CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

V. A Flavin Associated with the Purified Virus

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(Received for publication, May 21, 1941)

Elementary bodies of vaccinia exhibit uniformity in their chemical constituents and biological activity (1–3). Chemical, physical, and immunological studies have indicated a degree of complexity for this animal virus which is not shared by certain members of the group of crystallizable plant viruses. The view, therefore, that respiratory catalysts and certain specific growth substances, which are known to play an important rôle in bacterial metabolism, may function in the organization of elementary bodies of vaccinia is not untenable.

Modern researches by Warburg, Keilin, Meyerhof, and others, in the field of cellular catalysis, have led to the view that, although many substances may participate in a cellular reaction, certain well defined organic catalysts, often functioning as prosthetic groups of enzymes, take up key positions along the metabolic chain which begins with the oxidation of substrate and ends in many instances in the reduction of molecular oxygen. Of these catalysts, the flavins, the phosphopyridine nucleotides, and the cytochromes are important and have been well studied. Search for a cytochrome component of vaccine virus was unsuccessful (3). However, a copper constituent in constant amounts in purified elementary bodies of vaccinia has been found, indicating that a highly organized virus, such as vaccinia, may possess substances which function catalytically in biological oxidations.

Riboflavin is among the more important oxidative catalysts known to play a rôle in cellular metabolism. This substance when linked to phosphate and adenylic acid forms a nucleotide, and in this combination functions as the prosthetic group of a number of well defined enzymes concerned with the oxidation of coenzymes I and II, with the deamination of unnatural amino acids, and with the oxidation of xanthine and aldehydes (4). A new member of this group of flavins which is concerned with the oxidation of reduced cytochrome *c* has been recently reported by Haas, Horecker, and Hogness (5). In view of the ubiquity of flavin-containing enzymes in animal, plant, and bacterial cells, a search for components of this system in the elementary body

of vaccinia has been made. The results of the work are reported at this time.

EXPERIMENTAL

In the course of extraction of nucleic acid from elementary bodies of vaccinia it was observed that alkaline solutions of the purified virus gave a marked fluorescence in the presence of ultraviolet light. Increased alkalinization caused an irreversible destruction of the fluorescent property. Moreover, it was noted that suspensions of elementary bodies of vaccinia tended to lose this fluorescence to some extent on standing for prolonged periods of time in strong light. The fluorescence was partially regained, however, when the light-exposed virus was placed in the dark for several hours. Flavins are characterized in part by their instability in the presence of strong light (6). This reaction, known as photolysis, is greatly accelerated in an alkaline medium. It, therefore, occurred to us that the behavior of the virus in ultraviolet light could be explained in part, at least, if a flavin constituent were demonstrated.

Chromatographic Separation of Fluorescent Material from Virus

It was possible by treating vaccine virus with 50 per cent alcohol in a buffer at pH 4, to dissociate from the virus a substance which carried a major part of the total fluorescence. Moreover, it was possible by means of chromatographic methods to concentrate the material in a thin ring in an absorption tower of aluminum oxide from which it was subsequently eluted with sodium bicarbonate and methanol.

Construction and Use of Chromatographic Tower.—50 mg. of elementary bodies of vaccinia were suspended in 10 cc. of water, and 0.1 N hydrochloric acid added until the pH of the suspension was 4. The volume was brought to 15 cc. with distilled water, and 15 cc. of absolute alcohol were added slowly with vigorous stirring. The alcohol-elementary body mixture was heated for 5 minutes on a boiling water bath and filtered (Whatman No. 42 filter paper). The clear filtrate, examined with ultraviolet light, exhibited a marked bluish-green fluorescence. An absorption column was constructed, which consisted of soft glass tubing, 1.5 cm. in diameter and 35 cm. in length, with a constriction at one end. A small portion of glass wool was packed tightly against this constriction and Brockmann anhydrous aluminum oxide (Al_2O_3) was packed firmly against the glass wool until the column was filled to within 5 cm. of the top. Another portion of glass wool was packed at the top against the aluminum oxide. The tower was clamped to a stand and suction was applied at the constricted end, after which the filtrate from the heated elementary bodies was added slowly from above. The speed of flow of the filtrate through the aluminum column was controlled by the amount of suction applied. About 15 minutes was required for the passage of each 10 cc. of filtrate. It was necessary to pack the tower tightly enough for the fluid to descend slowly, wetting the aluminum oxide in an advancing sharp plane, otherwise

irregular channels appeared in the aluminum oxide and sharp separation of the zone was not obtained.

