CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

IV. Demonstration of Copper in the Purified Virus

By CHARLES L. HOAGLAND, M.D., S. M. WARD, JOSEPH E. SMADEL, M.D., and THOMAS M. RIVERS, M.D.

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

Plate 6

(Received for publication, May 8, 1941)

Previous studies have indicated that preparations of elementary bodies of vaccinia can be obtained which exhibit great constancy in their immunological, physical, and chemical properties. These studies have shown that the virus is composed chiefly of protein, thymonucleic acid, lipid, and carbohydrate, which occur in constant proportions in different lots of purified virus, and in concentrations not materially different from those found in bacterial cells (1, 2). No independent metabolism of elementary bodies has yet been discovered. Parker and Smythe (3), in 1935, were unable to demonstrate the utilization of oxygen by purified elementary bodies in the presence of hexose-monophosphate and "respiratory factor;" nor could they obtain evidence of acid production under anaerobic conditions with glucose-monophosphate, bicarbonate buffer, and an extract of tissue as a source of respiratory supplement.

The obligate parasitic nature of the elementary body of vaccinia makes it unlikely that an independent metabolism, apart from the host cell, exists. It is not unreasonable to assume, however, that the elementary body possesses an incomplete metabolic system, relying in part, or even in the main, on constituents within the host cell for its completion. With this hypothesis in mind we have searched for the presence in purified virus of substances which, in bacteria and more highly organized cells, are known to participate in oxidation-reduction chains. In this paper results of our search are recorded.

EXPERIMENTAL

A detailed description of the methods used in the concentration and purification of elementary bodies of vaccinia has appeared in an earlier publication (1). Freshly prepared, active virus, dried to constant weight, was used in each experiment recorded in this study.

Attempts to Demonstrate a Cytochrome System

Respiration in aerobic organisms is known to go in part, if not chiefly, through the cytochrome system, which acts as a reversible oxidation-reduction

link between oxygen and certain enzyme systems which have become reduced in the process of the oxidation of cell substrates (4, 5). So far as it is now known, all cells which are able to use molecular oxygen contain one or more members of a group of protein porphyrins with iron in tetra-pyrrolic combination. These substances are known collectively as the cytochromes, and have been shown to participate in reversible oxidation-reduction processes within the cell (5). An enzyme, cytochrome oxidase, concerned with the rapid oxidation of reduced cytochrome, has been studied in detail by Keilin and Hartree (6). Both spectroscopic and enzymatic methods are available for the detection of cytochrome *c* (7).

Spectroscopic Examination.—In selected biological material, suitably prepared, the bands of reduced cytochrome can often be seen clearly with the aid of a microspectroscope. A search for the cytochrome system in vaccine virus was first made spectroscopically by means of a small Zeiss microspectroscope which was substituted for the eyepiece of an ordinary monocular microscope. A hanging drop preparation, containing a thick suspension of elementary bodies in 5 per cent sodium hyposulfite was prepared and examined repeatedly with a strong light source for the bands of reduced cytochrome. No absorption of any type was evident within the visible range of the spectrum. Under the same conditions the bands of reduced cytochrome in a suspension of yeast were clearly observed. The opacity of the elementary body preparation, however, made it possible that faint bands of reduced cytochrome might well have been obscured, and consequently missed by this technique. We accordingly turned to the second method, employing cytochrome *c*, cytochrome oxidase, and a variety of hydrogen donators, in an attempt to demonstrate cytochrome oxidase and cytochrome *c*, respectively, in preparations of elementary bodies of vaccinia.

Examination of Virus for Cytochrome Oxidase.—Cytochrome *c* was prepared by the method of Keilin and Hartree (8). This substance, together with paraphenylenediamine and certain other hydrogen donators, can be used to demonstrate the presence of cytochrome oxidase in suitably prepared biological materials, as shown by oxygen uptake in the Warburg apparatus. Under carefully controlled conditions, the rate of oxidation of the paraphenylenediamine in the presence of pure cytochrome *c* is proportional to the concentration of the cytochrome oxidase which is contained in the substance tested.

