at that temperature. The virus stock was maintained in 2 ml ampules at sub-freezing temperatures.

Cultivation of Virus

The virus was cultivated in the chorio-allantoic fluids of 10-day

old, fertile hens eggs. The stock virus was diluted to 10^{-3} in sterile 0.85 per cent saline, and 0.1 ml of the 10^{-3} dilution was inoculated into the allantoic cavity through a hole punched in the air-sac end of the egg. The inoculated eggs were checked each day for deaths; all deaths occurring within a 24 hour period were considered to be traumatics, and these eggs were discarded. At the end of 72 hours the eggs were chilled for at least 5 hours and then harvested. The eggs were harvested at the end of 72 hours regardless of whether death had occurred. The strain of virus used gave very few deaths in 72 hours.

Harvesting of Fluids

The fluids were harvested by inserting a sterile wire screen into the allantoic cavity and then drawing off the fluids with a pipette. To prevent contamination of the virus fluids, a penicillin-streptomycin mixture was added to each volume of fluids collected. Collected fluids were placed in a refrigerator at a temperature of 2° - 5° C. An average of about 5 ml of fluid was harvested from 1200 virus-infected chicken embryos.

Titration by Hemagglutination

Hemagglutination titrations were run in order to determine the presence of virus in each group of fluids and to determine the titer of the virus. The titer obtained on all fluids ranged between 1:320 - 1:640.

The chicken red blood cells employed were prepared by withdrawing the blood from the bird into a physiological saline solution containing two per cent sodium citrate, in the ratio of nine parts of blood to one

part of sodium citrate. The cells were washed and centrifuged three times in physiological saline. The cells were finally resuspended in 0.5 per cent concentration in 0.85 per cent saline.

Hemagglutination titrations were run by setting up a series of 12 tubes and adding 1 ml of 0.85 per cent saline in the first tube and 0.25 ml in each of the other tubes. Twenty-five hundredths ml of the virus was added to the first tube and doubling dilutions were made in each of the subsequent tubes. Twenty-five hundredths ml of 0.5 per cent red blood cells were added to each tube and the tubes shaken. Titers of the virus were read after standing for forty-five minutes at room temperature.

Titration by Embryo Infectivity

An embryo infectivity titration was run on the collected fluids. Ten-fold dilutions were made of the virus fluids up to 10^{-10}, and 0.5 ml of the dilutions were inoculated into ten day old embryonated eggs. Five eggs were inoculated with each dilution of the virus. The eggs were incubated at 37° C for a total of 168 hours (7 days). The LD_{50} as determined by the Reed and Muench method (1938) was $10^{-5.86}$.

Table 1. Calculation of the 50 per cent endpoint according to the method of Reed and Muench.

Dilution	Alive	Dead	Total Alive	Total Dead	Per Cent Dead
10^{-1}	0	5	0	28	100
10^{-2}	0	5	0	23	100
10^{-3}	0	5	0	18	100
10^{-4}	0	5	0	13	100
10^{-5}	3	2	3	8	62.5
10^{-6}	3	2	6	6	50
10^{-7}	2	3	8	4	33.3
10^{-8}	4	1	12	1	8
10^{-9}	5	0	17	0	0
10^{-10}	5	0	22	0	0

Proportionate distance = $\frac{62.5 - 50}{62.5 - 33.3} = \frac{12.5}{29.2} = 0.43$

Difference in exponents = (-7) - (-5) = -2

LD_{50} = (-2) x (0.43) = -0.86 + -5 = $10^{-5.86}$

Purification of Virus

After collection of six liters of virus infected fluids the fluids were pooled and were allowed to stand at a temperature of 2° - 5° C for a period exceeding four weeks. The allantoic fluids were filtered through several thicknesses of sterile gauze to remove membranes and other large extraneous materials from the eggs

Differential Centrifugation. The virus was isolated and purified by two cycles of differential centrifugation. The allantoic fluids were placed in 50 ml steel centrifuge tubes, the tubes placed in a

frozen head and centrifuged in a Servall angle head, vacuum type centrifuge. The fluids were centrifuged at an average speed of 50,000 g for two hours. After centrifugation the temperature of the head was checked and was found to be about 37° C. This temperature was not great enough to cause a loss of infectivity in this strain of NDV.

The clear supernatant fluids were decanted leaving the bluish white pellet. Two ml of Ringer's solution was added to cover the virus pellet. After a minimum of 12 hours the pellets were suspended, to one-fourth the original volume of allantoic fluids, in Ringer's solution. Ringer's solution was prepared as follows:

0.7 grams of sodium chloride
0.035 grams of potassium chloride
0.0026 grams of calcium chloride
100 ml of distilled water

Hemagglutination titrations were run on both the clear supernatant and on the suspended pellets. The titer on the various groups of supernatants ranged from zero to a titer of 1-10. These titers indicated that the virus loss was very small in comparison to the volume of fluid. Hemagglutination titers on suspended pellets indicated a two fold increase in titer due to the first concentration by ultracentrifugation. A mixture of penicillin-streptomycin was added to the pellets suspended in Ringer's solution to lessen the chance of bacterial contamination.

The suspended pellets were subjected to a second cycle of centrifugation at a speed of approximately 2,000 g for 20 minutes. Centrifugation was repeated at this speed four times in an attempt to further purify the virus.

A second centrifugation was carried out in the Servall angle head centrifuge at an average speed of 59,000 g for one hour. The supernatant

was decanted and the pellets suspended in Ringer's solution to a volume one-twentieth as great as the original volume of allantoic fluids. The suspended pellets were subjected to an additional centrifugation at 2,000 g for 20 minutes. The virus particles at this point were considered to be partially purified.