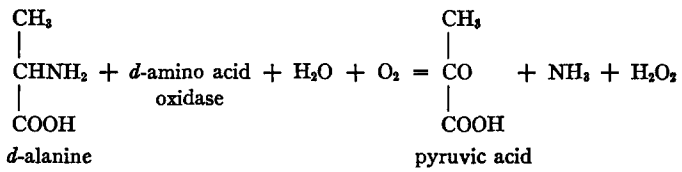
Development of the Chromatogram.—It was possible by frequent examination of the tower with a mercury arc light to note the changing position of the zone of fluorescence. 10 cc. portions of 4 normal hydrochloric acid-alcohol mixture were added until the ring had advanced 5 to 10 cm. below the top of the aluminum oxide column. By means of a file the absorbing column was broken cleanly just above the fluorescent ring and the aluminum oxide carrying the zone of fluorescent material was lifted out by means of a spatula and placed in a small beaker. 10 cc. of methanol-sodium bicarbonate solution were added to the aluminum oxide-absorption complex and the mixture shaken vigorously and filtered. The filtrate containing the fluorescent material was neutralized with 0.1 N hydrochloric acid and concentrated *in vacuo* to 5 cc. The material eluted from the ring of aluminum oxide was pale yellow in color and gave an intense bluish-yellow fluorescence in the presence of ultraviolet light.

Absorption Spectrum of the Fluorescent Material.—Attempts to identify the fluorescent substance by a study of the absorption spectrum failed. There was considerable end absorption in the ultraviolet, indicating the presence of extraneous material. Although marked fluorescence of the eluate was observed, there was insufficient concentration to render identification by absorption spectra possible, either in the visible or ultraviolet region of the spectrum. We accordingly turned to other methods for its identification.

Identification of Flavin

If the material responsible for any part of the fluorescence of the virus could be attributed to flavin, it was most likely bound in a flavoprotein combination, since free flavin would almost certainly have been washed away in the extensive process of virus purification.

In 1935 Krebs (7) described a technique for the estimation of flavin-adenine-dinucleotide, making use of the protein of the *d*-amino acid oxidase. *d*-Amino acid oxidase is a conjugated enzyme, with flavin-adenine-dinucleotide as its prosthetic group. It is readily prepared in large quantities from pig kidney. The intact enzyme specifically catalyzes the conversion of the unnatural amino acids to the corresponding alpha-keto acids, ammonia, and hydrogen peroxide. So far as it is known, all *d*-amino acids undergo oxidative deamination in the presence of *d*-amino acid oxidase, although great variation in rate of oxidation exists among the individual members of the *d*-amino acid series (7). For most purposes *d*-alanine has been used as a substrate because of its ready availability and the rapidity of its deamination. The over-all reaction may be written thus:



d-alanine pyruvic acid

Under carefully controlled conditions, worked out by Warburg and Christian (9), the above reaction can be made quantitative for the determination of flavin-adenine-dinucleotide. This is accomplished by dissociating the flavin of *d*-amino acid oxidase by lowering the pH to 4, and precipitating the protein portion of the enzyme from solution with ammonium sulfate. The protein alone is inactive, but becomes very active in the conversion of *d*-alanine to pyruvic acid when mixed with solutions containing flavin-adenine-dinucleotide. The oxygen uptake in the Warburg manometer in the presence of the specific protein and *d*-alanine is proportional to the flavin-adenine-dinucleotide in the test solution. According to Warburg and Christian (9) the reaction between the *d*-amino acid oxidase and *d*-amino acid, *i.e.*, the rate of oxygen uptake in the presence of an excess of *d*-amino acid, may be expressed by the equation: *Rate of* O_2 *uptake* $= k$ (*flavin*) (*protein*).