1000 gm. of fresh beef heart muscle were freed from fat and ligaments and ground finely in a meat chopper. The pulp was mixed with an equal volume of 0.15 N (2.5 per cent) trichloracetic acid. The mixture was allowed to stand at room temperature for 2 hours with occasional stirring, after which the fluid was pressed out, brought to pH 7 with sodium hydroxide, and centrifuged for 10 minutes. The clear supernatant fluid was drawn off by suction, and 50 gm. of ammonium sulfate for each 100 cc. of material was added. The precipitate was filtered off and discarded. The filtrate

was next treated with an additional 5 gm. of ammonium sulfate per 100 cc. and allowed to stand overnight in the cold. The material was then filtered, and while cold there was added 1/40 volume of cold 20 per cent trichloracetic acid. The fine red precipitate which appeared at this point in the procedure was collected by centrifugation. The precipitate was shaken with saturated ammonium sulfate and centrifuged again, after which it was transferred to a cellophane sac in a minimal amount of distilled water and dialyzed for 48 hours at 4°C. against 1 per cent sodium chloride. Finally the contents of the sac were shaken with a few drops of chloroform, filtered, and frozen and dried *in vacuo*.

An analysis performed on a sample of this material, dried to constant weight and ashed, revealed an iron content of 0.4 per cent which is close to the value given by Theorell and Åkesson for purified cytochrome *c* (9). The high activity of this material was demonstrated by oxygen uptake in the presence of paraphenylenediamine and cytochrome oxidase in the Warburg respirometer.

A sample of cytochrome *c* prepared by this technique was diluted with water until 1 cc. gave an oxygen uptake of 250 to 300 c.mm. per hour in the presence of an excess of hydroquinone and cytochrome oxidase. Freshly prepared elementary bodies, in 5 to 15 mg. lots, were suspended in 1.5 cc. of phosphate buffer, mixed with 0.5 cc. of the diluted cytochrome *c* solution, and placed in a Warburg respiration flask. In the side arm was placed 0.5 cc. of water containing 3.6 mg. of hydroquinone. The flask was attached to the manometer, cock sealed, and shaken in the water bath at 37°C. When temperature equilibrium had been reached, the hydroquinone solution was tipped into the elementary body-cytochrome *c* mixture, and the manometer read at frequent intervals for oxygen uptake. Control flasks, with cytochrome *c* and hydroquinone alone, were set up at the same time.

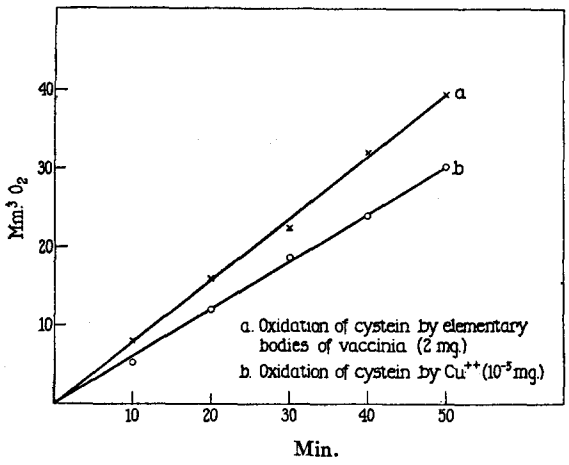
No significant oxygen uptake of the elementary body-cytochrome *c*-hydroquinone mixture, over that of the control flasks, was observed after 2 hours when the experiment was discontinued. If the virus contained cytochrome oxidase it was not possible to demonstrate it by this technique.

Examination of Virus for Cytochrome c.—A partially purified cytochrome oxidase for use in testing for cytochrome *c* was prepared from beef heart by the technique of Stotz and Hastings (10).

