CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

II. PROPERTIES OF NUCLEIC ACID OBTAINED FROM VACCINE VIRUS

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Highly purified preparations of elementary bodies of vaccinia contain constant amounts of fat, carbohydrate, and protein which occur in concentrations not materially different from those found in bacterial cells (1). In addition, evidence for the presence of nucleic acid in vaccine virus has been obtained through studies of ultraviolet absorption spectra of alkaline extracts of purified elementary bodies (2), and by means of the Feulgen reaction this acid has been identified as the thymus type. In order to establish more firmly the nature of the nucleic acid in vaccine virus and to extend the quantitative data obtained previously (2), the present study was carried out.

Material and Methods

The nucleic acid was obtained from elementary bodies which had already been extracted with ether and alcohol for studies of lipids (1). A complete description of the technique by which the purified elementary bodies were obtained has been recorded elsewhere (1, 3). A number of techniques for the isolation of nucleic acid from yeast and bacteria were compared before any attempt was made to isolate it from vaccine virus. The method of Levene (4), with certain minor modifications, proved most satisfactory for the study of the very small quantities of material at our disposal.

EXPERIMENTAL

Isolation of Nucleic Acid

After preliminary experiments nucleic acid from elementary bodies of vaccinia was isolated in the manner described in the following experiment.

548 mg. of dry, ether-alcohol extracted elementary bodies were treated with 50 cc. of 5 per cent sodium hydroxide. The mixture was heated for 30 minutes which resulted in the solution of the virus with the exception of a small residue. The hydrogen ion concentration was then adjusted to pH 7.5 with glacial acetic acid. 20 cc. of colloidal iron (5 per cent Fe₂O₃) were then added slowly with the immediate formation of a heavy precipitate. Filtration was carried out overnight at 4°C. The filtrate was reduced to 15 cc. in vacuo and treated with two volumes of absolute methyl alcohol containing 2 per
 cent hydrochloric acid. A white precipitate formed immediately and after 10 minutes was collected by centrifugation and washed with methyl alcohol until a negative test for chloride was obtained. The precipitate was dried in vacuo over calcium chloride. 15.6 mg. of a brownish white powder were obtained.

**Spectroscopic Characterization of the Nucleic Acid.**—Certain spectroscopic studies of alkaline extracts of elementary bodies of vaccinia have been previously reported (2). Caspersson (5), Lavin and Stanley (6), and others have recently extended the use of ultraviolet spectroscopy to the characterization of nucleic acid. The intense absorption of light by nucleic acids in the range from 2600 to 2650 Å has been shown to be due to purine and pyrimidine compounds, and is thus more or less unequivocal evidence of the presence or absence of nucleic acid if interfering substances are to some extent removed.

An absorption curve of nucleic acid isolated from vaccine virus, obtained

![Absorption curve of nucleic acid](image-url)
with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph, is shown in Fig. 1. The concentration of nucleic acid from vaccine virus calculated from the phosphorus content was in close agreement with the concentration determined by spectrophotometric methods (2). Moreover, the absorption curve of nucleic acid isolated from the virus is similar to that obtained from a solution of nucleic acid isolated from thymus gland. It must be remembered that ultraviolet spectroscopy under the conditions employed does not distinguish between ribo- and thymonucleic acid.

Chemical Characterization of the Nucleic Acid.—Tests made on the material isolated in the manner described above showed it to be soluble and insoluble in dilute alkali and acid, respectively. It gave a negative biuret, negative ninhydrin, and a negative test for inorganic phosphate. A small amount boiled with 10 per cent sulfuric acid for 2 minutes gave a negative test for ribose with orcinol (Bial’s test). Following hydrolysis a positive reaction was obtained by means of Schiff’s base (Feulgen reaction) used in accordance with the method of Widström (7). In the absence of lipid and certain extraneous aldehydes, a positive reaction with this test is presumptive evidence of the presence of desoxyribose, a constituent of thymonucleic acid. A blue color obtained with diphenylamine (8) was similarly indicative of the presence of this pentose. A test for desoxyribose, in which the tryptophane reaction of Thomas was employed, was also positive (9).

