

## CONSTITUENTS OF ELEMENTARY BODIES OF VACCINIA

### III. THE EFFECT OF PURIFIED ENZYMES ON ELEMENTARY BODIES OF VACCINIA

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PLATE 23

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The effect of various enzymes on the infectivity of animal and plant viruses has been the subject of frequent investigations (1-6). Many of the conflicting statements appearing in reports of these studies were explained by Pirie (7) who showed that most if not all the reported virucidal effects of pancreatic enzymes were due not to the enzymes themselves, but to the unsaturated fatty acids which contaminated the enzymatic materials. Indeed, she was able to duplicate many of the lethal effects attributed to enzymes by fatty acids alone in concentrations equal to those observed in the enzymatic preparations. It has become increasingly clear that the true effects of enzymes can be learned only through the use of highly purified or crystalline preparations of these active substances, and when possible by the use of equally pure viruses. Moreover, studies of chemical and physical changes in virus materials due to enzymatic activity should accompany studies of changes in infectivity.

Merrill (8), recognizing the importance of working with pure materials, undertook in 1936 to study the effect of crystalline proteolytic enzymes on various viruses and certain Gram-negative bacteria. Unfortunately, no chemical studies paralleled his studies of infectivity, so that the work as a whole remains to some extent equivocal. He concluded, however, that certain viruses behaved like proteins, in that they were inactivated by proteolytic enzymes, while others, like bacteria, were more resistant. Pirie (7), under the conditions of her experiment, found little or no effect of crystalline trypsin and chymotrypsin on pleuropneumonia organisms, fowl tumor virus, and vaccine virus; and Stanley (9) in studies of the action of crystalline trypsin on tobacco mosaic virus found that the effect of the enzyme was on the host and not on the virus. Pepsin (10) which has been found to inactivate tobacco mosaic virus, cannot be tested for its effect

on the infectivity of animal viruses, since the low pH required for activity of the enzyme in itself inactivates the viruses.

In view of the important relationship known to exist in general between the structure of a substance and its resistance or non-resistance to enzymes, the following studies on the effect of crystalline enzymes on elementary bodies of vaccinia were undertaken.

#### *Materials and Methods*

The preparation of elementary bodies of vaccinia has been dealt with extensively elsewhere (11, 12). Virus preparations, showing constant analytical data and a high degree of dermal infectivity in rabbits, were used throughout these studies. The activity of both purified and crystalline enzymes<sup>1</sup> toward a variety of specific substrates was clearly established before any attempt was made to use them in studies on vaccine virus.

Chemical methods are now available for the detection of activity of all crystalline enzymes. In many instances very slight activity can be determined with a high degree of reproducibility provided the external environment and concentration of reactants are kept constant (13). A variety of techniques are available for the estimation of the degree of hydrolysis by proteolytic enzymes. Most of these involve measurement of carboxyl or amino groups by titrimetric, electrometric, colorimetric, or manometric means. The estimation of amino nitrogen by the manometric technique of Van Slyke as an indication of proteolysis seemed most suitable for our work in view of the small quantity of virus used in each experiment.

In all instances histological changes in the elementary bodies were determined by Morosow's (15) staining reaction. Studies of the staining reaction of the virus paralleled all studies of chemical change and of changes in infectivity.

#### EXPERIMENTAL

*Effect of Crystalline Trypsin.*—Merrill (8) in 1936, testing elementary bodies of vaccinia prepared in this laboratory, found a slight amount of inactivation by trypsin only after prolonged incubation at ice box temperature. No chemical determinations were reported, however, so it is not possible to state whether any appreciable degree of hydrolysis of the virus was obtained. With a modification in the type of incubation we have repeated this experiment, making amino nitrogen determinations at regular intervals throughout the period of incubation.

<sup>1</sup> Crystalline pepsin was generously supplied by Dr. J. H. Northrop, crystalline chymotrypsin, crystalline carboxypeptidase, and purified cathepsin by Dr. J. S. Fruton, and crystalline ribonuclease by Dr. M. Kunitz. Crystalline trypsin was purchased from the Plaut Research Laboratories, Bloomfield, New Jersey. The crystalline papain was prepared in the laboratory of Dr. A. K. Balls. The crystalline ficin was prepared by Dr. A. Walti of Merck and Company Research Laboratories.

10 mg. of purified vaccine virus were suspended in 5 cc. of M/15 phosphate buffer solution at pH 7.8 by means of shaking with glass beads. When a milky suspension had been achieved, 5 cc. of buffer solution containing 1 mg. of crystalline trypsin were added; the tube was rotated to insure thorough mixing; and 1 cc. of the mixture was removed for amino nitrogen determination. The tube was sealed and incubated at 38°C. Samples of 1 cc. were removed every 2 hours for 6 hours and thereafter at longer intervals; these were inactivated by heat immediately upon removal, and used for amino nitrogen determination. Samples containing 0.005 mg. of virus were removed hourly, and after the pH was adjusted at 7.2 were inoculated at once into the shaved skin of chinchilla rabbits. At the same time smears for Morosow's stain were made.

Although a trace of amino nitrogen was released during the first few hours of incubation with crystalline trypsin, no important changes in staining reaction or infectivity were observed even after 72 hours of incubation. The slight release of free amino nitrogen noted during the first 2 hours of incubation was not increased during prolonged contact with trypsin, and may represent a slight contamination of virus material with rabbit tissue. At any rate, the amount was too small to be considered significant.