50 gm. of beef heart muscle were freed from fat and ligaments and ground twice in a meat chopper. The finely ground material was then placed in a bag made of two thicknesses of bandage gauze and washed in 1 liter of tap water at 40°C. for 10 minutes, with occasional squeezing. This procedure was continued through 4 washings at 40°C., alternating with 4 washings at 15°C. The mass of washed material, squeezed dry, was next placed in a mortar and ground with 60 mesh alundum in 100 cc. of M/15 K_2HPO_4 until a smooth paste was obtained. The paste was allowed to stand at room temperature 30 minutes with occasional stirring. The material was then centrifuged at 2000 R.P.M. and the sediment discarded. To the supernatant, which was allowed to stand at 4°C. overnight, was added an equal volume of 0.2 molar acetate buffer, pH 4.5. The mixture was then centrifuged and the supernatant

material discarded. The precipitate was resuspended in 10 cc. of M/15 K_2HPO_4. 0.5 cc. of this cytochrome oxidase preparation in the Warburg manometer was sufficient to yield an uptake of 20 to 30 c.mm. of oxygen per 10 minutes in the presence of an active preparation of cytochrome *c* and hydroquinone.

Purified elementary bodies, freshly prepared, in 5 to 15 mg. lots were suspended in 1.5 cc. of phosphate buffer and mixed with 0.5 cc. of an active suspension of cytochrome oxidase in the Warburg respirometer flask. In the side arm was placed 0.5 cc. of water containing 3.6 mg. of hydroquinone. When temperature equilibrium had been achieved, the hydroquinone solution was tipped into the elementary body-cytochrome oxidase mixture, and the manometers read at frequent intervals for



TEXT-FIG. 1. The oxidation of cystein by purified elementary bodies of vaccinia.

oxygen uptake. A control, with cytochrome oxidase and hydroquinone alone, was set up at the same time.

No appreciable oxygen uptake of the elementary body-cytochrome oxidase-hydroquinone mixture, over that in the control flasks, was observed after 2 hours when the experiment was discontinued. When the experiment was repeated, paraphenylenediamine being used as a hydrogen donator, a significant oxygen uptake occurred which lasted over a period of 2 hours.

A much greater and more consistent oxygen uptake, however, was demonstrated when cystein replaced paraphenylenediamine as a reducing agent. In this instance, the oxygen uptake was as great in the control system, which was composed of elementary bodies and cystein, without the addition of cytochrome oxidase (Text-fig. 1).

1 mg. of fresh elementary bodies was suspended in 0.15 M buffer and placed in the bottom of a Warburg flask. In the side arm was placed 1 cc. of an aqueous solution containing 6 mg. of cystein. Suitable controls, with elementary bodies and buffer

and cystein and buffer, were set up at the same time. After temperature equilibrium had been achieved, the cystein solution in the side arm was tilted into the respirometer flask; the manometers were read at 10 minute intervals for oxygen uptake. The rate of oxygen uptake from the oxidation of cystein by elementary bodies of vaccinia is shown in Text-fig. 1.

Identical rates of cystein oxidation by elementary bodies and by elementary bodies plus cytochrome oxidase indicated that no participation by cytochrome oxidase in this reaction was likely. With paraphenylenediamine, which is known from redox-potential considerations to be less specific than hydroquinone in the enzymatic reduction of cytochrome *c*, the case is not so clear, since no appreciable oxidation of paraphenylenediamine by elementary bodies of vaccinia without added cytochrome oxidase could be demonstrated. Results obtained by the use of paraphenylenediamine are also rendered ambiguous by the fact that its oxidation is catalyzed by traces of cytochromes *a* and *b* which in most cases contaminate preparations of cytochrome oxidase.

Search for a Metallic Catalyst

The catalytic effect of metals on the oxidation of paraphenylenediamine (8) and cystein (11) is well known. That a metallic component was responsible for the catalysis noted above was further indicated by the effect of potassium cyanide which in a concentration of 0.002 molar was effective in completely blocking the reaction. Aa′ dipyridyl, which is known to prevent oxidative catalysis by traces of ferric ion, was wholly without effect, indicating that inorganic iron did not play a part in this reaction. Sodium diethyl-dithiocarbamate, however, was effective in completely preventing the oxidation of cystein by elementary bodies of vaccinia. This substance has long been known to block copper catalysis by irreversible combination with the copper ion, and, because of this reaction, can be made specific for the detection of copper if iron is previously bound by pyrophosphate or dipyridyl (12).