A partial elementary analysis of a sample of the material, isolated from vaccine virus and dried to constant weight, revealed nitrogen, phosphorus, and carbon in very close agreement with analytical data presented by Levene for nucleic acid prepared from the thymus gland (4). The agreement of experimental values with the theoretical was quite satisfactory as indicated by the results recorded in Table I. Although too much importance cannot be attached to the results of elementary analysis of an amorphous substance, similarity of experimental and theoretical values together with the findings in preliminary qualitative tests described above,

<table>
<thead>
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<th>TABLE I</th>
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<td><strong>Elementary Analysis of Nucleic Acid Isolated from Vaccine Virus</strong></td>
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<tr>
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</tr>
<tr>
<td>Found in virus material</td>
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<td>Calculated for a tetrabase</td>
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were deemed indications of sufficient purity of the material under investigation to warrant further study.

A 5 mg. sample of the material was dissolved in a micro-reflux tube and heated on a boiling water bath with 10 per cent H$_2$SO$_4$ for 60 minutes. Concentrated ammonia was added gradually to a portion of the hot hydrolysate until the solution was quite alkaline. A fine white crystalline precipitate appeared almost immediately. This was separated by centrifugation and after being washed twice with hot ammonia water was dried in vacuo. Insufficient material was obtained for an elementary analysis, but qualitative tests for guanine were strongly positive. A larger sample of known thymonucleic acid, carried through the same procedure, gave similar crystals which analysis proved to be guanine.

The supernatant fluid from the guanine crystals obtained from vaccine virus was next boiled until free of ammonia. An excess of picric acid was then added and the mixture was allowed to stand; after several hours a few needle-like crystals separated from solution. There was insufficient quantity of crystals for elementary analysis, but qualitative tests for adenine picrate were positive.

Pyrimidine derivatives—cytosine, uracil, thymine—are separated from nucleic acid with great difficulty; consequently, the separation could not be accomplished on the minute quantity of material available. Nevertheless, certain color tests, introduced by Wheeler and Johnson (10a) and Harkins and Johnson (10b) are fairly satisfactory and were performed on the remaining portion of the hydrolysate obtained by the treatment of 5 mg. of nucleic acid with H$_2$SO$_4$. This portion was made neutral with NaOH after which bromine was added followed by an excess of barium hydroxide. A faint purple ring of barium dialurate was obtained. This color is given by cytosine and by uracil; hence, either one or both of these substances might have been present in the material examined.

The sample, on which the color test for uracil and cytosine was made, was next placed in a tube and distilled into a small quantity of water. Upon alkalinization, addition of $o$-aminobenzaldehyde, acidification with HCl, and realkalinization with sodium bicarbonate of the distillate, a faint blue fluorescence was obtained. This was due to 3-oxyquinaldine and indicated, according to Harkins and Johnson (10b), the presence of thymine in the material examined.

The experiment just described showed that guanine, adenine, and thymine were present in the nucleic acid examined. In addition, it was revealed that either one or both of the substances, cytosine and uracil, were also present. Inasmuch as thymine was present, it became obvious that the material under investigation contained thymonucleic acid; however, no quantitative estimation was possible. Uracil characterizes ribonucleic acid; but both uracil and cytosine react positively to the color tests used. Therefore, it was impossible to determine definitely from the results of the experiment whether yeast nucleic acid was present or not. Moreover, if it were present no idea of the amount was obtained. To clarify the situation and to get more information regarding the kind and amount of nucleic acid in our material, enzymatic studies were undertaken.
Enzymatic Characterization of the Nucleic Acid.—The use of enzymes in the differentiation of closely related substances is too well known to require emphasis. Jones (11), Dubos and Thompson (12), Schmidt and Levene (13), and Kunitz (14) have called attention to a heat stable enzyme in pancreas which depolymerizes ribonucleic acid. Nucleic acid containing desoxyribose, i.e., thymonucleic acid, is apparently unaffected. Methods for the use of ribonuclease in the quantitative study of yeast nucleic acid have been described in detail by Schmidt and Levene (13). Through the kindness of Dr. Kunitz a sufficient supply of crystalline ribonuclease was made available for a study of its effect on the nucleic acid isolated from vaccine virus.

