SOME CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

By JOSEPH E. SMADEL, M.D., GEORGE I. LAVIN, Ph.D., and RENÉ J. DUBOS, Ph.D.

(From the Hospital and the Laboratories of The Rockefeller Institute for Medical Research)

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The elementary body of vaccinia, which apparently constitutes the virus, has a complex antigenic structure. Infection with this agent gives rise to at least three types of antibodies (1), namely, neutralizing substances, antibodies against a heat-labile soluble substance (L), and antibodies against a heat-stable soluble substance (S). The last two types of antibodies precipitate with solutions of their homologous antigens and agglutinate elementary bodies but have little or no power to neutralize the virus (2, 3). Neutralizing substances are generally demonstrable by an *in vivo* technique; for example, when mixed with the virus they render it incapable of producing a lesion in animals; these substances have also been absorbed *in vitro* from immune serum with virus-infected tissue (4) and with active elementary bodies of vaccinia (2, 5).

If one substance in elementary bodies is responsible for eliciting the production of neutralizing antibodies by the infected animal then it would seem that the most direct approach to a study of such a material would be to break up the virus particle and examine the various component parts. Earlier attempts to disrupt elementary bodies by sonic vibration were unsuccessful (6); moreover, when the virus was subjected to grinding in a ball mill in the cold the results obtained in studies with neutral saline extracts of this material were not reproducible (7).

The present work which deals with the determination of certain constituents in intact elementary bodies of vaccinia extends the earlier observations reported from this laboratory (8). In addition, a method of obtaining extracts from elementary bodies is presented, together with observations on the extracted materials. At this time the evidence for the occurrence of thymus nucleic acid in the virus of vaccinia will be given in detail.

Materials and Methods

Elementary Bodies of Vaccinia.—Purified preparations of virus which had a close correlation between the number of infective units and the number of elementary bodies were employed in the present experiments. The methods of preparing the elementary

bodies and of estimating the purity of the resulting material have been recently described (9). In brief, the technique of preparation was that of Craigie (10), plus additional treatment designed to remove residual protein material adsorbed on the elementary bodies and also to remove excess salts, especially phosphates, which were present in the buffer solutions used in the early washings. The number of infective units in the pooled suspensions of elementary bodies was determined by the 50 per cent end point method before the preparations were dried from the frozen state. The number of elementary bodies was estimated by dividing the total weight of the desiccated material by the calculated weight of a single dehydrated elementary body, *i.e.*, 5.34×10^{-15} gm. The ratio of infective units to elementary bodies served as a means of estimating the purity of the virus preparations.

Precipitin Reaction.—Extracts of elementary bodies containing material soluble in the pH range near neutrality were tested for their precipitability with optimal amounts of anti-soluble substance antibodies. Hyperimmune rabbit serum absorbed free of S antibodies constituted the L antiserum used in the present experiments. S antiserum was obtained from rabbits hyperimmunized with heat-inactivated elementary bodies of vaccinia. 0.25 cc. amounts of varying dilutions of the solution to be tested were mixed with an equal volume of diluted antiserum in small pyrex tubes and were incubated overnight in closed racks at 56°C.

Analytical Methods.—Nitrogen was estimated by the method of Conway and Byrne (11), and phosphorus by a modification of the colorimetric method of Youngburg and Youngburg (12). Digestion with sulfuric acid and superoxol was carried out for 40 minutes both for nitrogen and for phosphorus determinations. The presence of yeast nucleic acid was investigated by testing for pentose with Bial's orcinol reagent. The Feulgen reaction for thymus nucleic acid was carried out according to the technique described by Widström (13). Sörensen's colorimetric method (14) was employed for the detection of glucosamine; in all cases the color developed was too faint to permit quantitative estimation of this substance.

Spectroscopic Methods.—Spectroscopic methods of analysis have been applied to the extracts of elementary bodies wherever possible. The desirability of identifying and estimating the quantity of a given substance by several different technical procedures need not be emphasized. Spectroscopic measurements are particularly well adapted to the present problem since previous work has shown that the purine and pyrimidine constituents of nucleic acid have an intense absorption band in the ultraviolet region of the spectrum with the maximum of absorption at about 2600 Å. This subject has been reviewed recently by Casperson (15). Furthermore, it has been found that proteins containing the aromatic amino acids tryptophane, tyrosine, and phenylalanine have an absorption band in the ultraviolet with the maximum of absorption at about 2800 Å (16). Finally, nucleoproteins in purified preparations of several plant viruses (17) have been shown to have an absorption band in the ultraviolet region of the spectrum with a maximum at about 2650 Å.

If it can be shown that the materials in question do not contain interfering substances, the method can be made quantitative. Such a procedure has been used to obtain an estimate of the nucleic acid concentration. Values of the absorption coefficient α are obtained on solutions of known concentration. The absorption coefficient is defined

$$\alpha = \frac{1}{d} \log \frac{I_0}{I}$$

in which c = concentration of absorbing substance in solution in mg. per cc., l = cell thickness in cm., I_0 = intensity of light entering the medium, I = intensity remaining after its passage through length l. When α is known it is only necessary to determine the value of $\log \frac{I_0}{I}$ for the unknown solution and substitute in the equation to find the value of c, the concentration. A Spekker spectrophotometer and a small Hilger quartz spectrograph were used to obtain these measurements.

The absorption curves were obtained with the aid of a spectrophotometer which has a high tension spark as its light source. More detailed absorption bands have been obtained by the use of a continuous light source instead of the line spectrum given by the high tension spark (18); photographs reproduced in this paper were taken with the hydrogen discharge tube as the light source. Spectroscopic studies were made immediately, or within a few hours, after extracts were obtained.