The fact that the oxidation of cystein by elementary bodies of vaccinia was blocked effectively by sodium diethyl-dithiocarbamate made it likely that we were dealing with a copper constituent. It must be remembered that in higher concentration sodium diethyl-dithiocarbamate will block the catalytic effect of certain other metals, such as iron, cobalt, and magnesium. That the catalytic effect was due entirely to a metallic component was indicated by the fact that the ash from elementary bodies of vaccinia gave even a higher rate of cystein oxidation than would have been given by the intact virus.

Metallic ions often contaminate reagents, and our first thought was that in the preparation of elementary bodies of vaccinia copper had been introduced as a contaminant. Careful tests of the buffers and other reagents used in each step in the preparation of virus, however, failed to reveal a source of copper as a contaminant. Stainless steel gauze was substituted for bronze for the

scarification process which precedes the seeding of virus on the skin of rabbits without affecting the concentration of copper in the final product. Moreover, repeated washing of the purified virus resulted in no appreciable change in the concentration of the catalytic substance. Since in no instance could steps in the method of purification of the virus be shown to be responsible for the introduction of the relatively large amount of the catalytic substance observed in the purified virus, it was decided to attempt identification of the compound and find whether it was linked with virus activity.

Spectroscopic Demonstration of Copper in Vaccine Virus.—For proof that the catalytic substance in the purified virus was copper, we turned to spectroscopic studies. Emission spectra procured for us by Dr. G. I. Lavin on several samples of purified virus revealed the lines of copper in each instance (Fig. 1).

The spectra were obtained by placing the dried materials in bored carbon electrodes which were then arced with 110 volts of direct current. The lower electrode, containing the test substance, was made the positive one in each case. The photographs were taken with a medium Hilger quartz spectrograph on 10 inch plates.

As a reference substance, 10 mg. of dried egg albumin, containing 0.05 per cent of added copper as copper sulfate, was likewise arced in a bored electrode. The resulting emission spectrum was compared with respect to position and intensity with that obtained from the purified virus. As a second reference substance 10 mg. of dried material separated from the virus in the last stage of purification (final horizontal sediment) was likewise arced, and the resulting emission spectrum was used for comparison.

When the emission spectrum of purified virus was photographed (Fig. 1 *a*) the only lines to increase in intensity, over the residual traces given by the electrodes, were the copper lines at 3247 and 3274 Å, and those given by phosphorus in the region of 2530 to 2555 Å. The copper lines in the purified virus matched in position and intensity those given by the copper added to the egg albumin (Fig. 1 *b*, *d*).

The spectrum obtained from the horizontal sediment (Fig. 1 *c*) ordinarily discarded in the final stage of virus purification is interesting in that it contains a number of lines not seen in the purified virus. The major line at 3302 Å is thought to be due to zinc. Although this line and others due to metallic trace substances are absent from the final virus product, no diminution in intensity of the copper lines occurred. The absence of iron and other metallic constituents of tissue from the purified virus is highly significant in view of the original source of the virus, from animal skin. This is additional evidence that there is no appreciable quantity of impurities in our virus preparations.

Demonstration of Copper by Chemical Means.—For quantitative determination of the copper constituent, several methods were available. The rate of cystein oxidation in the absence of other metallic substances can in itself be made quantitative. Comparison of the rate of cystein oxidation by elementary

bodies of vaccinia with the rate produced by known increments of copper ion revealed a copper content significantly over 0.03 per cent of the dry weight of the virus. For direct chemical determination we were able to employ successfully the method of Sachs *et al.* (12), which could be performed on as little as 15 mg. of elementary bodies.

