CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

VI. Studies on the Nature of the Enzymes Associated with 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)

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Elementary bodies of vaccinia, obtained by differential centrifugation of the dermal pulp of infected rabbits, have been shown to satisfy many of the criteria laid down by workers in protein chemistry for pure biological entities. Chemical examination of this purified virus has revealed the presence of many substances common to protoplasm, including certain of the vitamin catalysts, and at least one metallic component, copper, which seems to bear an intimate relationship to the final infective product (1–5).

Macfarlane and Salaman (6) have reported the presence of catalase and phosphatase in purified elementary bodies of vaccinia, but were unable to demonstrate dehydrogenase activity of any type, although the reaction of the virus with a variety of dyes and substrates was studied. The assumption by these workers that the catalase and phosphatase activity of their virus preparations was due to the virus, and not to possible tissue contaminants, was based on the observation that phosphatase and catalase were greatly concentrated during the process of virus purification, while dehydrogenases, which were present in high titer in the tissues and cell fragments from which the virus was separated, were absent from the final virus product. The possibility that the occurrence of phosphatase and catalase may have been due to surface adsorption was entertained, but was not considered likely because frequent washing of the virus failed to elute them.

As a continuation of previous studies on the nature of the constituents of purified elementary bodies of vaccinia, we decided to repeat the studies of Macfarlane and Salaman, and to extend them to certain other enzymes which are found in the host tissues from which the virus is prepared, and which might, therefore, be associated with the purified virus. The results of a search for oxidative enzymes have appeared in earlier communications (3, 4).

Materials and Methods

A detailed description of the technique for securing relatively large quantities of purified elementary bodies of vaccinia has been published previously (1). Elementary bodies prepared by this method show a high infectivity-elementary body ratio and a constant chemical composition. Although

hydrolytic enzymes are remarkably stable and, when dried from the frozen state and stored at low temperatures, retain their activity undiminished for many months, the dehydrogenases, on the other hand, are extremely labile and maintain their activity for relatively short periods of time. In order to rule out as far as possible the factor of enzyme lability, only those lots of freshly prepared virus which showed high infective titers were used in these studies. The methods employed for the quantitative determination of the enzymes in elementary bodies of vaccinia were tested on substrates and enzyme preparations of known purity and found to yield satisfactory results before being applied to studies on the enzyme constituents of the virus. Because of the limited quantity of virus available, certain of the methods had to be modified in order to permit quantitative determinations on small amounts of material.

Tests for Dehydrogenase Activity

Macfarlane and Salaman were unable to demonstrate dehydrogenase activity in their preparations of elementary bodies by the usual Thunberg technique at pH 7.2 and 37°C. There are certain disadvantages inherent in the use of the Thunberg technique, however, in that it is essentially a time reaction, with the end point depending on recognizable decolorization of a dye. Moreover, certain dehydrogenases may show marked inhibition by the concentration of methylene blue, or other dyes of suitable potential, which it is necessary to use in order to demonstrate reduction (7). Recently Quastel and Wheatley (8) have devised a method for the determination of dehydrogenase activity which differs materially from the Thunberg technique, and which lends itself to the use of smaller quantities of material. This method depends on the fact that electrons from hydrogen, which is taken up by coenzyme in the presence of a given dehydrogenase and its appropriate substrate, are transferred to a suitable metallic acceptor, such as ferric ion. The hydrogen ions, produced by this electron transfer, react in turn with bicarbonate buffer to yield carbon dioxide, which can be measured in the Warburg manometer. By the simple process of changing substrates, tests may be made with relative ease for a large series of dehydrogenases and the results expressed quantitatively in terms of carbon dioxide evolution. By means of this method, elementary bodies of vaccinia have been tested for malate, succinate, lactate, and pyruvate dehydrogenase activities.