The equation indicates that the rate of oxygen uptake will be increased in three ways: (1) by an increase in the flavin-adenine-dinucleotide component; (2) by an increase in the quantity of the specific protein; and (3) by an increase in both these constituents. Warburg and Christian (9) and others (10) have taken advantage of this equation to evolve a technique for the estimation of flavin-adenine-dinucleotide by determination of the constant of the protein in the presence of a known amount of pure flavin-adenine-dinucleotide. They found the relation between the activity of a fixed quantity of protein and *d*-amino acid, with varying amounts of flavin-adenine-dinucleotide, to be expressed by the equation $k = c\frac{V_o - V}{V}$ where c is the concentration of flavin-adenine-dinucleotide which will produce rate V, and V_o is the rate in the presence of an excess of flavin-adenine-dinucleotide. When $V = 0.5\ V_o$, k becomes equal to c, and can be expressed in micrograms of flavin-adenine-dinucleotide present in the test substance. By comparison of the rate produced by known amounts of flavin-adenine-dinucleotide with the rate produced by an unknown substance in the presence of a given quantity of the protein of the *d*-amino acid oxidase, the concentration of flavin-adenine-dinucleotide in the unknown substance can be determined.

Preparation of d-Amino Acid Oxidase.—*d*-Amino acid oxidase from pig kidney was prepared according to the method of Warburg and Christian (9), after which the specific protein was recovered free of prosthetic group by precipitation with ammonium sulfate.

To a fine mince of fresh pig-kidney cortex, 5 volumes of chilled acetone were added. The mixture was stirred vigorously for 5 minutes, after which it was filtered through a Büchner funnel. The precipitate was dried *in vacuo* at 4°C., then powdered, and kept in the cold as a source of starting material for the preparation of the specific protein.

To prepare the protein portion of the enzyme, 200 cc. of water were added to 10 gm. of the dried kidney powder. The material was next centrifuged and the sediment discarded. To 200 cc. of the clear supernatant solution at 0°C. were added 13.3 cc. of 1 molar acetate buffer, pH 3.8. The precipitate was collected by centrifugation and discarded. To the supernatant material kept at 0°C. was added ½ volume of saturated ammonium solution. The precipitate which formed slowly was collected by centrifugation in the cold, dissolved with 5 cc. of 0.1 molar sodium pyrophosphate buffer, pH 8.3, and made up to 100 cc. with water. To this dilute solution were added

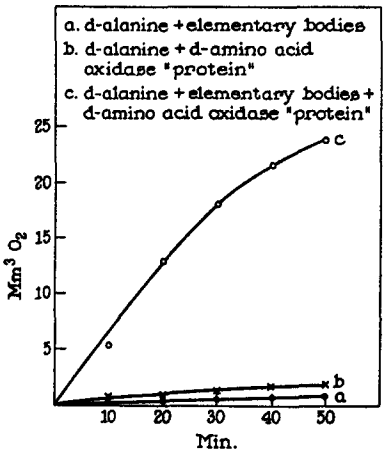
35 cc. of saturated ammonium sulfate, and while being shaken 40 cc. of 0.1 normal hydrochloric acid were added slowly. At this time the pH of the solution was 2.8, and the ammonium sulfate concentration was about 0.2 molar. The precipitate was collected by centrifugation, dissolved in 5 cc. of 0.1 molar sodium pyrophosphate, and made up to 100 cc. with water. Precipitation with ammonium sulfate and hydrochloric acid was repeated as before and the precipitate taken up in 2.5 cc. of 0.1 molar sodium pyrophosphate and made up to 25 cc. with water. The solution was clarified by centrifugation, after which it was frozen and dried *in vacuo*.

Qualitative Determination of Flavin-Adenine-Dinucleotide in Elementary Bodies.—The presence of flavin-adenine-dinucleotide in the elementary bodies was demonstrated in the following manner.