RESULTS

Observations on Intact Elementary Bodies

Feulgen Reaction.—Various observers (19, 20) have reported that elementary bodies of vaccinia lying within cells failed to stain by the Feulgen method, hence, presumably did not contain thymus nucleic acid. This was in contrast to the Guarnieri bodies which were found to give a positive reaction by Feulgen's technique. Furthermore, dry films of washed elementary bodies prepared on cover slips likewise failed to show any indication of the presence of thymus nucleic acid (20). When, however, the Feulgen technique was applied to a preparation of elementary bodies resuspended in aqueous media (21, 20), the virus particles gave a positive reaction. Our experience with washed elementary bodies has been in complete agreement with that of previous workers; moreover, we concur in the opinion of Bland and Robinow (20), who attributed the difference in the results obtained by slide and test tube methods to a quantitative factor.

A rough estimation of the amount of thymus nucleic acid in the intact elementary bodies was attempted by means of the Feulgen technique. A description of a typical experiment follows.

Two aliquots of 5 mg. each of dried elementary bodies were suspended in 1 cc. volumes of buffer solution, pH 2.3. One was chilled in cracked ice while the other was heated at 80°C. for 10 minutes. Both samples were then centrifuged quickly; the clear supernatant fluids were removed and the sediments resuspended in 1 cc. of buffer solution, pH 2.3. All four specimens were chilled to 0°C. and treated with 0.2 cc. of Schiff's reagent in the cold. 10 minutes later the heated sediment showed a strongly positive reaction but the other three materials were negative. The positive reaction was given by the particulate matter of the heated bodies, for, as the virus particles settled, the purple color became concentrated at the bottom of the tube and the supernatant fluid became colorless. During the next 20 minutes the positive reaction increased somewhat in intensity; furthermore, the unheated sediment took on a faint purple color.

The supernatant fluids which had been freed of elementary bodies before testing remained colorless. The intensity of the reaction of the hydrolyzed sediment was of the order of that given by a solution containing 0.1 mg. of thymus nucleic acid. Unheated elementary bodies which gave a slightly positive Feulgen reaction in the preceding experiment remained, on the contrary, completely negative when the elementary bodies were extracted with ethyl ether prior to the test.

In view of the results of the experiment recorded above, it is not unreasonable to assume that the positive test obtained with the hydrolyzed elementary bodies previously washed with ether was due to thymus nucleic acid since the Feulgen reaction is fairly specific for this compound when carried out according to Widström's method (13). All attempts to demonstrate yeast nucleic acid by means of the Bial reaction remained negative, even when as much as 30 mg. of elementary bodies were employed.

Phosphorus Content.—Determinations of total phosphorus were carried out on 10 mg. amounts of elementary bodies from each of four consecutive preparations. The elementary bodies were washed in dilute acetic acid and in distilled water to remove inorganic phosphates; 10 mg. samples of such washed virus preparations failed to give a test for inorganic phosphorus. Prolonged digestion (40 minutes) was necessary to obtain complete release of the organic phosphorus.

Under these conditions, the four lots of elementary bodies gave phosphorus analyses corresponding respectively to 0.38, 0.44, 0.45, 0.45 per cent of their dry weight. It may be worth recording that these values are well below those commonly obtained for bacteria; for instance, washed cells of *Diplococcus pneumoniae* (R variant), *Streptococcus hemolyticus* (group D), *Escherichia coli*, and *Klebsiella pneumoniae* were found to contain respectively 0.8, 1.25, 1.25, and 1.3 per cent of their dry weight as phosphorus when analyzed under similar conditions.

Presence of Glucosamine.—Six lots of elementary bodies were tested for the presence of glucosamine by Sörensen's method. In all cases, the amount of color developed from 7.5 mg. of virus preparation corresponded to that given by 0.15 to 0.45 mg. pure glucosamine. These amounts are far too small to permit of quantitative treatment. However, since no color developed in the control preparations, *i.e.*, when elementary bodies and acetyl acetone were not heated prior to addition of Ehrlich's reagent, the qualitative results seem to warrant the conclusion that the materials tested contained a small amount of glucosamine.

Extraction of Elementary Bodies

Preliminary observations suggested that elementary bodies of vaccinia were partially dissolved by concentrated solutions of sodium hydroxide.

This was indicated by a marked diminution in opacity of the virus suspension, by a change from jet black to tan in the staining of the virus particles when Morosow's silver technique (22) was applied, and, finally, by the release of nucleoprotein material into solution. Progressively milder methods of extraction were employed in the work until a procedure was evolved which seemed to be most useful for the present purposes. Protocols of two of the later experiments will be sufficient for illustration.

Experiment 1.—153 mg. of dry elementary bodies from a preparation having an infective unit-elementary body ratio of 1:2.9 were extracted at room temperature for 24 hours with 2 successive lots of 40 cc. of ethyl ether. 13.7 mg. of ether-extractable material were obtained after evaporaton and desiccation; this entire fraction contained 0.035 mg. of phosphorus. Residual ether was removed from the elementary bodies and 15 cc. of distilled water were added. The suspension was stored overnight at 0°C. in order to permit the virus particles to become completely hydrated; this measure facilitated the extraction of virus by decreasing the necessary time of exposure to alkali and heat. Sufficient amounts of distilled water and N/1 sodium hydroxide were added to bring the volume to 20 cc. and the concentration of alkali to N/20. The milky appearing suspension of virus was rapidly brought to a temperature of 56°C. and 15 minutes later it was quickly cooled to 0°C. The foregoing treatment changed the characteristics of the suspension; it was now only faintly opalescent and had become definitely viscid. When the pH was brought to about 8.5 by means of N/10 hydrochloric acid a slight increase in opalescence and in viscosity occurred. A small portion of this material was diluted a hundredfold with distilled water and examined microscopically. Morosow's stain (22) revealed (*a*) a large amount of amorphous tan material which was interpreted as indicating the presence of dissolved protein, (*b*) very many discrete round particles about the size of elementary bodies but tan in color, and (*c*) a few black spherical particles which occurred singly or in small aggregates and stained as do ordinary elementary bodies. A drop of this same lot of material, when subjected to dark-field examination, showed a few brilliantly illuminated particles which appeared to be typical elementary bodies, and a vast number of particles of uniform size which appeared smaller and less brilliant than elementary bodies.