Malate Dehydrogenase.—10 mg. of freshly prepared virus were placed in Warburg flasks and suspended in a mixture of 0.15 cc. of 0.2 molar sodium malate and 1.35 cc. of 0.9 per cent sodium chloride containing coenzyme prepared from red blood cells. To the side arm receptacle of the flask were added 0.1 cc. of 0.03 molar sodium cyanide, 0.4 cc. of 0.16 molar sodium bicarbonate, and 0.2 cc. of 10 per cent sodium ferricyanide. A small stick of yellow phosphorus was placed in the center inset to insure anaerobiosis, and the flasks were equilibrated with pure nitrogen gas. Appro-

priate controls of reagents, with saline replacing the virus, were set up at the same time. The flasks and contents were brought to 37°C. in the water bath, and the shaker set at 100 per minute. When temperature equilibrium had been achieved, the contents of the side arm were tilted into the reaction flasks and manometric readings taken at 10 minute intervals. No evolution of carbon dioxide, over that in the controls without virus, was observed at the end of 90 minutes, and the experiment was discontinued. Three lots of virus were tested for malic dehydrogenase with negative results in each case.

Succinate, Lactate, and Pyruvate Dehydrogenases.—Tests were performed for succinate, lactate, and pyruvate dehydrogenases after the manner described for malate dehydrogenase, with the substitution of 0.2 molar sodium succinate, lactate, and pyruvate, respectively, for sodium malate. Tests for each of these dehydrogenases were performed on two lots of virus, with negative results in each instance. If the virus initially possessed dehydrogenase activity toward any of the four substrates tested, such activity was very likely destroyed by the washing and centrifuging processes to which the virus was subjected during purification. Tests for other dehydrogenases were not performed. Macfarlane and Dolby (9), however, report negative tests on elementary bodies for triosephosphate and hexosemonophosphate dehydrogenases. These authors likewise were unable to obtain evidence of lactate dehydrogenase.

Tests for Phosphatase Activity

Macfarlane and Salaman tested seven preparations of elementary bodies for phosphatase activity (6) with positive results in every case. Elementary bodies prepared in our laboratories were found likewise to exhibit phosphatase activity, and to an extent approximating that reported by these authors for their material. Our studies have been extended, however, and show, we believe, that the presence of phosphatase in the elementary body may well be accounted for by its having been adsorbed from tissue detritus, and that it is not, as these authors suggest, necessarily to be regarded as an integral part of the virus.

β-Glycerophosphatase Activity.—To 5 mg. of freshly prepared elementary bodies were added 5 cc. of a β-glycerophosphate-buffer mixture containing a final concentration of 0.066 molar sodium barbital. The pH of the mixture was 8.9 as determined by the glass electrode. An appropriate control of pancreatic phosphatase was set up at the same time. The mixture was incubated for 1 hour at 37°C. and 0.5 cc. of 10 per cent trichloracetic acid was added to stop enzymatic action and to precipitate the virus. The mixture was then centrifuged and inorganic phosphorus determined in the supernatant fluid by the method of Fiske and Subbarow (10). The results of the determination of phosphatase activity on four lots of elementary bodies are given in Table I. The activity is expressed in terms of milligrams of phosphorus hydrolyzed per milligram of virus per hour. The results, although somewhat more constant from lot to lot, agree fairly well within a given pH range with those reported by Macfarlane and Salaman.

Adsorption of Phosphatase by Elementary Bodies of Vaccinia.—The presence of phosphatase in elementary bodies of vaccinia means either that the enzyme is an integral part of the virus, or that its presence in the virus is to be accounted for on the basis of adsorption of the enzyme from the host tissues, from which the virus was separated in the process of purification. The latter hypothesis seems likely when it is considered that large quantities of ruptured white cell elements and tissue detritus, rich in phosphatase, accompany the virus in its initial stages of preparation. This set of conditions, together

TABLE I

Phosphatase Activity of Elementary Bodies of Vaccinia

Lot	Virus taken for determination	Phosphorus hydrolyzed	Phosphatase activity mg. P/weight of virus
	mg.	*mg.*	
1	5	0.240	0.048
2	5	0.250	0.050
3	5	0.265	0.053
4	5	0.245	0.049

TABLE II

Phosphatase Activity of Elementary Bodies of Vaccinia Which Have Been Suspended 1 Hour in Dilute Phosphatase Solution and Washed with Buffers of Varying pH

Lot	Virus taken for determination	Phosphorus hydrolyzed	Phosphatase activity mg. P/weight of virus
	mg.	*mg.*	
1	5	0.618	0.121
2	5	0.510	0.102
3	5	0.780	0.156
4	5	0.492	0.098

with the large surface area possessed by the elementary body, renders the adsorption hypothesis highly tenable. To test this possibility, elementary bodies of known phosphatase content were suspended in a dilute solution of purified pancreatic phosphatase, washed repeatedly with buffer solutions of varying pH, and retested for phosphatase activity.