Extraction of Nucleic Acids

Precipitation of the Virus. The partially purified virus was precipitated by the addition of cold 30 per cent trichloroacetic acid. The virus was allowed to precipitate for ten minutes at 0° C and then collected by centrifugation. Approximately 0.4 ml of packed wet virus was obtained from each 3-liter lot of allantoic fluid. Precipitated virus was suspended in a minimum amount of water and was dialyzed against running water for 17 hours at room temperature. After dialysis the water was evaporated from the virus particles. Evaporation was speeded by placing the dialysing membrane in front of a fan.

Defatting of the Virus. After drying, the virus was defatted by the addition of chloroform-methanol in a ratio of 2:1. The fat and the chloroform-methanol layers were removed after centrifugation.

Extraction with Hot 10 per cent Sodium Chloride. Nucleic acid was quantitatively extracted from the defatted virus by boiling in a 10 per cent sodium chloride solution for 20 minutes. The suspension was stirred occasionally during the extraction process. This process was carried out three times. After each extraction the suspension was centrifuged; the nucleic acid remained suspended in the clear supernatant. Supernatants from the three extractions were combined.

Precipitation of Nucleic Acid. Nucleic acid was precipitated by

the addition of two volumes of ethanol and collected by centrifugation. The precipitated nucleic acid was washed with 66 per cent ethanol to remove any salt which might be present and then washed again with 95 per cent ethanol. Finally, the nucleic acid was washed with ethyl ether. The nucleic acid and ether suspension were added to a 5 ml vial and the ether was allowed to evaporate to dryness.

Hydrolysis

The hydrolytic agent used in this study was N-HCl. Sixty λ of N-HCl was added to the vial containing the nucleic acid. The vial was sealed and placed in a boiling water bath for one hour. The hydrolyzed material was added to a strip of filter paper and allowed to dry. The vial was rinsed with 10 λ of N-HCl and this 10 λ was placed on another strip of filter paper and allowed to dry.

Detection of Nucleic Acids

Chromatography. Nucleic acid components were determined by the use of paper chromatography and by use of the Beckman spectrophotometer. The chromatography procedure was carried out in a large chromatocab which had sufficient space to hold as many as 30 two-inch filter strips. Thus knowns and unknowns could be run simultaneously.

Strips of filter paper were prepared which were two inches in width and 22 inches long. The material to be tested was placed on a marked spot three inches from the top of the filter paper.

Solvent System. The solvent used was isopropanol-HCl-water, prepared in the proportions:

170 ml of isopropanol
41 ml of conc. HCl
39 ml of distilled water

The solvent was placed in the chromatocab 48 hours prior to the introduction of the filter paper to allow the cab to become saturated. After saturation of the cab the filter strips containing the known and unknowns were fastened in the troughs, and the troughs were placed in the chromatocab. One hundred ml of solvent was placed in each trough. The chromatocab was sealed, and the solvent was allowed to proceed by capillary action for a distance of at least 38 centimeters. The filter strips were removed from the chromatocab and allowed to dry before examination under the ultraviolet light.

Detection of Nucleic Acids under Ultraviolet Light. Dried filter strips containing the knowns and unknowns were placed under a source of ultraviolet light, and the spots were located. The spots appeared as dark purple areas. The entire spot was circled and the center of the spot marked. The R_F values for each spot were determined by the following equation:

$$R_F = \frac{\text{distance moved by spot}}{\text{distance moved by solvent}}$$

Spectrophotometric Determination of Nucleic Acids. The Beckman DU spectrophotometer was used in this study in an attempt to substantiate the results obtained from chromatography. After detection of the spots under the ultraviolet light the spots were cut out and eluted in 0.1 N HCl. The spots from known bases were eluted in 4 ml of 0.1 N HCl and the unknowns were eluted in 2 ml. After elution for at least 12 hours the tubes containing filter paper and hydrochloric acid were centrifuged at 1500 rpm. The supernatants containing the nucleic acids were decanted into chemically clean test tubes. The maximum and minimum wave lengths were determined for knowns and unknowns by the spectrophotometric

readings.

Preparation of Knowns

In order to have an experimental control it was necessary to prepare knowns of each of the purine and pyrimidine bases to be run with the unknowns. Dilutions of the following known bases were made at a concentration of 100 milligrams of base in 50 ml of 0.1 N HCl:

Adenine
Cytosine
Cytidylic Acid
Uridylic Acid
Uracil
Thymine
Hypoxanthine
Thymidine
Guanine
Xanthine
5-methyl cytosine

Strips of filter paper were prepared as with the unknowns and 10 λ of the known base was placed on the filter strip at a distance of three inches from the top of the paper. The knowns were placed in the chromatocab with the unknowns. Duplicates of the known bases were run.

Microbiological Assay of Nucleic Acid

A portion of the dried defatted virus, representing 3 liters of virus infected allantoic fluids, was used for the determination of nucleic acids by a microbiological assay technique (Miller, 1956).

Stock Cultures. The bacterium used in this determination was Thermobacterium acidophilus R-26, for which the deoxyribonucleosides are essential growth factors. The organism will grow equally well on each of at least five different deoxyribonucleosides. Stock cultures of Thermobacterium acidophilus R-26 were maintained by weekly transfers

in a skimmed milk medium prepared as follows:

100 ml of skimmed milk
0.1 gram of cysteine
0.5 gram of Difco yeast extract
autoclaved for 10 minutes at 15 lb.
stored at 10° C.