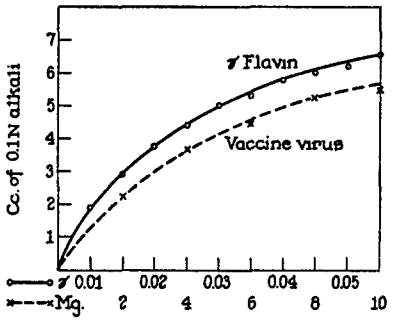
11 mg. of freshly prepared elementary bodies of vaccinia were suspended in 1.1 cc. of water and heated for 10 minutes at 80°C. Fluid lost by evaporation was replaced, and 1.0 cc. of the suspension containing 10 mg. of virus was placed in the bottom of a conical Warburg flask. The central well of the flask contained a small roll of filter paper and 0.2 cc. 5 N NaOH. 2 mg. of specific *d*-amino acid oxidase "protein" in 0.5 cc. of 0.1 molar sodium pyrophosphate buffer, pH 8.3, were added to the elementary body suspension and thoroughly mixed by gentle rotation. 1 mg. of *d*-alanine in 0.5 cc. of the pyrophosphate buffer was added to the side arm. The flask was attached to the manometer and the manometer-flask system equilibrated with pure oxygen. The manometers were then shaken in the water bath until equilibrium had been achieved and the *d*-alanine in the side arm was tilted into the reaction flask. The manometers were shaken and oxygen consumption observed at 10 minute intervals for a period of 2 hours. Control flasks, containing specific "protein" and *d*-alanine, elementary bodies and *d*-alanine, and specific "protein" and elementary bodies were set up at the same time.

No reaction was observed in the control flasks. A steady oxygen uptake in the flask containing elementary bodies, "specific protein," and *d*-alanine of about 4 to 5 mm. of oxygen per 10 minute interval was noted consistently. Oxygen consumption was likewise shown on successive lots of elementary bodies prepared in the same manner. A graph for a typical reaction rate is given in Text-fig. 1. When unheated elementary bodies were used in the set-up described above, oxygen utilization was likewise observed, but at a significantly lower rate. The increased rate of oxygen uptake observed in the case of elementary bodies heated to 80°C. can be explained in part by the fact that for maximum activity it is necessary first to release flavin-adenine-dinucleotide by denaturation of the virus, and in part by the fact that heat inactivates the trace of catalase which, as Macfarlane and Salaman have shown, is associated with virus preparations (8). This catalase, which we have also found in our elementary body preparations, unless inactivated by heat or cyanide, served to release oxygen from the hydrogen peroxide which is formed in the reaction mixture, thereby lowering the apparent oxygen uptake in the Warburg manometer.

Quantitative Determination of Flavin-Adenine-Dinucleotide in the Virus by Enzymatic Reaction.—An attempt was made to estimate the amount of flavin-adenine-dinucleotide in elementary bodies by comparison of the rate of reaction of the protein of *d*-amino acid oxidase and heated virus, with that of the protein and flavin-adenine-dinucleotide prepared from yeast. For this purpose it was necessary to prepare pure flavin-adenine-dinucleotide according to the method of Warburg and Christian (9).



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIG. 1. Oxidation of *d*-alanine by elementary bodies of vaccinia in the presence of the "protein" of *d*-amino acid oxidase.

TEXT-FIG. 2. Growth response of *Lactobacillus casei* E in a basal medium to pure riboflavin and to elementary bodies of vaccinia.

10 kilos of fresh yeast were mixed with 20 liters of hot water and held at 75–80°C. for 10 minutes with stirring, after which the residue was removed by centrifugation. To the supernatant fluid were added 11 kilos of ammonium sulfate and the dinucleotide extracted from the precipitated material by three successive extractions with 2.5 liters of phenol (U.S.P., 88 per cent). The phenol mixture was next extracted successively with a total of 6 liters of ethyl ether and 1.5 liters of water. The aqueous solution of dinucleotide, freed from ether by distillation *in vacuo*, was made acid to Congo red with 140 cc. of 2 normal nitric acid and the dinucleotide precipitated with 20 cc. of 30 per cent silver nitrate. The silver precipitate was next collected by centrifugation and washed with 100 cc. of water, suspended in 50 cc. of water, and decomposed with hydrogen sulfide. The dinucleotide was extracted from the silver sulfide by washing the precipitate eight times with 25 cc. of molar/100 barium acetate. This solution, containing the barium salt of the dinucleotide, was made acid with 6 cc. of normal sulfuric acid, and the barium sulfate precipitate centrifuged and washed with water. To the aqueous extract were next added 150 gm. of ammonium sulfate,