Phosphatase, partially purified by fractional precipitation from a sodium chloride extract of dried pancreatin, was dried from the frozen state and the activity found to be approximately ten times that of the original pancreatin, on the basis of dry weight. 1 mg. of the crude phosphatase was dissolved in 10 cc. of 0.9 per cent sodium chloride, with the formation of a perfectly clear solution. Elementary bodies, in 5 mg. lots, were suspended in 5 cc. of this solution and allowed to remain for 1 hour at 20°C. At the end of this period the virus was recovered by centrifugation, washed alternately

by centrifugation and resuspension in successive 5 cc. portions of buffer solutions of pH 6 and 8, respectively. Four individual washings were performed, two with each buffer, and the virus resuspended in 5 cc. of the β-glycerophosphate substrate-buffer mixture previously described. At the end of a 1 hour period of incubation at 37°C. inorganic phosphorus determinations were made. The results are recorded in Table II.

The virus, in some instances, showed a threefold increase in phosphatase value after treatment with a dilute solution of crude phosphatase. That the increase in phosphatase activity was real, and not an apparent one from phosphatase which might have been carried over in solution by dilution in the washing process, was shown by the failure to obtain measurable phosphatase activity in the third and fourth buffer solutions from which the phosphatase-treated virus was recovered. This experiment indicates that the phosphatase activity shown by purified elementary bodies of vaccinia may well be due to adsorption, and that failure to remove the enzyme by repeated washing is not sufficient evidence to prove that it was an integral part of the virus. On the other hand, it does not constitute positive evidence that the phosphatase activity originally shown by the virus was due solely to adsorption of enzyme. At the moment an experiment which would offer an unequivocal solution of this problem does not suggest itself.

Catalase Activity of Elementary Bodies

In our laboratory all preparations of elementary bodies tested for catalase have thus far yielded positive results. This fact is not surprising when it is considered that the virus is in contact with tissue detritus and cell fragments, rich in catalase, in its earlier stages of preparation. That the enzyme is not carried over by dilution of tissue juices accompanying the virus has been shown by the fact that the successive buffers in which the virus is washed in the course of its purification yield negative tests for catalase. In order to show that the catalase is an integral part of the virus, however, it is first necessary to rule out the possibility of adsorption. This, as the following experiment shows, it is difficult to do. The fact that elementary body preparations will adsorb and hold tenaciously large amounts of catalase from dilute solutions makes tenable the explanation that the catalase in the purified virus has been adsorbed.

Determination of Catalase.—2 cc. of a 0.1 per cent hydrogen peroxide solution in 0.15 molar potassium acid phosphate buffer, pH 7.0, were placed in a Warburg respiration flask. To the side arm were added 5 mg. of elementary bodies suspended in 0.5 cc. of dilute phosphate buffer. Appropriate controls, with buffer solution replacing the virus, were set up at the same time. The flasks were attached to the manometers and shaken in a water bath at 20°C. When temperature equilibrium had been achieved the virus was tilted from the side arm into the reaction flask and

changes in oxygen tension read at 5 minute intervals. The results for five preparations of elementary bodies are recorded in Table III. The catalase activity has been expressed as the *Kat. f.* value, which is the conventional term for ratio of the velocity constant, *k*, to the dry weight of the test material, or virus, on which the determination was performed.

The velocity constants, and the consequent *Kat. f.* values, for our preparations are significantly lower than those reported by Macfarlane and Salaman. It is possible that this discrepancy is to be explained by certain differences in the methods used for the determination of catalase activity. As may be seen from Table III, different lots of elementary bodies vary widely in their catalase content. Lot 3, for example, shows over ten times the activity of lot 2. This may be explained in part by the lability of catalase to the conditions imposed by the process of virus purification, and perhaps in part by the differences in catalase content of the cell fragments from which the virus was prepared.