15 mg. of elementary bodies, dried to constant weight, were ashed at 600°C. in a vitreosil thimble with the aid of 0.25 cc. of reagent nitric acid. When ashing was complete, as revealed by the complete disappearance of carbon, the ash was extracted with 3 cc. of 6 N hydrochloric acid in 1 cc. amounts by warming the mixture to insure solution. The material was then transferred quantitatively to a 25 cc. volumetric cylinder, and followed by 2 additional rinses of the vitreosil thimble with 1 cc. of distilled water. A blank determination on reagents, including the ashing procedure, was run at the same time. 2 cc. of concentrated ammonia water was added and the solution cooled. 1 cc. of a 0.2 per cent aqueous solution of sodium diethyl-dithiocarbamate was next added and mixed thoroughly with the test solution by rotation of the tube. 10 cc. of isoamyl alcohol were next added, and the mixture shaken thoroughly for 1 minute. The yellow complex of copper and diethyl-dithiocarbamate was extracted quantitatively by the isoamyl alcohol and appeared in the isoamyl alcohol layer which rose slowly to the top and formed a sharp interface with the aqueous layer below. The colored layer was removed by means of a pipette and transferred to a colorimeter cup and compared with a known copper standard treated in an identical manner. All reagents were tested for copper and made up in triple glass-distilled water. No test for copper could be obtained on the reagents alone.

TABLE I

Copper Content of Elementary Bodies of Vaccinia

Lot	Virus taken for analysis	Copper
	mg.	*per cent*
1	15	0.051
2	15	0.056
3	15	0.052
4	15	0.048

The results of copper determinations made on four different lots of purified elementary bodies are given in Table I.

Nature of Copper Constituent

The fact that the copper constituent of our virus preparations was not appreciably altered by repeated washing of the purified virus indicated that the substance was held more firmly than by mere adsorption of inorganic copper ion.

Ultrafiltration of Elementary Bodies of Vaccinia.—Ultrafiltration, in which the volume of wash water could be kept small, failed to lower significantly the

copper constituent, indicating that it was held firmly to the virus. Copper sulfate, as a source of copper ion, added in amounts 4 to 5 times that present in the virus, was readily removed in two washings.

10 mg. of elementary bodies was suspended in 10 cc. of distilled water and placed in a copper-free cellophane bag made of Visking sausage casing. The upper end was knotted, and the bag placed in an ultrafiltration tube similar to that described by Coolidge (13). The tube was then placed in a centrifuge and spun for 2 hours at 3500 R.P.M. After that time a measurable quantity of ultrafiltrate had appeared in the bottom of the tube below the glass constriction on which the cellophane tube rested. This ultrafiltrate was tested for copper by means of the cystein oxidation technique and resultant oxygen uptake in the Warburg respirometer. No significant trace of copper ion could be detected in the ultrafiltrate, and no diminution in copper concentration was detectable in the elementary bodies from which the ultrafiltrate had been removed. Added copper in amounts twice that already present in the virus, however, was readily removed by such treatment and could be recovered quantitatively in the ultrafiltrate.

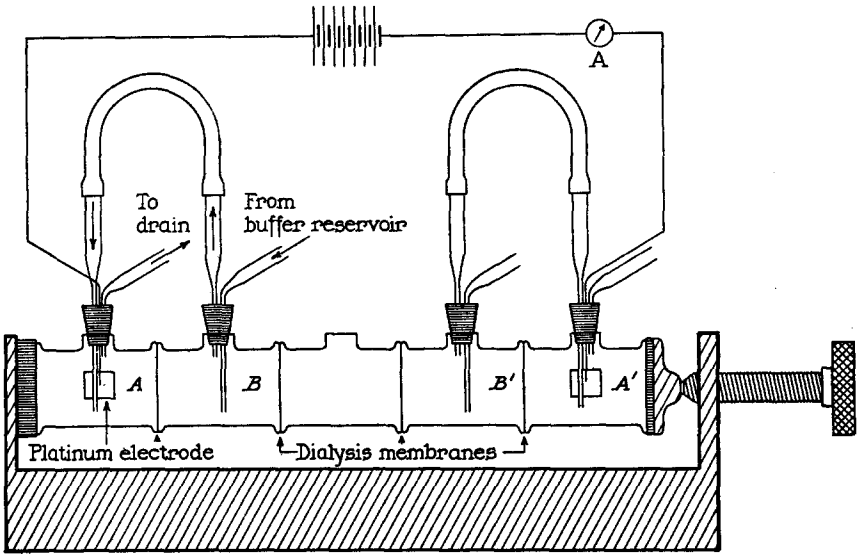
Electrodialysis of Elementary Bodies.—Electrodialysis is one means of insuring removal from proteins of metallic substances which are not held in organic combination. The copper in certain of the purified copper proteins, such as tyrosinase and laccase, is not removed by this procedure. In our experience, the usual methods of electrodialysis render vaccine virus inactive. With ordinary electrodes this can be traced to rapid and uncontrollable pH changes due to electrolysis of buffer. Moreover, since the removal of metallic substances by electrodialysis is also affected greatly by small pH changes, it was necessary to devise some technique whereby pH could be carefully controlled and shifted easily by a variety of buffers. An electrodialysis unit, devised several years ago by Dr. J. H. Bauer and Dr. T. P. Hughes of the International Health Division of The Rockefeller Foundation, was modified according to the accompanying illustration and found quite satisfactory for our purposes.