After storage overnight at 0°C., the pH of the alkali-treated suspension had dropped to 8.0 and approximately 1 cc. of white precipitate had appeared. Horizontal and angle centrifugation failed to yield a clear supernatant solution; consequently, the material was run in the concentration centrifuge (23) at 30,000 R.P.M. for 20 minutes. 26 cc. of water-clear supernatant fluid and approximately 0.2 cc. of firm light gray semi-translucent gelatinous sediment were obtained. The former was labeled extract A. The pellet of sediment was removed in one piece and suspended in 20 cc. of a N/50 concentration of sodium hydroxide. After storage at 0°C. for 18 hours ultracentrifugation was again employed. The unneutralized clear supernatant fluid was designated extract B; the sediment was again extracted in a manner identical with that employed for the first alkaline extract. 20 cc. of water-clear supernatant fluid, pH 8.0, were obtained and labeled extract C. About 0.15 cc. of sediment having the firm dry gel-like appearance of the first ultra-sediment were suspended in 20 cc. of N/40 sodium hydroxide and stored overnight in the cold. Ultracentrifugation of the unneutralized suspension at 30,000 R.P.M. for 20 minutes yielded a clear supernatant fluid, extract D, and approxi-

mately 0.4 cc. of a soft semigelatinous sediment. This sediment, designated alkali-insoluble residue, was readily resuspended in 10 cc. of distilled water, forming a uniformly opalescent suspension which was stable. No change in appearance of the suspension of insoluble residue was noted when the pH was lowered to 1.2 with N/1 hydrochloric acid; however, when the pH was raised to 4.5 opalescence increased and at pH 5.0 marked flocculation occurred. Precipitated material and fluid were placed in a cellophane sack and dialysed at 0°C. against repeated changes of distilled water for several days; the pH after dialysis was 6.8. One drop of this material was shaken vigorously in 7 cc. of distilled water and then dry films were prepared. Morosow's stain revealed only slight amounts of amorphous tan material which formed a background in the preparation. Large brown masses scattered throughout the microscopic field seemed to be made up

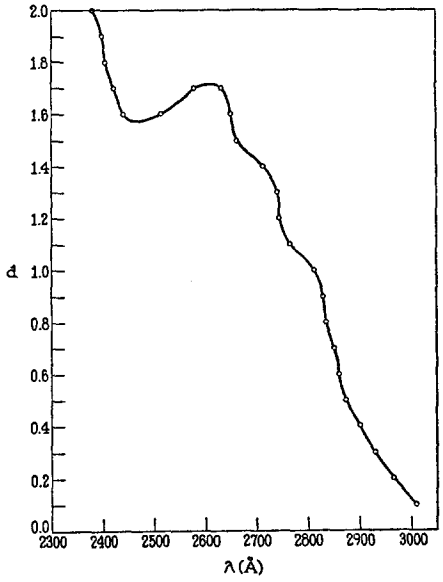

FIG. 1. Absorption curve of extract A, Experiment 1. d (optical density) = $\log \frac{I_0}{I}$.

of particles of uniform magnitude which were tan in color and about the size of elementary bodies. In addition, single spheres of light brown color the size of elementary bodies were numerous, but no virus particles with the typical black appearance were noted. The dialysed alkali-insoluble residue after thorough desiccation was recovered as 24.8 mg. of tan colored powder; it contained 0.34 per cent phosphorus and 10 per cent nitrogen. An estimation of non-combustible ash made on 8 mg. of the material was 7.5 per cent.

5 to 10 cc. amounts of each of the four extracts were taken to pH 5.0 with hydrochloric acid and then dialysed at 0°C. in cellophane bags for several days against repeated changes of distilled water. The dialysed materials were dried from the frozen state and further desiccated by storage under vacuum over phosphorus pentoxide at 50°C. for 18 hours, followed by several days at room temperature. The resultant materials

were weighed on a micro balance and the total amount of dry material in each extract was calculated.

The following data were obtained on the four extracts.