TABLE III

The Catalase Activity of Purified Elementary Bodies of Vaccinia

Lot	Virus taken for determination	*k* (velocity constant)	Catalase activity *Kat. f.* (*k*/gm. of virus)
	mg.		
1	5	0.00173	0.346
2	5	0.00087	0.174
3	5	0.00903	1.810
4	5	0.00130	0.260
5	5	0.00260	0.520

Adsorption of Catalase by Elementary Bodies of Vaccinia.—Catalase was prepared in crystalline form from ox liver by the method of Sumner and Dounce (11). A solution of the crystals was diluted so that 1 cc. gave an oxygen uptake, with a hydrogen peroxide substrate in the Warburg flask, of approximately 3 to 4 cm. per minute. 5 mg. of elementary bodies, of known catalase activity, were suspended in 5 cc. of this solution for 1 hour at 20°C. At the end of this period the virus was collected by centrifugation and washed repeatedly with dilute buffer until the supernatant solution gave a negative test for catalase. The virus was reclaimed by centrifugation and dried from the frozen state. 2 mg. quantities of the catalase-treated virus were set up in the Warburg apparatus, and the oxygen tension developing from the catalase action on hydrogen peroxide determined in the manner previously described. The activity was so great as to render accurate rate determinations impossible. Within 1 minute a tension of 200 cm. and more of oxygen was developed. A determination of the velocity constant at this rate of oxygen evolution was impossible.

The experiment showed clearly that the virus possessed great surface affinity for catalase, and that relatively large amounts of the enzyme had been ad-

sorbed, and, moreover, that it had not been removed by frequent washing. In the light of these facts, the conclusion that the original catalase action of the virus may have been due to adsorbed enzyme is hard to escape.

Lipase Activity of Elementary Bodies of Vaccinia

Most tissues are rich in enzymes capable of hydrolyzing fatty acid esters of glycerol. These enzymes, which are referred to as lipases or esterases, are extremely stable, and would be expected to withstand the washing and centrifuging procedures to which elementary bodies are subjected in the course of their purification. Lipase determinations were accordingly made on several lots of the purified virus.

Determination of Lipase Activity.—A number of methods for the determination of lipase activity exist. The methods most commonly employed are based on the values obtained from the titration of the fatty acid released by the hydrolysis of suitable fatty acid esters. A manometric method, devised by one of us, in which the carbon dioxide released by the reaction of the fatty acid with a bicarbonate buffer is employed as a measure of lipase activity, has been used successfully in this laboratory for some time (12). The method gives accurate reaction rates and makes use of the slope of the linear portion of the curve, obtained by plotting carbon dioxide evolution against time, as a measure of the activity of the enzyme.

5 mg. of elementary bodies, suspended in 0.5 cc. of saline, were placed in the side arm of a Warburg respiration flask. To the flask were added 1 cc. of a 10 per cent emulsion of tributyrin in water, neutralized to a phenol red end point with sodium hydroxide, and 1 cc. of a bicarbonate buffer solution, containing 4.5365 gm. of sodium bicarbonate per liter. When this mixture was equilibrated with 5 per cent carbon dioxide, and diluted 1:1 with the tributyrin emulsion, a lipase substrate of pH 7.8 was obtained. The flask was equilibrated with a stream of pure carbon dioxide until the substrate became acid to phenol red, at which time a 5 per cent carbon dioxide-95 per cent nitrogen mixture was substituted for pure carbon dioxide and equilibration continued until a 5 per cent concentration of carbon dioxide in the flask was assured. Appropriate controls on the reagents, with saline replacing the virus suspension, were set up at the same time and likewise equilibrated. The flask, attached to the manometer, was then shaken in a water bath at 37°C. When temperature equilibrium had been achieved, the contents of the side arm were tilted into the reaction vessel and the increase in carbon dioxide tension read at 5 minute intervals. The results of a typical reaction are given in Fig. 1.

Five lots of elementary bodies have been tested for lipase activity with positive results in each instance. From Fig. 1 it may be seen that the hydrolysis of tributyrin by elementary bodies of vaccinia is effected readily and at a constant rate over a long interval of time.

It was next decided to test the adsorptive capacity of elementary bodies for lipase in a manner analogous to that described for the adsorption of phosphatase and catalase.