and the resulting mixture extracted with 15 cc. of paracresol. The paracresol solution, containing the dinucleotide, was washed many times with a 0.2 normal solution of sulfuric acid containing 50 per cent, by weight, of ammonium sulfate. 500 cc. of ethyl ether were added to the paracresol solution and the mixture shaken with water. The aqueous layer, recovered by means of a separatory funnel, was made acid to litmus and sufficient 2 molar barium acetate added to remove the sulfuric acid as barium sulfate. A phosphorus analysis on the aqueous material was performed, and for each gram atom of phosphorus found, one mole of barium acetate was added. The mixture was concentrated *in vacuo* to about 10 cc. and 2 moles of ammonium sulfate added for each gram atom of phosphorus, after which the mixture was dried *in vacuo* and the ground dried residue extracted several times with 95 per cent alcohol. The alcohol was discarded, the residue extracted at 40°C. with 2.5 cc. of water, and the aqueous extract discarded. The brown residue was then dissolved in 10 cc. of water at 60°C. On cooling to room temperature crystals of the barium salt of flavin-adenine-dinucleotide were precipitated. These were washed with absolute alcohol and dried *in vacuo* at 0°C.

The quantitative determinations of the amount of flavin-adenine-dinucleotide in vaccine virus were conducted in the following manner.

Flavin-adenine-dinucleotide in amounts less than 0.5 microgram was dissolved in 0.5 cc. of water and placed in the bottom of a Warburg respiration flask. 2 mg. of specific *d*-amino acid oxidase protein, in 0.5 cc. of 0.1 molar sodium pyrophosphate buffer, pH 8.3, were added and mixed thoroughly by gentle rotation. 1 mg. of *d*-alanine in 0.5 cc. of pyrophosphate buffer was added to the side arm. The flask was attached to the manometer, and the system equilibrated with pure oxygen. The manometer cock was closed and when temperature equilibrium had been achieved in the water bath at 38°C. the *d*-alanine in the side arm was tilted into the reaction flask and the manometer observed at 10 minute intervals for oxygen uptake. Control flasks containing the specific protein and *d*-alanine, and flavin-adenine-dinucleotide and protein were set up at the same time. In another set of flasks, heated elementary bodies, as a source of flavin-adenine-dinucleotide, and appropriate controls were set up in the manner previously described, using a 2 mg. aliquot of the specific protein for each 10 mg. lot of heated elementary bodies.

The results are given in Table I. 0.0002 and 0.0001 mg. of flavin-adenine-dinucleotide yielded rates respectively slightly higher and slightly lower than the rate given by 10 mg. of elementary bodies of vaccinia. Although the identification of flavin-adenine-dinucleotide by this technique was possible, absolute quantitative results were hard to secure for three reasons: (1) it is difficult to know by any standards thus far brought forward when one has obtained a pure flavin-adenine-dinucleotide from yeast; (2) it is a laborious task to prepare, and keep standardized, active solutions of the specific protein from *d*-amino acid oxidase, since the material becomes irreversibly inactivated upon standing in solution; and (3) it requires several determinations on each dilution of material to locate accurately the point in the equation where $V = 0.5\ V_o$.

Moreover, when amounts of virus greater than 10 mg. were used for the determination of flavin-adenine-dinucleotide by this procedure an unexplained inhibition of the reaction seemed to occur so that total activity was actually less than that observed with smaller quantities of virus. When amounts of virus smaller than 10 mg. were used, the rates were often too low for accurate manometric measurement. Under the best of conditions the quantitative results obtained by this technique can be regarded only as an approximation. For these reasons we turned to yet another procedure for the quantitative determination of flavin in our virus material.

Quantitative Determination of Riboflavin in Elementary Bodies of Vaccinia by Microbiological Assay.—In 1939, Snell and Strong (11) described a relatively simple method for the quantitative determination of riboflavin. The method is based on the requirements of *Lactobacillus casei* E for this substance as a growth factor. To a basal medium, in which all the growth requirements of

TABLE I

Oxidative Deamination of d-Alanine by the "Protein" of d-Amino Acid Oxidase Together with Elementary Bodies of Vaccinia and with Flavin-Adenine-Dinucleotide Prepared from Yeast