Extract A.—The entire extract contained 14.89 mg. of dry material and of this 0.131 mg. or 0.88 per cent was phosphorus. The absorption spectrum curve given by the ultracentrifuged extract, pH 8.5, is shown in Fig. 1. Maximum absorption was just to the long wave length side of 2600 Å; there were also weak maxima at about 2750 Å and at 2800 Å. Photographs taken with the continuous light of the hydrogen discharge

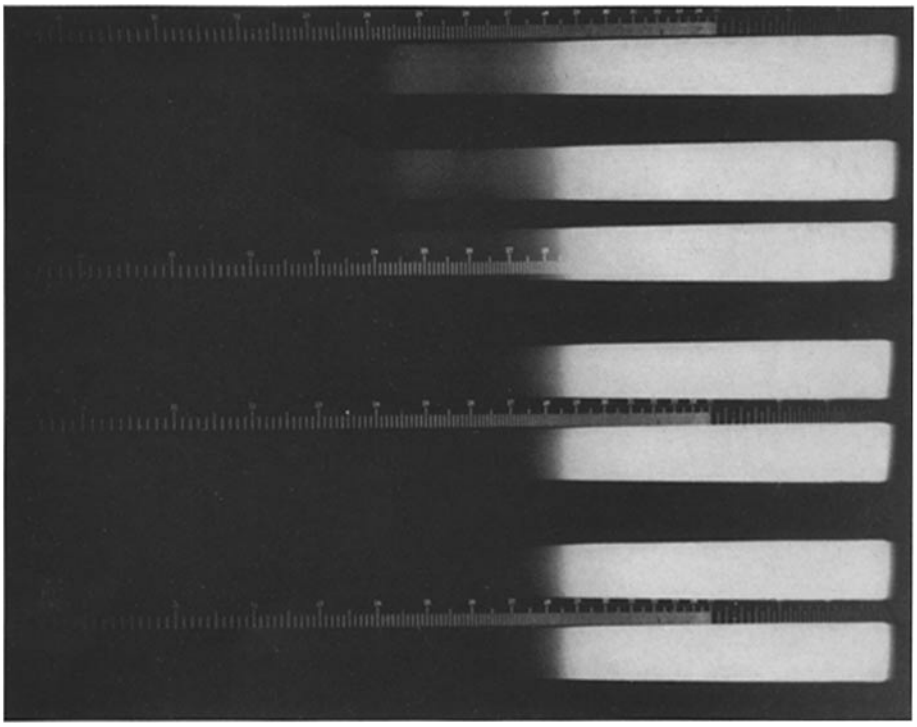

FIG. 2. Photograph of extract B, Experiment 1. A quartz Baly cell and a continuous light source were employed. Photographs taken through different thicknesses of the cell were used to bring out all the bands.

tube likewise showed that the main absorption was at about 2600 Å; in addition, a narrow band in the tyrosine region was present. The band usually associated with the presence of tryptophane could not be detected. These data indicated that the material was a nucleoprotein. An estimated value of 0.11 mg. of thymus nucleic acid per cc., or 2.86 mg. for the entire extract, was obtained by comparing the absorption coefficients of this material with those of known concentration of thymus nucleic acid.

A 1:16 dilution of extract A gave a good precipitin reaction in the presence of an optimal amount of S antiserum but no reaction was demonstrable with optimal amounts of L antiserum.

Extract B.—The dry weight was 63.65 mg. and the total phosphorus 0.414 mg. or 0.65 per cent. Photographs taken of the unneutralized extract, *i.e.*, in N/50 sodium hydroxide solution, with the continuous light source showed non-specific end absorption and in addition very weak bands in the tryptophane and tyrosine regions. A slight amount of white insoluble material appeared when the extract was brought to pH 8; after this was removed, band absorption in the nucleic acid region was apparent in photographs of the solution (see Fig. 2). Moreover, faint bands in the tryptophane,

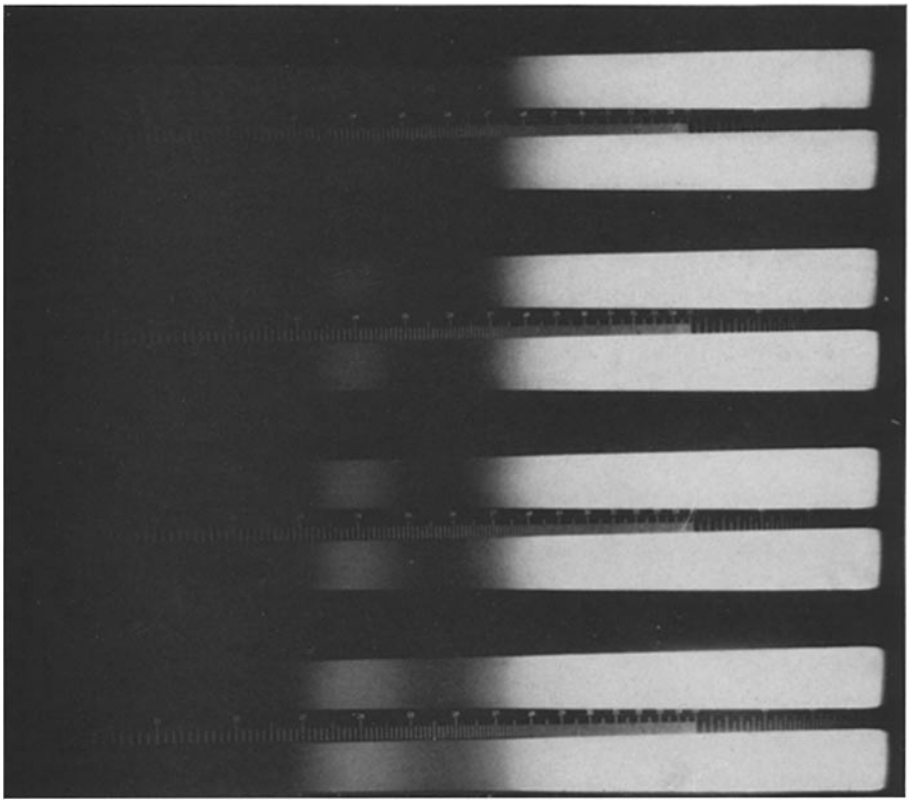

FIG. 3. Photograph of solution of nucleic acid isolated from extract A, Experiment 2. Taken by the same technique employed for illustration in Fig. 2.

tyrosine, and phenylalanine regions were noted on the original plate but are not brought out in the reproduction. The absorption curve of the material soluble at pH 8.0 was in general similar to that illustrated in Fig. 1. The thymus nucleic acid content, estimated spectroscopically, was 0.55 mg. per cc., or 11.00 mg. in the total extract.