Preparation of Virus. Dried defatted virus samples were triturated in 0.25 ml of 5 per cent sodium hydroxide. This suspension was allowed to incubate for 24 hours at 37° C after which it was neutralized with dilute acetic acid and the volume was brought to 3.0 ml with distilled water. One tenth ml of 0.1M magnesium sulfate and about 1 milligram of deoxyribonuclease were added and the mixture was again incubated at 37° C for 24 hours. The mixture was adjusted to pH 6.5, diluted to 6.0 ml with distilled water and assayed at four different concentrations.

Inoculum. A loopful of stock skimmed-milk culture was transferred to a tube of inoculum medium and incubated at 37° C for 24 hours. The bacteria were sedimented, washed with 5 ml of saline, and resuspended in a second portion of saline. Assay tubes were inoculated with one drop of the above suspension dispensed from a hypodermic syringe equipped with an 18-gage needle.

Inoculum Medium. The inoculum medium was prepared as follows:

0.5 ml of 10^{-3} M thymidine
0.38 ml of DNA (200 microgram/ml)
3.50 ml of double strength assay medium
1.62 ml of distilled water
autoclaved for 10 minutes at 15 lb.

Standards. Thymidine standards were prepared as controls, in the following manner:

Stock solution (10^{-4} g Mol. deoxyriboside)

24.2 mg thymidine
100 ml 25 per cent ethanol

Working standard (5×10^{-9} g Mol. deoxyriboside per ml)

0.05 ml stock solution
9.95 ml water

The working standard was added to screw cap tubes in amounts of 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 ml. The volume was brought up to 1.0 ml with distilled water, making a standard series from 5×10^{-10} to 5×10^{-9} gram moles of thymidine per tube. One ml of the assay medium (Table 2)was added to each tube. The tubes were autoclaved for five minutes at 15 pounds, cooled and inoculated with one drop of the washed inoculum. The standards were incubated for 24 hours at 37° C, and their turbidities were measured in a Beckman DU spectrophotometer at a wave length of 500 millimicrons. All standards were run in duplicate.

A standard thymidine curve was plotted from the turbidity measurements for comparison of growth response of Thermobacterium acidophilus R-26 to thymidine and for calculation of the amounts of deoxyriboside in the unknown sample.

Assay Procedure. Virus samples were added to screw cap tubes in amounts of 1.0, 0.8, 0.4 and 0.2 ml. Each of the tubes were brought to 1.0 ml with distilled water and 1 ml of assay medium was added to each tube. The tubes were shaken, autoclaved for five minutes at 15 pounds, cooled and inoculated with one drop of the inoculum dispensed from an 18-gage needle.

Uninoculated blanks were run for every concentration of the unknown so that correction could be made for the turbidity in the unknown. The optical densities of the unknown virus samples were determined in a Beckman DU spectrophotometer at a wave length of 500 millimicrons. The results were calculated from the standard thymidine curve.

Table 2. Composition of assay medium (Miller, 1956)

	Quantity per liter		Quantity per liter
DL-Alanine	400 mg	Thymine	20 mg
DL-Isoleucine	400 mg	Xanthine	20 mg
DL-Methionine	400 mg	Cytidylic acid	50 mg
DL-Norleucine	400 mg	Ammonium sulfate	6 g
DL-Norvaline	400 mg	Tween 80	1 g
DL-Phenylalanine	400 mg	4% Tryptic digest of casein*	125 ml
DL-Serine	400 mg		
DL-Threonine	400 mg	$MgSO_4 \cdot 7H_2O$	400 mg
DL-Tryptophan	400 mg	$MnSO_4 \cdot 4H_2O$	20 mg
DL-Valine	400 mg	$FeSO_4 \cdot 7H_2O$	20 mg
Glycine	200 mg	NaCl	20 mg
L-Arginine·HCl	200 mg	Sodium acetate	12 g
L-Cystine	200 mg	KH_2PO_4	2 g
L-Histidine·HCl·H_2O	200 mg	K_2HPO_4	2 g
L-Hydroxyproline	200 mg	Glucose	30 g
L-Leucine	200 mg	Thiamine	2 mg
L-Lysine·HCl	200 mg	Riboflavin	2 mg
L-Proline	200 mg	Niacin	2 mg
L-Tyrosine	200 mg	Calcium pantothenate	2 mg
L-Aspartic acid	800 mg	Pyridoxal	0.4 mg
L-Glutamic acid	2 g	p-Aminobenzoic acid	0.4 mg
Uracil	40 mg	Biotin	0.02 mg
Adenine sulfate	20 mg	Folic Acid	0.02 mg
Guanine·HCl	20 mg		

Final pH 6.5

*(Roberts and Snell, 1946)

EXPERIMENTAL RESULTS

Chromatographic Analysis

The results represent a chromatographic analysis for the nucleic acids of the Leavenworth strain of NDV, run on three liters of virus infected allantoic fluids.

R_F Values of Known Bases. The results from chromatograms of known bases of the nucleic acids are shown in Table 3. The R_F values were calculated from the distance moved by the nucleic acid spot and the distance moved by the solvent and are an average of duplicate chromatograms.

Table 3. R_F values of known bases.