Test material	Amount	"Protein" of d-amino acid oxidase	O_2 uptake
	mg.	*mg.*	*mm.³ per hr.*
Elementary bodies of vaccinia	10.0	2.0	22.0
Flavin-adenine-dinucleotide	0.0001	2.0	17.9
" " "	0.0002	2.0	24.2

this bacillus except riboflavin are present in excess, is added a finely divided suspension of the material to be assayed for riboflavin. The mixture of medium and substance to be tested is then autoclaved, and when cool inoculated with a fresh, washed culture of *Lactobacillus casei* E. The culture is allowed to incubate 72 hours, or until no further growth of the bacilli occurs. A set of control tubes containing the basal medium to which known increments of crystalline riboflavin have been added is prepared at the same time, autoclaved, inoculated with *Lactobacillus casei* E, and incubated for the same period. Since riboflavin in the test material is the factor limiting growth of the organism, both the number of organisms, as measured by turbidity, and the amount of lactic acid produced, as measured titrimetrically, bear a relationship to the quantity of riboflavin present. By this technique purified vaccine virus has been shown to contain riboflavin, and the amount has been expressed in micrograms of riboflavin per unit weight of the dry virus.

A set of culture tubes containing 10 cc. of basal medium prepared according to the directions given by Snell and Strong (11) were set up in duplicate and increments of riboflavin added in amounts of 0.0, 0.05, 0.1, 0.15, 0.20, 0.25, 0.30, and 0.40 micro-

grams. A set of tubes with the basal medium containing freshly prepared vaccine virus, in amounts of 2.0, 4.0, 6.0, 8.0, and 10.0 mg. were set up at the same time. Both the standard medium containing increments of riboflavin and the medium containing vaccine virus, in lieu of riboflavin, were autoclaved at 20 pounds for 15 minutes, cooled to room temperature, and inoculated with 0.05 cc. of a 24 hour culture of *Lactobacillus casei* E. This culture had been grown on a medium containing 0.1 microgram of added riboflavin, washed twice with phosphate buffer, and resuspended in 20 cc. of sterile physiological saline. The tubes containing the standard and those with the virus material being tested were then incubated at 38°C. for 72 hours. At the end of this time the growth turbidity was measured by means of a photocell colorimeter of the Evelyn type, and the lactic acid was titrated with 0.1 normal sodium hydroxide to a brom-thymol blue end point. In general, the results of duplicate samples obtained by titration agreed more closely than results obtained

TABLE II

Flavin Content of Purified Elementary Bodies of Vaccinia as Determined by Assay with Lactobacillus Casei E

Lot	Virus taken for assay	Flavin, per 100 gm. of virus
	mg.	*mg.*
1	2	1.5
2	2	1.5
3	2	1.4
4	2	1.5
5	2	1.4
6	2	1.5

by turbidity measurements, and were finally used exclusively as a measure of growth and consequent metabolic activity of the culture.

Determinations by means of *Lactobacillus casei* E done on 10 lots of virus reveal, from lot to lot, a fairly constant content of riboflavin, ranging from 1.1 and 1.5 mg. per 100 gm. of virus. The results obtained by microbiological assay are given in Table II. A graph of the growth response of *Lactobacillus casei* E to pure riboflavin and to elementary bodies of vaccinia is given in Text-fig. 2.

Separation of Flavin Constituent from Elementary Bodies of Vaccinia

All attempts to separate the flavin from purified elementary bodies of vaccinia by procedures which in themselves do not inactivate the virus have thus far failed; namely, repeated washing with buffers ranging from pH 6 to pH 8, ultrafiltration by means of the Coolidge apparatus (12) or electrodialysis.

Attempt to Separate Flavin from Virus by Repeated Washing in Dilute Buffers.—On the assumption that the flavin-adenine-dinucleotide present in purified virus might have been adsorbed from the extraneous animal material

during the process of preparation of the virus, we set about to see if it could be eluted by methods which are of recognized value in the separation of adsorbed substances. For flavin constituents, certain of the factors influencing adsorption and elution are known. Among these is the factor of pH. In general, adsorption of flavin is favored at a low pH level and elution favored within higher pH ranges.