Serological investigations were carried out with the whole extract at pH 8.5, since at this point the extract was only faintly opalescent in the undiluted state. The precipitin titer was 1:256 with S antiserum but no flocculation occurred in the presence of optimal amounts of L antibodies.

Extract C.—The dry weight was 6.7 mg. Evidence of small amounts of tryptophane, tyrosine, and phenylalanine in the extract was detected spectroscopically but no absorption in the nucleic acid range was noticed. This extract precipitated to a dilution of only 1:4 in the presence of S antibodies.

Extract D.—This specimen contained nothing that was considered to have been extracted from elementary bodies.

The method of extraction was modified somewhat in the next experiment. Furthermore, apparently pure nucleic acid was separated from one of the extracts containing nucleoprotein.

Experiment 2.—212.0 mg. of dry elementary bodies from a preparation having an infective unit-elementary body ratio of 1:3.7 were extracted with ethyl ether as in the previous experiment. 14.2 mg. of dry material were recovered from the ether; this entire fraction contained 0.060 mg. of phosphorus. Residual ether was removed from the elementary bodies, 10 cc. of distilled water were added, and the suspension was stored overnight in the cold. Sufficient distilled water and N/1 sodium hydroxide were added to bring the volume to 30 cc. and the concentration of alkali to N/20. The heavy opaque suspension of virus became faintly opalescent and moderately viscid after heating at 56°C. for 15 minutes. The material was further diluted with sufficient distilled water and N/1 hydrochloric acid to bring the volume to 41.5 cc. and to reduce the concentration of alkali to N/80; no change in the appearance of the suspension was noted. Ultracentrifugation at 30,000 R.P.M. for 20 minutes brought down approximately 0.5 cc. of semigelatinous, semitranslucent gray-white sediment and left a colorless limpid supernatant fluid having approximately the viscosity of normal serum. The sediment was saved and the supernatant fluid was further diluted to 60 cc. with distilled water and then treated with N/10 hydrochloric acid. A portion of the extract, neutralized to pH 8.5, was used immediately for serological studies. It had titers of 1:16 and 1:128 with optimal amounts of L antibodies and of S antibodies, respectively, but did not precipitate in the presence of normal rabbit serum. The main portion of the solution became increasingly viscid as pH 9 was approached and at about pH 8.0 a moderate opalescence appeared. Turbidity of the extract was greater after storage for a few hours. Horizontal centrifugation failed to clear the supernatant fluid completely but brought down about 2 cc. of white sediment which had the tenaciousness of thick mucus. Ultracentrifugation resulted in a clear supernatant fluid, which was designated extract A, and a small amount of firm gelatinous sediment. The latter was added to the mucoid horizontal sediment and designated pH 8.0 insoluble material.

The original ultra-sediment, obtained after the first alkaline extract, was treated with 30 cc. of N/40 sodium hydroxide and stored overnight at 0°C. The supernatant fluid removed after ultracentrifugation was neutralized and labeled extract B; the sediment was designated alkali-insoluble residue. The latter was washed with distilled water, brought to pH 3.0, and dialysed against repeated changes of distilled water. 17.2 mg. of residue were recovered after thorough desiccation and this contained 0.35 per cent phosphorus and 7.8 per cent nitrogen; an ash determination was not made.

The following data were obtained on the two extracts and on the pH 8.0 insoluble material. Portions of these materials used for determination of dry weight were acidulated and dialysed as in the previous experiment.

Extract A.—The entire extract contained 40.56 mg. of dry material. Sufficient phosphorus and nitrogen were detected to account for 0.32 and 15.6 per cent, respectively, of the dried material. Some of the phosphorus was undoubtedly lost during the treatment preceding drying of the above material, since 0.0040 mg. were present in each cc. of the original extract, whereas only 0.0032 mg. per cc. were found after dialysis. Faint bands indicating the presence of tyrosine, phenylalanine, and tryptophane, in addition to slight absorption in the nucleic acid region of the spectra, were found on spectroscopic analysis. The presence of a certain amount of material, which absorbed non-specifically, interfered somewhat with the interpretation of the photographs.

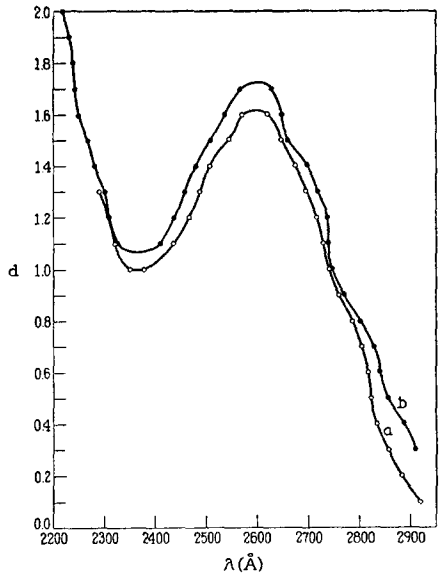

FIG. 4

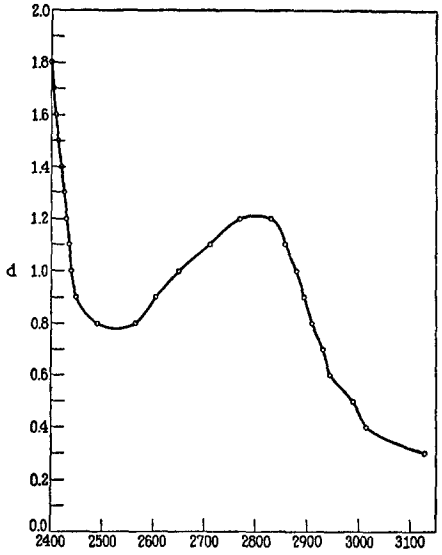

FIG. 5

FIG. 4. Absorption curve (*a*) of thymus nucleic acid (0.07 mg. per cc.) compared to (*b*) that given by a solution of nucleic acid from extract A, Experiment 2.