Base	Solvent distance (cm)	Spot distance (cm)	R_F Value
Adenine	49.15	13.35	0.2716
Cytosine	42.55	17.60	0.4170
Cytidylic Acid	43.50	24.50	0.5632
Guanine	47.65	8.00	0.1678
Hypoxanthine	39.90	10.40	0.2606
5-Methyl Cytosine	45.60	19.75	0.4331
Thymidine	44.00	8.1	0.8273
Thymine	44.25	31.0	0.7009
Uracil	46.20	26.6	0.5757
Uridylic Acid	41.55	29.05	0.6997
Xanthine	45.75	8.25	0.1804

R_F Values of Unknown Nucleic Acid. The results from chromatograms of unknown nucleic acid are shown in Table 4. The R_F values obtained from the unknown were compared with the R_F values of the known bases. It was concluded that spot no. 1 was guanine or xanthine; spot no. 2 was adenine or hypoxanthine; spot no. 3 was uridylic acid and spot no. 4 was thymidine.

Table 4. R_F values of unknown nucleic acid.

Spot no.	Solvent distance	Spot distance	R_F value
1	44.8	8.5	0.1897
2	44.8	12.0	0.2679
3	44.8	31.2	0.6964
4	44.8	36.5	0.8147

The size of spots no. 3 and no. 4 were considerably smaller than spots no. 1 and no. 2. There is a possibility that these spots were artifacts and not nucleic acid. However, they did give the characteristic color of nucleic acid under the ultraviolet light.

Spectrophotometric Analysis

An attempt was made to substantiate the results obtained by chromatography by use of the Beckman DU spectrophotometer. Due to the small amounts of nucleic acid recovered the unknown spots were eluted in only 2 ml of 0.1N hydrochloric acid, the minimum which could be read in the spectrophotometer. The data obtained from the spectrophoto-

metric readings is recorded in Table 5.

Table 5. Spectrophotometric readings of unknown nucleic acid spots eluted in 2 ml of 0.1N hydrochloric acid.

Spot no.	Wave length	Optical density
1	210	0.547
	215	0.420
	220	0.333
	225	0.272
	230	0.256
	235	0.255
	240	0.273
	245	0.267
	250	0.258
	255	0.249
	260	0.224
	265	0.207
	270	0.204
	280	0.176
	290	0.127
	300	0.073
2	215	0.260
	220	0.375
	225	0.298
	230	0.250
	235	0.237
	240	0.254
	245	0.214
	250	0.206
	255	0.211
	260	0.229
	265	0.231
	270	0.229
	275	0.194
	280	0.177
	290	0.155
	300	0.084

The spectrophotometric reading of unknown spots no. 3 and no. 4 showed that there was insufficient nucleic acid to be detected. The unknown spots no. 1 and no. 2 had maximum absorption peaks at 240 and

265 millimicrons respectively. Spectrophotometric readings of the known bases gave the following absorption maximums: (1) Adenine, 265 millimicrons; (2) Guanine, 250 millimicrons; (3) Hypoxanthine, 250 millimicrons and (4) Xanthine, 265 millimicrons. These absorption maximums are in good agreement with those found in the literature for the same compounds (Beaven, et al. 1955). Figures 1 and 2 show the type of curve obtained with each of the unknowns when the wave length is plotted against the optical density.

Microbiological Assay

The optical densities obtained from the thymidine standards after 22 hours of incubation are shown in Table 6.

Table 6. Thymidine standards showing optical density for each concentration of thymidine.

Thymidine (gram moles per tube)	Optical density*
5×10^{-9}	0.186
4×10^{-9}	0.154
3×10^{-9}	0.123
2×10^{-9}	0.088
1×10^{-9}	0.0400
5×10^{-10}	0.0185
0	0.0

* Optical densities represent an average of duplicate readings.

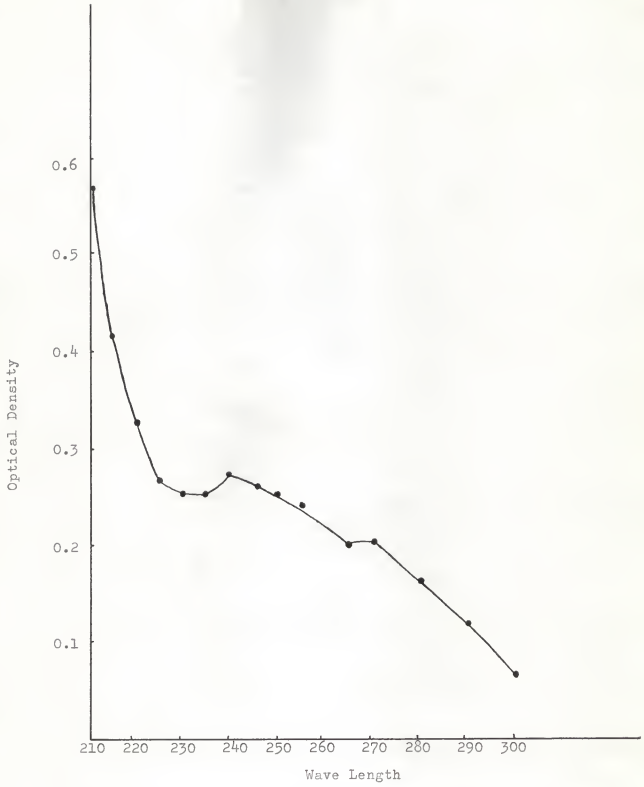

Fig. 1. Graph of unknown spot no. 1 showing point of maximum absorption.