To 20 mg. of purified elementary bodies of vaccinia were added 20 cc. of 0.025 molar citrate buffer of pH 4. The virus was removed by means of centrifugation and resuspended and washed twice in distilled water. The virus was finally collected by centrifugation, dried at 0°C. *in vacuo*, and assayed for flavin by means of the microbiological method previously described. The experiment was repeated, but citrate buffers of pH 5 and 6.5, and 0.075 molar phosphate buffers at pH 7, 7.5, and 8.0 were used. No significant loss of flavin could be detected following these repeated washings with buffers of varying pH.

Attempt to Separate Flavin Constituent by Ultrafiltration.—For ultrafiltration a simple device described by Coolidge (12) was used. By this method Coolidge was able to show that bilirubin was attached to the albumin fraction of human serum and could not be removed by ultrafiltration.

10 mg. of purified virus were suspended in 10 cc. of dilute buffer at pH 7.2 and placed in a cellophane bag. The end was knotted, and the bag placed vertically in a small tube of the Coolidge type, supported by a porous plate which rested in turn on a partial constriction at the middle of the tube. The assembled tube was centrifuged at 2000 R.P.M. for 1 hour. After this time about 5 cc. of ultrafiltrate were obtained. The ultrafiltrate, tested microbiologically, did not contain riboflavin. Moreover, no fluorescence of this material was observed in ultraviolet light.

Attempt to Separate Flavin Constituent by Electrodialysis.—An apparatus and procedure for electrodialysis which does not inactivate vaccine virus has been described previously (3). By this technique it is possible to control the pH at any desired level by means of dilute buffer which flows steadily through the protecting cells placed between the virus and electrodes.

10 mg. of virus were suspended in dilute buffer in the center cup of a 5-compartment electrodialysis cell, and dialyzed with a potential of 110 volts and a current of 50 milliamperes for 48 hours at 20°C. Aseptic precautions were insured by autoclaving the cell before use and the cautious introduction of bacteria-free virus. That no bacterial contamination or growth occurred was shown by cultures made on blood agar plates at frequent intervals during the course of the dialysis.

Electrodialysis produced no significant drop in the flavin content of the virus over that noted in a control lot of virus kept at 20°C. and at the same pH.

DISCUSSION

All attempts to separate the flavin component from elementary bodies of vaccinia by means which did not result in inactivation and denaturation of the virus failed, namely, repeated washing with buffers ranging from pH 6 to 8, ultrafiltration by means of the Coolidge apparatus, and electrodialysis. The only successful means thus far found to remove the flavin constituent has been to lower the pH to 4 and precipitate the virus from suspension by means of heat and alcohol, methods which are known to dissociate flavin-adenine-dinucleotide from its protein component.

With respect to the concentration of riboflavin in vaccine virus, it is interesting to note that it compares favorably with that found in muscle, but it is significantly below that in yeast which is an exceptionally rich source of riboflavin. The published values also show a greater concentration of riboflavin in bacteria than that found in vaccine virus, although a great range of variability in microorganisms is known to occur.

The demonstration that elementary bodies of vaccinia contain constant amounts of flavin-adenine-dinucleotide is in itself an interesting fact, but proof that this substance is an integral part of the elementary body and that it functions in the metabolism of the virus obviously constitutes a much larger problem. It would be a most attractive hypothesis to assume that its presence there permits the elementary body to divert a certain portion of the energy derived from oxidative reactions, begun in the host cell, through its own flavin oxidation-reduction system, thereby enabling it to live as an obligate parasite. So far, no direct evidence can be offered for this point of view, except that the presence of flavin-adenine-dinucleotide constituent, if upon further work it is definitely demonstrated to be an active component of the virus, would indicate that the virus possesses in part a metabolic system akin to that of its host cell.

CONCLUSION

Suspensions of purified elementary bodies of vaccinia exhibit fluorescence in the presence of ultraviolet light. This fluorescent constituent can be separated by chromatographic methods provided the virus is first denatured by acid and heat. By means of the specific protein of *d*-amino acid oxidase it has been possible to identify the flavin constituent as flavin-adenine-dinucleotide and show that it can participate in the oxidative deamination of *d*-alanine. By means of microbiological assay the flavin component has been quantitatively measured and shown to compare favorably in concentration with that observed in animal cells and in some bacteria; its concentration in virus is lower than that observed in yeast. The demonstration that it exists as an integral portion of the virus is not conclusive. So far, however, it has been

separated from the elementary bodies only by means which in themselves inactivate the virus.

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