FIG. 5. Absorption curve of solution of heat-stable soluble antigen of vaccinia.

The titer of this solution was 1:16 with optimal dilutions of S antiserum.

20 cc. of extract A were treated by the technique (24) employed for the isolation of pure nucleic acid from nucleoprotein derived from *Streptococcus hemolyticus*. The extract was taken to pH 6.0, 5 cc. of chloroform and 2 cc. of iso-amyl alcohol were added, and the emulsion was shaken on a machine for 6 hours. Protein gel was removed by horizontal centrifugation and the entire procedure was repeated on the clear supernatant fluid. 16 cc. of liquid were recovered after the third chloroform extraction. A reproduction of a photograph of this solution taken with the continuous light source is shown in Fig. 3. The band with the maximum in the region of 2600 Å was attributed to the presence of nucleic acid and, since bands indicating the presence of aromatic amino acids were not observed, the solution was considered to be practically free of protein. The absorption curve of this treated extract is presented in Fig. 4 and for

reference the curve of thymus nucleic acid is added. The difference between the values of the maxima and minima of these almost identical curves was in contrast to that observed with nucleoproteins, see Fig. 1, in which absorption in the aromatic amino acid region was superimposed on the absorption by nucleic acid. Estimation of thymus nucleic acid in this treated extract by the spectroscopic method gave a value of 0.073 mg. per cc. Furthermore, 0.033 mg. of total phosphorus were found in 6.0 cc. of this solution and since no inorganic phosphorus was detected in a similar amount of solution, each cc. contained 0.0055 mg. of organic phosphorus.

pH 8.0 Insoluble Material.—This fraction, insoluble in the pH range between 6.0 and 8.0, dissolved readily in 30 cc. of N/40 sodium hydroxide. 2 cc. of this solution were used to investigate the solubility of the material in the acid range; the precipitate which was at its maximum around pH 7.0 began to dissolve in the region of pH 5.0 and at pH 3.0 it was almost completely redissolved.

A portion of the solution of this fraction in N/40 sodium hydroxide was shown to contain 0.0154 mg. of phosphorus and 0.326 mg. of nitrogen per cc. 19.5 mg. of dry material were obtained from 10 cc. of the alkaline solution of the pH 8 insoluble material after it had been acidulated and dialysed. On the basis of these values, the entire fraction was made up of 58.5 mg. of material which contained 0.79 per cent phosphorus and 16.7 per cent nitrogen. These percentage estimations are probably slightly higher than they should be since at least some material was lost from this sample during dialysis. This was indicated by the fact that chemical analysis of the 19.5 mg. of dried material resulted in estimations of 0.48 per cent phosphorus and 14.2 per cent nitrogen.

Spectroscopic examination of the pH 8.0 insoluble material dissolved in N/40 sodium hydroxide showed end absorption which could have masked any absorption by nucleic or aromatic acids; however, a suggestion of band absorption in the region of 2450 Å was noted. The failure to demonstrate nucleic acid by spectroscopic methods in the alkaline solution of this material which possessed considerable amounts of phosphorus was reminiscent of a similar experience with extract B in Experiment 1. In an attempt to eliminate the material responsible for the end absorption, a portion of the N/40 sodium hydroxide solution of this fraction was taken to pH 2.0 with N/1 hydrochloric acid. The slight precipitate which formed was removed and redissolved in N/40 solution of sodium hydroxide. The material soluble at pH 2.0 was examined spectroscopically at this hydrogen ion concentration and was found to give an absorption curve similar to that illustrated in Fig. 1 and considered to be typical for nucleoprotein. The fraction insoluble at pH 2.0, when redissolved in alkali and studied, gave marked end absorption; moreover, a suggestion of a band of absorption in the region of 2450 Å was again observed.

Serological studies were not undertaken with the pH 8.0 insoluble material.

Extract B.—The 30 cc. of extract remained water-clear when neutralized to pH 7.0. Spectroscopic examination indicated the presence of tryptophane, tyrosine, and phenylalanine but little if any nucleic acid. The precipitin titer with S antibodies was 1:8. Only 4.0 mg. of dried material were present in the entire fraction.

The data just presented show that nucleoprotein material was extracted from elementary bodies of vaccinia by treatment with dilute solutions of sodium hydroxide. In addition, the results indicate that a substance was extracted which was serologically indistinguishable from the heat-stable

soluble antigen of vaccinia. This antigen is a protein not in combination with nucleic acid (see later discussion). Furthermore, the recovered alkali soluble material made up slightly more than half the total weight of the original virus preparation.

The nitrogen-phosphorus ratios of different extracts varied considerably; a value of 21.0 was calculated for the pH 8.0 insoluble fraction in Experiment 2, whereas in one of the early experiments a value of 8.3 was obtained for a solution extracted from elementary bodies by treatment with N/2 sodium hydroxide solution for 18 hours at 0°C. In none of the extracts, however, did the nitrogen-phosphorus ratios reach a value around 2 which has been reported for fractions derived from *Streptococcus hemolyticus* (25) and *Diplococcus pneumoniae* (26).