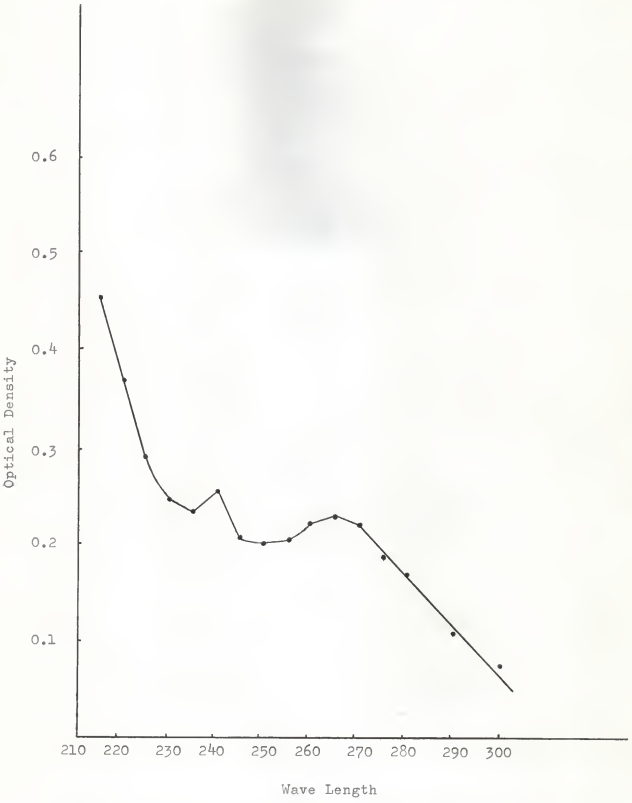

Fig. 2. Graph of unknown spot no. 2 showing point of maximum absorption.

Figure 3 shows the standard growth response curve obtained from plotting the optical densities against the concentration of thymidine in each tube.

The results obtained from the turbidity measurements of the unknown sample of nucleic acid solution after 22 hours incubation are listed in Table 7. The growth response curve obtained from various concentrations of the unknown nucleic acid sample is given in Fig. 4.

Table 7. Optical densities for various concentrations of unknown nucleic acid solution.

Unknown sample (ml)	Optical density	Optical density (uninoculated blank)	Optical density (corrected)
1.0	0.545	0.129	0.416
0.8	0.525	0.091	0.434
0.4	0.480	0.025	0.455
0.2	0.450	0.0	0.450

The optical densities obtained from the turbidity measurements of the unknown sample were greater than those for the standard growth curve for thymidine. Therefore, an interpolation of the growth response of the unknown sample to the standard growth curve could not be made. It was concluded that the amount of the deoxyribonucleosides present in the smallest concentration of the sample was greater than 5×10^{-9} gram moles.

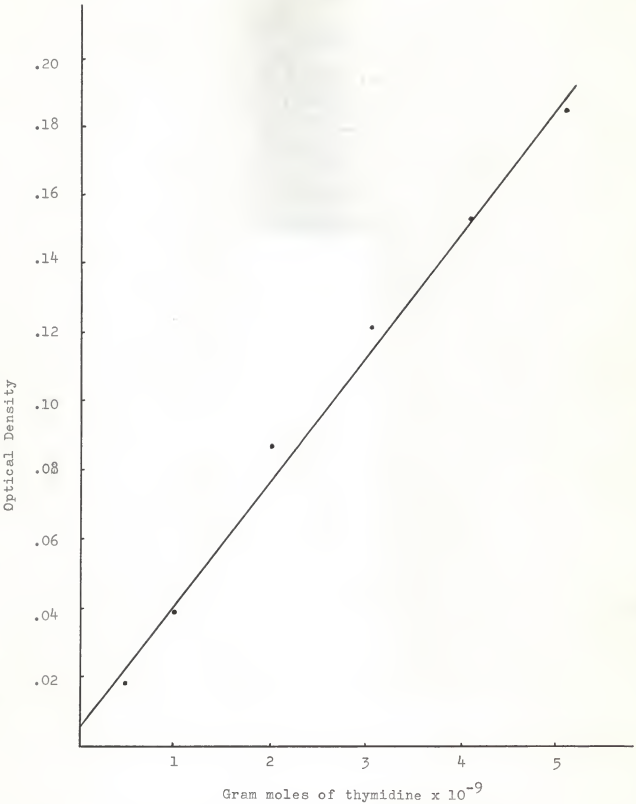

Fig. 3. Standard growth response curve for thymidine.

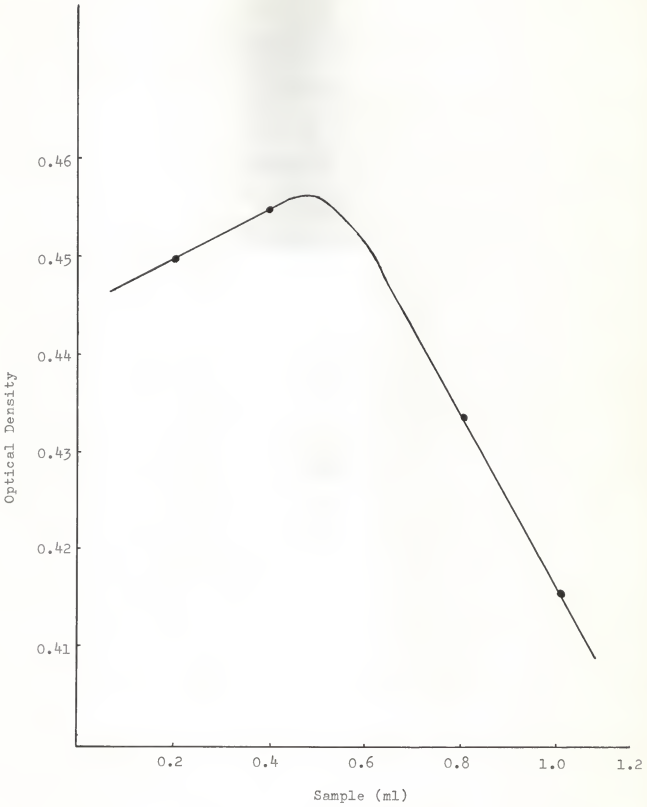

Fig. 4. Growth response for unknown nucleic acid sample

DISCUSSION

Previous studies on the nucleic acid content of influenza virus and of Newcastle disease virus indicate conflicting results both in types of nucleic acid present and in quantities of nucleic acid. The reasons for these conflicting results may be due in part to the techniques available for analysis and to the methods of purification of the virus.