Adsorption of Lipase by Elementary Bodies of Vaccinia.—Lipase was prepared by ammonium sulfate precipitation of the globulin in a 10 per cent sodium chloride extract of dried pancreatin. The crude lipase globulin obtained from the sodium chloride extract was dialyzed for 24 hours against distilled water at 4°C. to remove salts and finally dried from the frozen state. The lipase activity of this preparation was approximately 25 times that of the original pancreatin. 20 mg. of elementary bodies were suspended in a 0.1 per cent solution of the crude lipase preparation and allowed to remain 1 hour at 20°C. The virus was reclaimed by centrifugation and

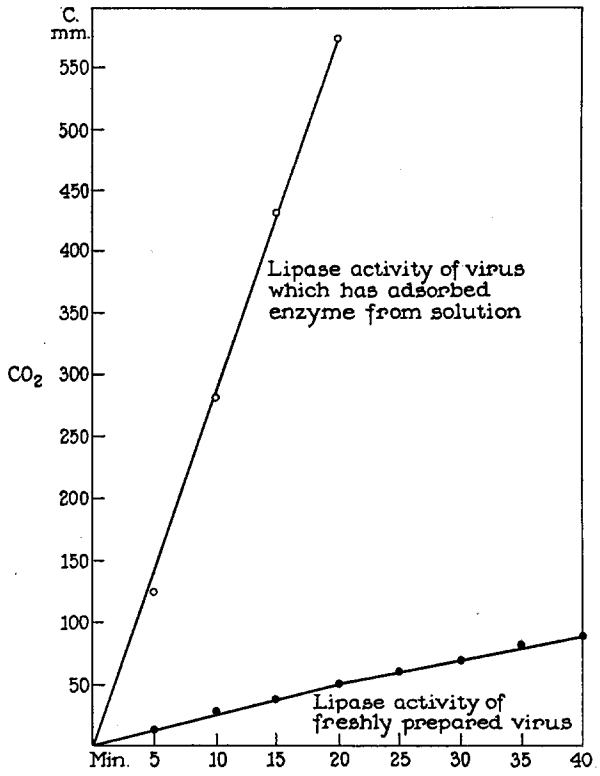

FIG. 1. Hydrolysis of tributyrin by elementary bodies of vaccinia.

washed by thorough resuspension and centrifugation several times in 7 to 10 cc. quantities of distilled water until the supernatant waters no longer gave a test for lipase. The virus was dried from the frozen state and lipase determinations performed on 5 mg. aliquots. Values for the rate of hydrolysis of tributyrin by lipase-treated virus are plotted in Fig. 1.

The great capacity of purified elementary bodies to adsorb lipase is conclusively demonstrated by the foregoing experiment. From a relatively dilute solution the virus is able to effect an enormous concentration of this enzyme as shown by activity measurements before and after adsorption.

Adsorptive Specificity of Elementary Bodies

The adsorptive experiments, performed with phosphatase, catalase, and lipase, show that the elementary body of vaccinia has a highly active surface capable of adsorbing certain enzymes from solution. That the adsorptive power of the virus is an exceptionally strong one is shown by the failure to remove these enzymes by frequent washing with saline and dilute buffers. It therefore became of interest to know if this property of the elementary body was exhibited indifferently to all enzymes, or if a certain degree of specificity in the adsorption could be demonstrated. Since the enzymes used in the adsorption experiments described above were of animal origin, it was of interest to know if an enzyme of vegetable origin, such as urease, might likewise be adsorbed.

Urease Adsorption Experiment with Elementary Bodies.—30 mg. of elementary bodies were suspended in a 1 per cent aqueous solution of Squibb's urease for ½ hour at 38°C. The virus was reclaimed by centrifugation and washed by resuspension and centrifugation until the supernatant wash waters no longer gave a positive test for urease. The virus was dried from the frozen state and urease determinations made on 5 mg. quantities of the dried urease-treated virus. The quantitative determination of urease activity was performed by the method of Krebs and Henseleit (13). This method was devised for the determination of urea, but can be used equally well for the determination of urease if a substrate of urea is supplied. 5 mg. of urease-treated elementary bodies were suspended in 0.5 cc. of saline and added to the side arm of a Warburg respiration flask. In the bottom of the flask were placed 1.5 cc. of a 1 per cent solution of urea and 0.5 cc. of sodium acetate-acetic acid buffer, pH 5. Enzyme controls, with 0.2 cc. of 1 per cent urease solution in the place of the virus, and reagent controls, with saline replacing the virus, were set up at the same time. The flasks were attached to the manometers and shaken in a water bath at 37°C. When temperature equilibrium had been effected, the virus was tilted into the reaction mixtures and changes in carbon dioxide tension read at 5 minute intervals for 1 hour. No urease activity of the treated virus could be demonstrated, although good activity of the urease preparations in the control flasks was noted consistently.