Residual material left after repeated alkaline extraction of elementary bodies was of special interest because of its morphological resemblance to the "ghost particles" that Craigie and Wishart (27) observed in suspensions of elementary bodies which had been stored for a long period of time. Chemical studies have not yet been carried far enough to hazard a guess as to the identity of this residual material or mixture of materials; however, in view of the present observations a mixture of unextracted nucleoprotein and a substance low in phosphorus and nitrogen might be postulated. The results obtained in Experiments 1 and 2 by chemical methods of study are summarized in Table I.

Correlation of Spectroscopic and Chemical Estimations of Nucleic Acid.—It has been shown earlier in this report that intact elementary bodies of vaccinia gave a positive Feulgen test when the reaction was carried out under conditions which rendered the test fairly specific for thymus nucleic acid. When the virus preparations were treated with dilute sodium hydroxide the soluble materials obtained were also found to give both by chemical and by spectroscopic analysis the reactions of thymus nucleic acid.

Qualitatively, these alkaline extracts failed to give a test with Bial's orcinol reagent; they did not contain, therefore, the ribo sugar characteristic of yeast nucleic acid. On the contrary, the same extracts always gave a marked Feulgen reaction after mild hydrolysis at pH 2.3, but failed to react without hydrolysis; these findings suggested very strongly the presence of thymus nucleic acid. In several cases, the intensity of the Feulgen reaction shown by the different extracts was compared with that of solutions containing known amounts of thymus nucleic acid. Although the Feulgen reaction does not lend itself to quantitative interpretation, it was evident that on the basis of this comparison much of the organic phosphorus present in the alkaline extract could be accounted for as nucleic acid.

Attempts were also made to determine the amounts of nucleic acid present in the extracts by comparing their absorption in the ultraviolet with that of known concentrations of thymus nucleic acid. In Experiment 2 for instance, spectroscopic analysis of the portion of extract A purified by Sevag's method indicated the presence of 0.073 mg. nucleic acid per cc.; the same fraction was found to contain 0.0055 mg. of phosphorus per cc. Furthermore, the fraction of Experiment 2 which was insoluble at pH 8.0 and soluble at pH 2.0 was estimated to contain 0.163 mg. nucleic acid per cc. on the basis of spectroscopic analysis and found to contain 0.0154 mg. phosphorus per cc. These two examples indicated a satisfactory agreement between the concentrations of nucleic acid determined from the results of spectroscopic analysis and the concentrations computed from the total organic phosphorus present in the extract.

TABLE I

Summary of Chemical Data on Two Preparations of Elementary Bodies of Vaccinia

Experiment No.	Dry weight of E.B.	Total phosphorus in intact E.B.	Ether-extractable material		Alkali-extractable material				Residue after alkaline extraction					
					Weight		Phosphorus		Weight		Phosphorus		Nitrogen	Ash
	mg.	*per cent*	*mg.*	*per cent*	*mg.*	*per cent of virus*	*mg.*	*per cent of virus*	*mg.*	*per cent of virus*	*mg.*	*per cent of residue*	*per cent of residue*	*per cent of residue*
1	153	0.43	13.9	9.0	86.4	56.5	0.55	0.36	25.0	16.3	0.09	0.36	10.0	7.5
2	212	0.38	14.2	6.7	113.1	53.3	0.70	0.33	17.2	8.1	0.05	0.29	7.8	Not done

However, it must be admitted that it was not always possible to reconcile the results obtained by different techniques. Some extracts rich in organic phosphorus and positive to the Feulgen test failed to show the absorption characteristic of nucleic acid; this was in some cases due to the presence in the unneutralized extract of an unknown substance capable of absorbing very strongly in the ultraviolet range. This substance was insoluble in ethyl ether and precipitated at neutral or acid (pH 2–3) reaction.

In other cases, spectroscopic analysis indicated amounts of nucleic acid much larger than could be accounted for by the total organic phosphorus present in the extracts. Only unproved assumptions can be offered to explain these discrepancies, *e.g.* (*a*) overestimation by the spectroscopic method because of the presence in the extracts of substances giving continuous absorption in the ultraviolet, (*b*) underestimation by chemical methods because of loss of split products of nucleic acid during dialysis.

Granted these unexplained difficulties, the results seem to warrant the conclusion that alkaline extraction released from the elementary bodies of vaccinia a substance, free or combined to a protein, which belonged to the group of desoxyribonucleic acids.

Estimation of Heat-Stable Soluble Antigen.—No precipitation was noted early in the work when antivaccinal serum was mixed with extracts of elementary bodies obtained by treatment with concentrated solutions of sodium hydroxide, *i.e.*, N/2 to N/10. In retrospect it is obvious that this was to be expected, for it was found that a solution of pure heat-stable antigen after being heated at 56°C. for 90 minutes in a concentration of N/20 sodium hydroxide was no longer precipitated by its specific antibody. Treatment under the same conditions for 30 minutes reduced the precipitin titer of the solution of antigen from 1:1024 to 1:128; even treatment for only 15 minutes reduced the titer approximately 50 per cent. In order to extract as much material as possible from elementary bodies and still employ a comparatively mild method that would not completely inactivate the heat-stable soluble antigen, in Experiments 1 and 2 the virus was initially exposed to a N/20 concentration of sodium hydroxide at 56°C. for 15 minutes. Precipitin titers of various extracts in these two experiments have been recorded.