The discovery of chromatography provided a means of detecting the nucleic acids on a qualitative and a quantitative basis. The adaptation of the microbiological assay procedure utilizing Thermobacterium acidophilus R-26 (Hoff-Jorgensen, 1952) has provided investigators with a sensitive method for detecting deoxyribonucleic acid. The present studies have compared the two methods of analysis and have given an indication of the specificity and sensitivity of each.

It has been shown that the microbiological assay method utilizing the organism Thermobacterium acidophilus R-26, which has a growth requirement for the deoxyribonucleosides, is more sensitive than the method employing paper chromatography and ultraviolet spectrophotometry. The growth requirements for this organism cannot be replaced by any preparation other than the deoxyribonucleosides. Therefore, it is a good organism for the assay of deoxyribonucleic acid. The method allows for the detection of microgram quantities of deoxyribonucleic acid with an accuracy of about 10 per cent (Hoff-Jorgensen, 1952).

With the chromatographic methods employed the only nucleic acid components which could be determined were adenine or hypoxanthine and guanine or xanthine. These compounds were not obtained in large enough quantities to give good readings in the spectrophotometer. No conclusions

as to the type of nucleic acid or the quantity could be drawn from these results. The apparent peaks which were observed at 240 millimicrons were caused by interference with some compound which absorbs ultraviolet in the same range as the nucleic acids. It was concluded that this interference could have been caused by trichloroacetic acid. It has been reported (Wyatt, 1955) that approximately 0.5 mg of nucleic acid is required for the detection of the nitrogenous bases with paper chromatography.

Purification by means of differential centrifugation was used in this investigation in preference to methods utilizing red blood cells or aluminum phosphate columns. By use of the latter methods large quantities of viral materials are lost. This must be considered as an important factor when a limited amount of virus is available. The problem of purification is still a major one, and it is doubtful that any procedure available will yield a pure animal virus particle.

For chromatographic and spectrophotometric analyses a slight amount of impurity may be of little significance since the techniques are of limited sensitivity. However, when microbiological methods are used the purity of the virus is a real factor. To illustrate this, an experiment was run which showed that small amounts of deoxyribonucleosides present in commercially prepared ribonucleic acid can be detected by use of the microbiological assay procedure described.

The apparent inhibition of the growth of Thermobacterium acidophilus R-26 at greater concentrations of the unknown nucleic sample (Fig. 2) cannot be explained. There was an insufficient amount of viral material to run duplicate analyses of the unknown.

SUMMARY

Recent advances in methods for the nucleic acid analysis of viruses have provided investigators with several acceptable procedures.

In this study the Leavenworth strain of Newcastle disease virus was purified by several cycles of differential centrifugation. The virus was collected by precipitation with trichloroacetic acid. After defatting the virus the dried virus particles were divided into two portions. The nucleic acids of one portion were extracted by treatment with hot 10 per cent sodium chloride, and the nitrogenous bases of the nucleic acids were determined by paper chromatography and ultraviolet spectrophotometry. The other portion of the virus was treated with deoxyribonuclease, and the nucleic acids were collected. A microbiological assay method using the organism Thermobacterium acidophilus R-26 was used for detection of the amount of deoxyribonucleosides present in the virus sample.

Results indicate that the microbiological assay method is much more sensitive than paper chromatography and that it should be the method of choice when limited quantities of virus are available.

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BIBLIOGRAPHY

Ada, G. L. and B. T. Perry
The nucleic acid content of influenza virus. Aust. J. Expt. Biol. Med. Sci. 32:453-468. 1954.

_____.
Specific differences in the nucleic acids from A and B strains of influenza virus. Nature. 175:854-855. 1955.

_____.
Influenza virus nucleic acid: Relationship between biological characteristics of the virus particle and properties of the nucleic acid. J. Genl. Microbiology. 14(3):623-633. 1956.

Bang, F. B.
Filamentous forms of Newcastle virus. Proc. Soc. Exptl. Bio. Med. 63:5-7. 1946.

Beard, J. W.
The chemical, physical and morphological properties of animal viruses. Phys. Rev. 28:349-367. 1948.

Beard, J. W., W. R. Bryan, and R. W. G. Wyckoff.
The isolation of the rabbit papilloma virus protein. J. Infect. Dis. 65:45-52. 1949.

Beaven, G. H., E. R. Holiday, and E. A. Johnson
"Optical properties of nucleic acids and their components." The Nucleic Acids. Vol. I. Academic Press. New York, N. Y. 1955.

Brachet, J.
"The biological role of the pentose nucleic acids." The Nucleic Acids. Vol. II. Academic Press. New York, N. Y. 1955.