5 glass cells, with ground contact joints, were separated by cellophane discs which were sealed to the contact joints by rubber cement. A platinum electrode was placed in each of the end cells and buffer of any desired molarity and pH, from a reservoir above, was allowed to run slowly into cells B and B′ and over into A and A′ by means of glass U-tubes, as shown in Text-fig. 2. From the end cells, the buffer was allowed to drain away at a rate which was controlled by two screw clamps attached to the outlet tubes. The material to be dialyzed was placed in the middle cell, separated from the electrode cells by cells B and B′ which were slowly but continuously rinsed with new buffer. pH determinations, done at frequent intervals with the glass electrode, revealed no changes in pH, either in the center or two adjacent cells, over a period of 3 to 4 days of continuous electrodialysis.

10 mg. of freshly prepared elementary bodies of vaccinia were suspended in M/20

citrate-NaOH buffer, and submitted to continuous electrodialysis for 36 hours at 20°C., with a potential of 110 volts and a current of 20 milliamperes. Samples of the suspension of virus were removed for copper analyses and infectivity studies at 6 hour intervals.

With M/20 citrate-NaOH buffers, over a pH range of 6 to 8.5 no drop in copper concentration was observed Moreover, no drop in infectivity of the virus over control samples of virus kept at the same temperature and pH was noted. Copper added to egg albumin in one instance and washed suspensions



TEXT-FIG. 2. Modified Hughes-Bauer electrodialysis apparatus in which the pH is controlled by means of circulating dilute buffer.

of *Lactobacillus casei* in another, in amounts equivalent to that found in vaccine virus, was readily and completely removed within 4 to 6 hours over the same pH range. Finally, copper ion added to virus suspensions could likewise be removed by this technique.

Concentration of Copper Constituent with Purification of Virus.—A study of the virus material at various stages of purification has yielded certain information concerning the constituents which make up the purified virus. The virus constituents are necessarily concentrated as the purification proceeds, while the constituents representing contaminants arising from the skin of the rabbit tend to disappear (1). A study of the virus material during the process of purification showed a striking increase in the amount of copper.

A portion of the dermal pulp scraped from the skin of rabbits infected with vaccinia was separated from the virus by differential centrifugation, as described in an

earlier publication dealing with the purification of elementary bodies of vaccinia (1). This material was washed several times in dilute buffer and finally sedimented by prolonged centrifugation and dried from the frozen state. Successive sediments, ordinarily thrown away in the course of virus purification, were likewise retained, washed in successive changes of dilute buffer, dried from the frozen state, and analyzed for copper. The values for copper obtained after ashing of the dried sediments are recorded in Table II, together with the copper content of the purified virus separated from these successive sediments.

The successive analyses (Table II) show that a 25-fold increase in copper concentration occurred during the process of purification of elementary bodies of vaccinia. The relatively high copper content of the final sediment, which is ordinarily discarded during the process of virus concentration and purification, is not surprising since it is known to contain large amounts of the active agent.