The maximum dilution at which pure heat-stable soluble antigen of vaccinia (S), will react with optimal amounts of antibody has been shown to be approximately 1:640,000 (28); expressed another way, a specific precipitin reaction can be obtained under the proper conditions with about 0.0000016 gm. of dry antigen. The amount of S substance in a given solution was roughly estimated by employing this value as the approximate weight of antigen that resulted in visible precipitation. For example, 1:256 dilution of extract B in Experiment 1 gave a precipitate when incubated with an optimal amount of S antibody. Since 0.25 cc. of a 1:256 dilution contained one dose of antigen, then there were 20,480 doses in the entire 20 cc. of extract. This number of doses of pure S antigen would have a dry weight of approximately 32.8 mg. Certain extracts appeared to consist principally of S antigen; the estimated amount of S substance in extract B in Experiment 2 was 3.1 mg. while the dried material in the extract weighed 4.0 mg. Results of the two experiments recorded in this paper might be regarded as indicating that about one-fourth of the dry weight of vaccine virus is in the form of heat-stable soluble antigen; such a conclusion would not be warranted, however, because of the inaccuracies inherent in the methods employed. Nevertheless, the results are sufficiently clear-cut to show that a significant amount of material serologically similar to S antigen was present in extracts of elementary bodies.

Attention should be called to the fact that some of the material in these extracts which precipitated specifically with S antibodies, and which we have considered to be S antigen, differed in at least one respect from the S antigen encountered in crude dermal filtrate or prepared in a purified form from dermal filtrate. The largest amounts of S antigen occurred in extracts which were also rich in nucleoprotein. One would be justified in assuming that such extracts represented a mixture of substances since S antigen is not a nucleoprotein. The heat-stable soluble antigen obtained from dermal filtrate has been shown to be a protein which is soluble in alcohol, is obtained in the pseudoglobulin fraction and has an isoelectric point near pH 4.5 (28). Recent work has demonstrated that it was also insoluble at about pH 1.2 but redissolved between pH 2 and 3 and that it retained its serological activity after such treatment. The antigen gave positive tests for glucosamine and glucuronic acid and on spectroscopic analyses it was shown to contain tryptophane, tyrosine, and phenylalanine but little or no nucleic acid. Its absorption curve is illustrated in Fig. 5. Therefore, S antigen, as ordinarily found, was soluble in aqueous solution in the pH range near the neutral point. This was not observed with much of the serologically active material in extracts of elementary bodies. Thus, in Experiment 2 the S titer of the first alkaline extract neutralized to pH 8.5 was 1:128 but after removal of the fraction insoluble at pH 8 which contained nucleoprotein the S titer dropped to 1:16. A similar observation was made in Experiment 1 with extract B; here, the original titer was 1:256 but after removal of the material insoluble at neutral reaction the titer was 1:2. The interpretation of these observations must await the results of additional studies, for in the present experiments the identification of S antigen was not accomplished in the protein residue which was left after separation of nucleic acid from nucleoprotein.

DISCUSSION

Nucleoprotein and S antigen were recovered in solution after extraction of preparations of dry elementary bodies of vaccinia with dilute solutions of sodium hydroxide. Elementary bodies contained approximately 0.4 per cent of inorganic phosphorus and most of this could be accounted for as a constituent of the desoxyribonucleic acid which was present in the virus preparations. Qualitative reactions suggesting the presence of small amounts of glucosamine were obtained in all cases.

A percentage concentration of total phosphorus higher than that observed by us has been reported for elementary bodies of vaccinia (29, 21); however, the amount of thymus nucleic acid (21) liberated from the virus, after extraction of lipids, by treatment with solutions of sodium carbonate

was about the same as that obtained by our estimations. These aspects of the problem are now being investigated further, and attempts to correlate the two sets of findings will be postponed. It may be pointed out at this time, however, that certain significant differences in the apparent purity of virus preparation employed for study in the two laboratories have already been recorded (9). It is of interest to note that McFarlane and Macfarlane (21) also detected small amounts of glucosamine in their preparations of vaccine virus.

All of the viral agents so far studied in a purified state have been reported to contain nucleic acid of the yeast or thymus type. The former type has been found in preparations of a number of plant viruses (30), of the agent of Rous' sarcoma (31), and of several types of bacteriophage (32). It should be mentioned, however, that the importance of phosphorus-containing compounds in one of these bacteriophages has been recently questioned (33). Vaccine virus, on the other hand, is the only agent to date from which the thymus type of nucleic acid has been obtained. Elementary bodies of psittacosis, observed in smears made from spleens of infected mice, also apparently contained thymus nucleic acid since they gave a positive Feulgen reaction (34).

Our interest in the serological identification of material extractable from elementary bodies has already been stated. The observation that a significant proportion of the dry weight of virus preparations could be accounted for by the presence of heat-stable soluble antigen adds new evidence for the belief (27, 35) that the soluble antigens arise from the virus of vaccinia and are not a response of the host cells to infection. Investigations on the various substances extractable from vaccine virus are in a preliminary stage; it is obvious that still more gentle methods of extraction are needed and that complete serological and immunological data on the materials of the type already obtained are necessary.

SUMMARY

Treatment of elementary bodies of vaccinia with dilute solutions of sodium hydroxide resulted in the extraction of certain soluble materials accounting for half of the dry weight of the virus. Elementary bodies contained about 0.4 per cent inorganic phosphorus, practically all of which occurred in the form of a nucleoprotein containing thymus nucleic acid. In addition, a substance was recovered that reacted with S antibodies. From past experience one is led to believe that S antigen, as ordinarily encountered, is a protein which is not in combination with nucleic acid.

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