The method of Schmidt and Levene (13) for the determination of the activity of ribonuclease, useful as it is as a macro-analytical technique, was not found satisfactory for tests on the minute amounts of nucleic acid available. A technique developed by MacFadyen (15), however, in which unchanged nucleic acid is precipitated with uranyl chloride, was found to precipitate minute amounts of the unchanged acid from solution at pH 2. Nucleotides are precipitated only at higher pH values. By this technique it is possible to effect complete precipitation of less than 0.5 mg. of nucleic acid when the dilution is carefully controlled. Therefore, by means of MacFadyen’s method and ribonuclease, a study of our material was made in the following manner.

2.5 mg. of nucleic acid isolated from vaccine virus were made up to 5 cc. with a buffer solution, pH 6.7, and incubated at 37°C. with 0.5 mg. of crystalline ribonuclease. Samples of 1 cc. were taken every 30 minutes for 2 hours, precipitated with 0.2 cc. of uranyl chloride-trichloracetic acid reagent, allowed to stand 10 minutes at room temperature, and then centrifuged at 2000 R.P.M. The supernatant fluid was decanted and the inside of the tube and precipitate were washed with 1 cc. of 10 per cent trichloracetic acid; centrifugation was again carried out at 2000 R.P.M., after which the supernatant fluid was discarded. The precipitate was digested and the phosphorus was determined according to the method of Kirk (16). The amounts of unchanged nucleic acid, i.e., nucleic acid precipitable with the uranyl chloride-trichloracetic acid reagent, expressed in terms of milligrams of phosphorus have been plotted against time of enzymatic action as shown in Fig. 2. As a control on the activity of the enzyme, a known sample of yeast nucleic acid, purified according to the method of Levene, was used. The rapid depolymerization of the known yeast nucleic acid is indicated by the decreasing amounts of nucleic acid precipitable by uranyl chloride reagent which have also been plotted in Fig. 2.

The slight decrease in the amount of virus nucleic acid precipitable with the uranyl chloride-trichloracetic acid reagent after digestion with ribonuclease for 90 minutes (Fig. 2) may indicate the presence of a small amount of yeast nucleic acid. On the other hand, some spontaneous depolymeriza-
tion of the vaccine virus nucleic acid may have occurred independently of enzymatic activity. Known solutions of thymonucleic acid do not change appreciably, however, under similar conditions of incubation and precipitation. In any event, the results of the experiment recorded in Fig. 2 clearly indicate that at least 90 per cent of the material subjected to the action of ribonuclease was not yeast nucleic acid. It remained, however, to determine whether this 90 per cent was thymonucleic acid or not. This was accomplished by the use of diphenylamine reagent.

**Quantitative Estimation of Nucleic Acid in Vaccine Virus**

The carbohydrate released by the hydrolysis of thymonucleic acid gives an intense blue color with diphenylamine in acid solution under definite conditions (8). The color produced is stable and is proportional to the concentration of thymonucleic acid in the original solution. According to Bielschowsky and Klein (17) the color reaction is due to the thyminose content and is given only by the carbohydrates bound to the purine bases in the thymonucleic acid molecule (18). A modification of existing techniques, in which diphenylamine for the quantitative determination of thymonucleic acid is employed, resulted in a method suitable for the micro-determination of nucleic acid in vaccine virus as is indicated by the following experiment.