Cunha, Raymundo, M. L. Weil, Dorothy Beard, A. R. Taylor, D. G. Sharp, and J. W. Beard. Purification and characters of NDV (California strain). J. Immunol., Virus Res. and Exptl. Chemotherapy. 55(1):69-89. 1947.

Davidson, J. N.
The biochemistry of the nucleic acids. London: Methuen and Co. New York: John Wiley and Sons. 1953.

Dorner, R. W. and C. A. Knight.
The preparation and properties of some plant virus nucleic acids. Jour. Bio. Chem. 205:959. 1953.

Franklin, R. M., H. Rubin and C. Davis.
The production, purification, and properties of Newcastle disease virus labeled with radiophosphorus. Virology. 3:96-114. 1957.

Fries, N. and B. Frosman
Quantitative determination of certain nucleic acid derivatives in pea root exudate. Physiologia Plantarum. 4:410. 1951.

Frisch-Neggemeyer, W. and L. Hoyle.
The nucleic acid and carbohydrate content of influenza virus A and of virus fractions produced by ether disintegration. J. Hyg. 2:201-211. 1954.

Grahn, A. F.
The chemical analysis of purified influenza virus A (PR8 strain) containing radioactive phosphorus. Canada J. Res. 28:186-195. 1950.

Hanson, R. P., E. Upton, C. A. Brandley and N. S. Winslow.
Heat stability of hemagglutinin of various strains of Newcastle disease virus. Pro. Soc. Exptl. Biol. Med. 70:283-287. 1949.

Hoagland, D. L.
The chemistry of viruses. Am. Rev. Biochem. 12:615. 1943.

Hoff-Jorgensen, E.
A microbiological assay of deoxyribonucleosides and deoxyribonucleic acids. Biochem. J. 50:400-403. 1952.

Hotchkiss, R. D.
The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J. Biol. Chem. 175:315-332. 1948.

_____.
"The biological role of the deoxypentose nucleic acids." The Nucleic Acids. Vol II. Academic Press. New York, N.Y. 1955.

Knight, C. A.
The preparation of highly purified PR8 influenza virus from infected mouse lungs. J. Exptl. Med. 83(1):11-24. 1946.

_____.
The nucleic acid and carbohydrate of influenza virus. J. Exptl. Med. 85:99. 1947.

_____.
Constituents of viruses. Annual Rev. Microbic. Vol. III. George Banta Publishing Co. 1949.

_____.
The chemical constitution of viruses. Advances in Virus Res. Academic Press. New York, N. Y. 1954.

Lauffer, M. A. and W. M. Stanley.
The physiological chemistry of tobacco mosaic virus protein. Chem. Rev. 24:303-321. 1939.

Loring, H. S.
Properties and hydrolytic products of nucleic acid from tobacco mosaic virus. J. Bio. Chem. 130:251. 1939.

Loring, H. S. and C. E. Schwerd.
Isolation of a macromolecular constituent with properties of the Lansing strain of Poliomyelitis viruses. Pro. Soc. Exptl. Biol. & Med. 62:291-292. 1946.

Markham, R. and J. D. Smith
A technique for the identification and estimation of purine and pyrimidine bases, nucleosides, and related substances. Biochem. J. 45:294. 1949.

_____ _____.
Chromatographic studies on nucleic acids. Biochem. J. London. 46:513. 1950.

_____ _____.
Chromatographic studies of nucleic acids. Biochem. J. London. 49:401. 1951.

Markham, R.
"Virus Nucleic Acids." Advances in Virus Res. Vol. I. Academic Press, New York, N. W. 1953.

Marshak and Vogel.
Microdetermination of purines and pyrimidines in biological materials. J. Biol. Chem. 189:597. 1951.

Miller, H. K.
The nucleic acid content of influenza virus. Virology, 2:312-320. 1956.

Miller, H. K. and W. Schlesinger.
Differentiation and purification of influenza viruses by adsorption on aluminum phosphate columns. J. Immunol. 75(2):155-160. 1955.

Pirie, N. W. and others
Purified preparation of tobacco necrosis virus (Nicotania virus II). Parasitology. 30:543-551. 1938.

Reed, L. J. and H. Muench.
A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27:493-497. May, 1938.

Sharp, D. G., A. R. Taylor, I. W. McLean, Jr., D. Beard, J. W. Bear, A. E. Feller, and J. H. Dingle. Isolation and characterization of influenza virus B virus (Lee Strain). J. Immunol. 48:129-153. 1944.

Sheffield, F. W., Wilson Smith, and G. Belyin.
Purification of influenza virus by red-cell adsorption and elution. British J. Exptl. Pathol. 35(3):214-222. 1954.

Smith, J. D. and R. Markham
Hydrolysis of ribonucleic acids. Biochem. J. 46:509. 1950.

Stanley, W. M.
The isolation and properties of tobacco ring spot virus. J. Biol. Chem. 129:405-428. 1939.

_____.
An evaluation of methods for the concentration and purification of influenza virus. J. Exptl. Med. 79:235-266. 1944.

Stanley, W. M. and Max A. Lauffer.
"Chemical and Physical Procedures." Viral and Rickettsial Infections of Man. 2nd edition. J. B. Lippincott Co. 1952. London, Philadelphia, Montreal.

Taylor, A. R. and others
Nucleic acid of the papilloma virus protein. J. Infect. Dis. 71:110. 1942.

Taylor, A. R.
Chemical analysis of the influenza viruses A (PR8 strain) and B (Lee strain) and the Swine Influenza virus. J. Biol. Chem. 153:675-686. 1944.