The experiments with urease show that the adsorptive capacity of elementary bodies for enzymes is to be regarded as somewhat specific. Although urease is known to have a high affinity for adsorbents, similar to that of enzymes in general, no adsorption of urease on elementary bodies could be demonstrated under the conditions of the experiment. Whether adsorption could have been demonstrated at another pH was not tested, since it was desired to learn only of the possibility of enzyme adsorption under the conditions of virus purification which is carried out at neutral pH.

DISCUSSION

The experiments described above show that elementary bodies of vaccinia, which have been prepared by washing and differential centrifugation, show

enzymatic activity toward a number of substrates. The enzymes which have been found thus far, however, are those which are known to occur in high concentration in white cells and tissue detritus. The fact that the virus is originally in intimate contact with various enzyme constituents in the dermal pulp from which the virus is prepared, makes the hypothesis that certain of these enzymes may be adsorbed on the virus, during the process of purification and concentration, a very tenable one. Dehydrogenases, and other redox enzymes which occur in cells generally and which might be expected to be adsorbed on virus particles, appear to be absent. If present initially, the lability of these enzymes might well preclude their surviving the several steps employed in the purification of the virus. Although it has not been possible to design experiments which prove that the enzymes found in the purified virus are adsorbed from tissue elements, the fact that the virus will take up large quantities of certain enzymes from a dilute solution makes a strong case for the adsorption hypothesis. Until methods can be devised which are capable of distinguishing between enzymes which represent an integral part of the virus and those which exist as contaminants, it would seem that the problem is impossible of solution. So far, no enzyme protein has been found in purified elementary bodies which is not at the same time a known constituent of normal tissue and the presence of which might not be accounted for on the basis of its adsorption from the host cells.

In the light of the above facts concerning the adsorption of enzymes by elementary bodies, what may we conclude with reference to certain other constituents which have been reported in this virus, namely, lipid, carbohydrate, nucleoprotein, copper, flavin, and biotin (1–5)? With respect to the first three substances, the fact that these occur in almost stoichiometric proportions in different preparations of virus and that they account for a major share of the mass of the virus, goes far to preclude their being accounted for on the basis of adsorption. With respect to the presence of copper, the fact that this element withstands electrodialysis, that it is released only upon hydrolysis of the virus, that it is concentrated at least 25 times in the process of virus purification, and that it occurs in constant amounts from lot to lot of elementary bodies, speaks strongly for its being an integral part of the virus. In the case of flavin adenine dinucleotide, the discovery that this substance likewise occurs in near stoichiometric relationship to other virus components, and that elementary bodies do not adsorb additional amounts of this material from a dilute solution of flavin adenine dinucleotide, speaks convincingly in favor of its close relationship to virus structure (14). As for biotin, the observation that this substance is released in relatively large amounts during the process of hydrolysis (5) of the virus likewise points to an intimate association of this material with elementary body structure. Moreover, elementary bodies show no appreciable adsorption of biotin from solution under a variety of conditions (14).

CONCLUSIONS

Purified elementary bodies of vaccinia have been tested with a variety of substrates and found to possess phosphatase, catalase, and lipase activity. Tests for malate, succinate, pyruvate, and lactate dehydrogenases were negative.

Interpretation of these results is complicated by the observation that elementary bodies of vaccinia adsorb relatively large quantities of certain enzymes from dilute solutions. These enzymes are not eluted by procedures of washing and centrifuging similar to those carried out in the preparation of the virus. For this reason, the presence of phosphatase, catalase, and lipase in the purified virus may well be accounted for on the basis of adsorption from the host tissues which are known to be rich in these particular enzymes. That some degree of specificity in this adsorption is to be recognized is shown by the failure of the virus to adsorb urease, an enzyme of vegetable origin.

Until some method can be devised which will distinguish between the enzymes of the host cell and those which may be integral parts of the virus it would seem that the problem of the enzyme constituents of vaccine virus is incapable of definite solution.

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