10 mg. of purified elementary bodies suspended in 2 cc. of 0.1 N HCl containing 1 per cent crystalline pepsin\(^1\) were placed in a graduated centrifuge tube, shaken occasionally,

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\(^1\) The crystalline pepsin used in this study was generously supplied by Dr. J. H. Northrop. A study of the effects of crystalline enzymes on elementary bodies of vaccinia will be published soon.
and incubated at 37°C for 2 hours. At the end of this time, a milky solution of the virus was obtained; no formed particles remained in suspension as demonstrated by Morosow's stain. 1 cc. of 5 N NaOH was next added; this resulted in complete clarification of the virus. The solution was next heated in a boiling water bath, the water lost by evaporation being replaced. The solution was then cooled, and 0.5 cc. of glacial acetic acid were added, followed by three volumes of methyl alcohol. A flocculent precipitate settled out on standing in the cold for 1 hour. The material was centrifuged for 5 minutes at 2000 r.f.m. and the supernatant fluid was decanted. To the precipitate were added 2 cc. of a reagent containing 1 per cent diphenylamine in 1:40 sulfuric acid-glacial acetic acid mixture and 1 cc. of water. The precipitate went into solution instantly, after which it was heated on a boiling water bath for 10 minutes, water being added to replace that lost on evaporation. A deep blue color appeared on cooling which was maximum after 30 minutes. This color was compared in a colorimeter with that given by a known concentration of thymonucleic acid treated in the same manner with

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>Amount of virus</th>
<th>Nucleic acid by color produced with diphenylamine</th>
<th>Phosphorus</th>
<th>Nucleic acid calculated from phosphorus content</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>10.0</td>
<td>5.2</td>
<td>0.58</td>
<td>6.4</td>
</tr>
<tr>
<td>57</td>
<td>10.0</td>
<td>6.0</td>
<td>0.58</td>
<td>6.4</td>
</tr>
<tr>
<td>66</td>
<td>10.0</td>
<td>5.6</td>
<td>0.59</td>
<td>6.6</td>
</tr>
<tr>
<td>69</td>
<td>10.0</td>
<td>5.5</td>
<td>0.62</td>
<td>6.8</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>5.6</td>
<td>0.59</td>
<td>6.6</td>
</tr>
</tbody>
</table>

The values for phosphorus given in this table have been previously reported (1). From the data summarized in Table II it appears that 5.6 per cent of the elementary body of vaccinia consists of thymonucleic acid. If one assumes that the major part of the phosphorus from elementary bodies comes from the nucleic acid fraction, another set of calculations for nucleic acid is possible. Nucleic acid values calculated on this assumption are shown in Table II and agree favorably with direct estimation by means of the diphenylamine reagent.

**DISCUSSION**

Employing a relatively large amount of material, we have been able to obtain unequivocal evidence that the nucleic acid in vaccine virus is largely of the thymus variety. Failure of the major portion of isolated nucleic
NUCLEIC ACID FROM VACCINE VIRUS

acid to undergo depolymerization in the presence of crystalline ribonuclease demonstrates conclusively that not more than 10 per cent can be of the yeast type. The evidence for the presence of a small amount of ribonucleic acid in the vaccine virus rests solely thus far on the fact that there is a slight decrease in the nucleic acid precipitated with uranyl chloride-trichloracetic acid reagent following incubation with ribonuclease. In this study, as in previous ones, negative tests for ribose with Bial's reagent were obtained. It is true that ribonucleic acid is more soluble than thymonucleic acid, a fact which may have resulted in proportionally greater losses of the ribonucleic acid in the process of isolation of our material.

Although on previous occasions it has been stated that thymonucleic acid occurs in the viruses of vaccinia and psittacosis (2, 19), the data presented at this time, so far as we are aware, represent the most conclusive evidence that this type of nucleic acid occurs in a virus. This information is particularly interesting in view of the extensive work on nucleic acid in other viruses (6, 20–23) in which the ribonucleic type was found. The biological significance of our findings is not at the moment obvious.

SUMMARY

It has been possible by means of classical chemical methods to isolate and to characterize to some extent the nucleic acid of elementary bodies of vaccinia.

Determination by means of diphenylamine reagent revealed that the major part of the nucleic acid was of the thymus type. This was further substantiated by its stability in the presence of ribonuclease, less than 10 per cent undergoing depolymerization during prolonged incubation at 37°C.

By the technique employed, at least 5.6 per cent of the virus was shown to be thymonucleic acid. This amount agreed favorably with the value calculated from the non-lipid organic phosphorus of elementary bodies on the assumption that the phosphorus bound in the organic form was derived principally from nucleic acid.

BIBLIOGRAPHY