TABLE II

A Comparison of the Copper Content of Vaccine Virus with That of Materials Discarded during Purification of the Virus

Material	Material taken for analysis	Copper
	mg.	*per cent*
Crude dermal pulp	20	0.002
"First horizontal sediment"	20	0.005
"Final horizontal sediment"	20	0.040
Purified virus	20	0.050

Dialysis of Elementary Bodies against Potassium Cyanide Solution.—Kubowitz (14) has claimed to show that the copper constituent of certain copper protein enzymes can be removed by dialysis against a dilute solution of potassium cyanide, with resultant loss of activity of the protein. Upon addition of copper in the form of inorganic salts, the activity of the protein is restored. An attempt to remove the copper from vaccine virus by dialysis against potassium cyanide was unsuccessful.

20 mg. of freshly prepared elementary bodies of vaccinia were suspended in dilute phosphate buffer in a cellophane sac and dialyzed with frequent agitation against 0.1 molar potassium cyanide at pH 7.2 for 48 hours at 4°C. The virus was next dialyzed against distilled water until free of cyanide, after which the virus suspension was frozen and dried to constant weight *in vacuo*.

An analysis of this preparation by means of ashing and color produced with sodium diethyl-dithiocarbamate revealed no significant loss of copper. The infectivity of this material, although dropping somewhat, was not less than that observed in a comparable virus control dialyzed for the same period of

time, but against phosphate buffer alone. The reduction in titre in both instances may have been due to agglutination occurring as a result of increased electrolyte concentration.

Studies of Enzymatic Activity of the Copper Constituent.—A great many copper proteins are now known (5). Hemocyanin, hemocuprein, hepatocuprein, polyphenol oxidase, laccase, and tyrosinase are representatives of a group of proteins containing copper in organic combination. The three latter substances are enzymes and are active in the oxidative catalysis of certain benzenoid compounds. On the basis of the possibility that a similar enzymatic rôle could be ascribed to the copper constituent in the elementary body of vaccinia, the effect of the intact virus on a number of substrates of the benzenoid series was studied.

1.5 cc. of M/150 catechol in phosphate buffer, pH 7.7, were placed in the side arm of a Warburg respiration flask. 2 mg. of freshly prepared elementary bodies of vaccinia suspended in phosphate buffer were placed at the bottom of the vessel. Controls of substrate and buffer without virus were set up at the same time. The manometer cocks were closed and the shaking apparatus set at 80 per minute. When temperature equilibrium had been achieved, the catechol substrate was tilted into the elementary body suspension and the manometers observed at 10 minute intervals for oxygen uptake. No reaction was observed in 2 hours when the experiment was discontinued.

A similar attempt to demonstrate the catalytic oxidation of other benzenoid compounds was made. No oxygen uptake was detected when paraphenylenediamine, hydroquinone, orcinol, phloroglucinol, benzidine, or tyrosine was used as a substrate with 2.0 mg. of freshly prepared virus in a system similar to that described in detail for catechol.

DISCUSSION

It is interesting that a certain degree of parallelism between virus purification and copper concentration can be demonstrated. This is particularly significant in view of the fact that other metallic constituents present in the early stages of virus purification are absent from the final product in so far as can be demonstrated spectroscopically. The fact that no significant spectroscopic traces of iron compounds exist in the purified virus indicates, we believe, that no appreciable contamination of the purified virus with cellular debris is likely.

The demonstration of a copper constituent associated with the purified elementary body of vaccinia further emphasizes the complexity of the elementary body of vaccinia. That the material is not one of the known copper-protein enzymes is fairly certain, as evidenced by its lack of reactivity with the known substrates of these substances. Failure to be removed by electrodialysis over a pH range in which the virus remains active indicates that the

copper is bound with a degree of firmness exhibited by known copper proteins, such as tyrosinase and hemocyanin. It is tempting to assume that a metallic group such as copper, by virtue of its known rôle as an oxidative catalyst, may possibly function in the respiratory activity of the virus. No proof of such a function can be furnished at this time.