Taylor, A. R., D. G. Sharp, D. Beard, J. W. Beard, J. H. Dingle and A. E. Feller. Isolation and characterization of influenza A virus (PR8 strain). J. Immunol. 47:261-282. 1943.

Taylor, A. R., D. G. Sharp, D. Beard, and J. W. Beard
Isolation and properties of the equine encephalomyelitis virus. J. Infect. Dis. 72:31-41. 1943.

Taylor, A. R., D. G. Sharp, I. W. McLean, Jr., D. Beard, and J. W. Beard. Concentration and purification of influenza virus for the preparation of vaccines. J. Immunol. 50:291-316. 1945.

Vischer, E. and E. Chargaff.
The seperation and quantitative estimation of purines and pyrimidines in minute amounts. J. Biol. Chem. 176:703. 1948.

Watson, J. D. and F. D. Crick.
Genetial implications of the structure of deoxyribonucleic acid. Nature. 171:963-996. 1953.

Wyatt, G. R.
The purine and pyrimidine composition of deoxypentose nucleic acids. Biochem. J. 48:584. 1951.

_____.
The nucleic acids of some insect viruses. J. Genl. Physiol. 36(2): 201-205. 1952.

Wyatt, G. R.
"Separation of Nucleic Acid Components by Chromotography on Filter Paper." The Nucleic Acids. Vol. I. Academic Press, New York, N. Y. 1955.

Wyatt, G. R. and S. S. Cohen.
A new pyrimidine base from bacteriophage nucleic acids. Nature. 170:1072. 1953.

A COMPARISON OF THE CHROMATOGRAPHIC AND THE MICROBIOLOGICAL ASSAY METHODS FOR THE DETERMINATION OF THE NUCLEIC ACIDS IN NEWCASTLE VIRUS

by

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The purpose of this study was to compare the chromatographic and the microbiological methods for the determination of certain bases of nucleic acid present in Newcastle disease virus (NDV).

Previous studies have given conflicting results as to the amount and types of nucleic acid present in animal viruses. The discrepancies are attributed to the difficulty of obtaining a highly purified virus (investigators used virus of varying degrees of purity) and the difficulty of obtaining sufficient quantities of virus to perform analyses.

The Leavenworth Kansas Strain of NDV was grown in the chorio-allantoic cavity of 10-day old hens eggs and the fluids harvested after 72 hours incubation at 37° C. The virus was purified by first passing the fluids through several thicknesses of gauze, to remove heavy membraneous material, and then the virus was concentrated and purified by differential cycles of high and low speed centrifugation at 59,000 g for 2 hours and 2,000 g for 20 minutes. After centrifugation at high speed the virus particles were suspended in Ringer's solution.

Hemagglutination titrations indicated that the original virus infected allantoic fluids had a titer of 1:320 to 1:640; a two fold increase in titer was obtained after the first centrifugation.

The virus was extracted from the concentrated saline solution by treatment with an equal volume of cold 30 per cent trichloroacetic acid and was collected by centrifugation. After dialysis for 17 hours in running water, the virus particles were dried and defatted by use of a mixture of chloroform and methanol. At this point the total quantity of virus was divided into equal parts, one portion used for a

chromatographic analysis and the other for microbiological assay.

For the chromatographic analysis, the nucleic acids were extracted from the virus particles by thrice boiling the virus particles in 10 per cent sodium chloride and were precipitated from the supernatants by the addition of ethanol. After washings with 66 per cent ethanol, 95 per cent ethanol and ethyl ether, the nucleic acid was evaporated to dryness.

The nucleic acids were hydrolysed by boiling with 1 N-HCl for one hour. The hydrolysed material was placed on a strip of filter paper and allowed to dry. After saturation of the chromatocab with the solvent, isopropanol-HCl-water, the strip of filter paper was placed in the chromatocab and the bases of the nucleic acids present were separated by capillary action of the solvent. The solvent was allowed to move a minimum of 38 centimeters on the filter paper before being removed. The nucleic acid spots were detected under an ultraviolet lamp.

Spectrophotometric determinations were made of the nucleic acids by first eluting the spots detected under the ultraviolet lamp in 1 N-HCl and then reading their absorption to ultraviolet in a Beckman DU spectrophotometer.

The nucleic acids for the microbiological determination were extracted from the defatted virus by treatment with deoxyribonuclease. The solution of nucleic acid was assayed in four different concentrations.

The organism used for the microbiological assay, Thermobacterium acidophilus R-26, has a specific growth requirement for the deoxyribosides. It was prepared by growth in an inoculum medium consisting of thymidine,

deoxyribonucleic acid, assay medium and water. After growth for 24 hours the bacteria were centrifuged and suspended in saline.

The unknown nucleic acid solution prepared from the virus was added to screw cap tubes in the following amounts: 1.0 ml, 0.8 ml, 0.4 ml and 0.2 ml. The volume of each was brought to one ml with distilled water and two ml of assay medium was added to each tube; the tubes were autoclaved, cooled and inoculated with one drop of the suspension containing the bacterium, Thermobacterium acidophilus R-26. After 22 hours incubation at 37° C the turbidities were read in a Beckman spectrophotometer. The concentration of deoxyriboside present in each concentration of the unknown was determined by interpolation of the results on a standard growth curve for thymidine.

The results indicate that the microbiological method of analysis is much more specific and sensitive than the chromatographic analysis. There was an insufficient amount of virus in the chromatographic analysis to draw any conclusions as to the type or quantity of nucleic acid present. However, with the microbiological method the presence of deoxyribonucleic acid was confirmed.