SUMMARY

A search by means of spectroscopic and enzymatic techniques has failed to demonstrate either cytochrome or cytochrome oxidase in purified elementary bodies of vaccinia. A constituent of the virus which catalyzes the oxidation of cystein has been found and identified as copper in a concentration amounting to 0.05 per cent of the dry weight of the virus. The copper constituent was not removed by repeated washing, ultrafiltration, dialysis against 0.1 molar potassium cyanide, or by electrodialysis over a pH range which did not inactivate the virus. During the process of purification of the virus a 25-fold increase of the copper constituent was observed. Emission spectra obtained from the dry virus also revealed copper but no significant traces of other metallic substances. No biological rôle can yet be ascribed to the copper component of virus.

BIBLIOGRAPHY

1. Hoagland, C. L., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1940, **71**, 737.
2. Hoagland, C. L., Lavin, G. I., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1940, **72**, 139.
3. Parker, R. F., and Smythe, C. V., *J. Exp. Med.*, 1937, **65**, 109.
4. Haas, E., *Naturwissenschaften*, 1934, **22**, 207.
5. Green, D. E., Mechanisms of biological oxidations, Cambridge, England, Cambridge University Press, 1940.
6. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, 1938, **125**, 171.
7. Keilin, D., *Proc. Roy. Soc. London, Series B*, 1930, **106**, 418.
8. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, 1937, **122**, 298.
9. Theorell, H., and Åkesson, Å., *Science*, 1939, **90**, 67.
10. Stotz, E., and Hastings, A. B., *J. Biol. Chem.*, 1937, **118**, 479. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., *J. Biol. Chem.*, 1938, **124**, 733.
11. Warburg, O., *Biochem. Z.*, 1927, **187**, 255.
12. Sachs, A., Levine, V. E., Andersen, A. C., and Schmit, A., *J. Lab. and Clin. Med.*, 1941, **26**, 734.
13. Coolidge, T. B., *J. Biol. Chem.*, 1940, **135**, 541.
14. Kubowitz, F., *Biochem. Z.*, 1938, **299**, 32.

EXPLANATION OF PLATE 6

FIG. 1. Emission spectra of purified elementary bodies of vaccinia (*a*), of egg albumin to which copper was added to 0.05 per cent concentration (*b*, *d*), and of material removed from elementary bodies of vaccinia in final stage of purification (*c*).

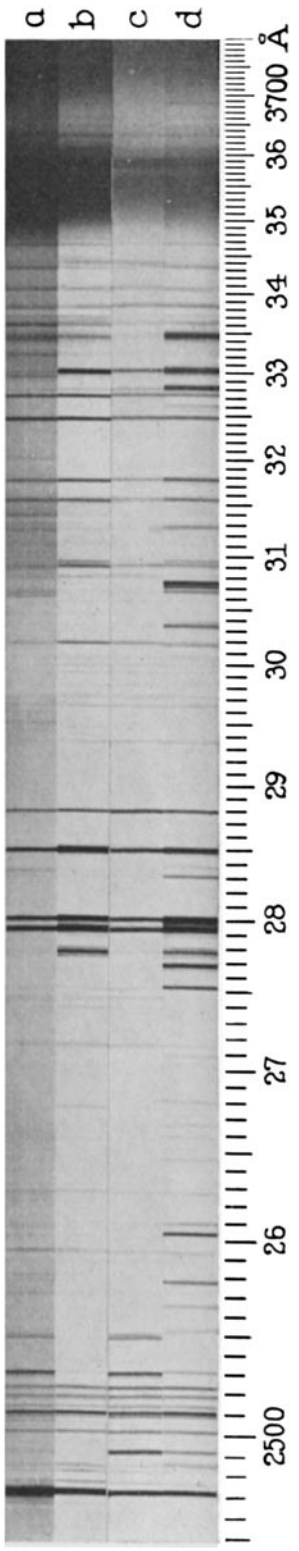


FIG. 1

(Hoagland *et al.*: Copper constituent in vaccine virus)