*Effect of Crystalline Chymotrypsin.*—Merrill (8) has likewise tested the effect of crystalline chymotrypsin on four animal viruses. Although he believed that the virus of equine encephalomyelitis was affected slightly by the enzyme, no effect was demonstrable on the viruses of pseudorabies and swine influenza virus or on the elementary bodies of vaccinia. As outlined below, the experiment with chymotrypsin and elementary bodies of vaccinia has been repeated, with amino nitrogen determinations and stains by Morosow's technique being made at intervals at which infectivity was determined.

10 mg. of vaccine virus were suspended in M/15 phosphate buffer at pH 7.8 and 1 mg. of crystalline chymotrypsin added. After thorough mixing samples were removed for controls. The remainder was incubated at 38°C., and samples were taken at regular intervals for estimation of amino nitrogen, and for studies of staining reaction and infectivity.

No demonstrable effect of crystalline chymotrypsin on vaccine virus was observed during a period of 24 hours, as judged by infectivity, release of amino nitrogen, or changes in staining reaction (Table I).

*Effect of Carboxypeptidase.*—Carboxypeptidase, crystallized by Anson in 1935 (16), attacks the peptide link adjacent to a free carboxyl group, and accordingly works with trypsin and chymotrypsin to produce a complete hydrolysis of proteins. No effect of this enzyme on vaccine virus has been recorded, although it should be manifest in those studies in which crude pancreatic enzymes have been used.

10 mg. of vaccine virus were treated at pH 7.8 in the manner described for trypsin and chymotrypsin and incubated with 1 mg. of crystalline carboxypeptidase. The activity of this enzyme had been previously established by Dr. J. S. Fruton on a synthetic peptide substrate. Samples were removed for investigation of the content of amino nitrogen, staining reaction, and infectivity as described for the experiment with trypsin.

No effect of carboxypeptidase on vaccine virus was evident after 24 hours, as judged by amino nitrogen release, loss of infectivity, or changes in staining reaction (Table I).

TABLE I  
*Effect of Crystalline Enzymes on Elementary Bodies of Vaccinia*

Enzyme	Infectivity after 24 hrs. incubation	Staining reaction	Release of amino nitrogen from virus	Release of amino nitrogen from control substrate
Trypsin	++++	Unchanged	±	++++
Chymotrypsin	++++	"	-	++++
Carboxypeptidase	++++	"	-	++++
Mixture:	++++	"	±	++++
Trypsin				
Chymotrypsin				
Carboxypeptidase				
Pepsin	Inactivated by low pH	Destroyed by pH of solution	++++	++++
Ribonuclease	++++	Unchanged	-	-
Papain (partially purified)	-	Lost	++++	++++
Ficin	++++	Unchanged	-	++++
Cathepsin (partially purified)	++++	"	-	++++

*Effect of a Mixture of Crystalline Proteolytic Enzymes.*—It has become evident from the work of Northrop and others (13) on the reaction kinetics of proteolytic enzymes, that the complete hydrolysis of a protein is achieved in stages, through the intermediation of several enzymes. For instance, more complete hydrolysis of casein is achieved by mixtures of trypsin and chymotrypsin than by either enzyme alone, while an even greater degree of hydrolysis is observed if carboxypeptidase is added to such mixtures. Merrill (8) believed that the slight inactivation of vaccine virus which he observed after prolonged incubation was greater with mixtures of trypsin and chymotrypsin than with either enzyme alone. Carboxypeptidase was not available at the time his studies were made.

10 mg. of vaccine virus were suspended in phosphate buffer at pH 7.8 and incubated at 38°C. with a mixture containing 1 mg. each of trypsin, chymotrypsin, and carboxypeptidase. This mixture of enzymes at the same pH was exceedingly active in the hy-

hydrolysis of casein and gelatin substrates as indicated by a rapid release of amino nitrogen during the first few hours of incubation. Samples of the suspension of vaccine virus in the mixture of enzymes were taken at intervals during incubation for amino nitrogen determinations, studies of the staining reaction, and infectivity.

No effect on infectivity or staining reaction was observed after 24 hours' incubation at 38°C. A slight amount of amino nitrogen was released initially, but did not increase after the first few hours of incubation (Table I).

*Effect of Trypsin, Chymotrypsin, and Carboxypeptidase on Elementary Bodies Inactivated by Heat.*—Resistance to proteolysis by pancreatic enzymes may be a function of the active virus. Northrop and others (13) have commented at length upon the inability of proteolytic enzymes to attack native protoplasm for, although certain proteolytic enzymes of plants may attack surviving tissue, apparently this capacity is not shared by proteolytic enzymes of animal origin. Accordingly, the action of crystalline trypsin, chymotrypsin, and carboxypeptidase on heat-inactivated virus was next observed.

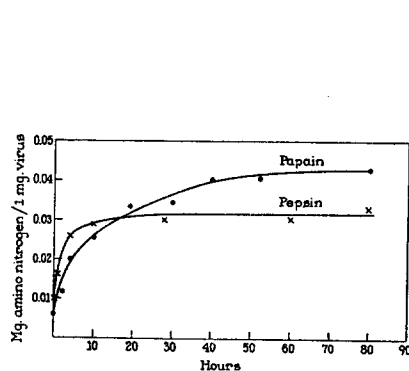
10 mg. of vaccine virus were suspended in phosphate buffer solution at pH 7.8 and heated to 90°C. for 30 minutes. The material was cooled, and loss of volume by evaporation made up with distilled water. A mixture of trypsin, chymotrypsin, and carboxypeptidase was then added and the material incubated at 38°C. Samples for amino nitrogen determinations and investigation of the staining reaction and infectivity were removed at regular intervals.

Most of the characteristic staining reaction of the virus was destroyed by heat, although a certain amount of structure was seen to persist in the elementary body. Complete inactivation of the virus material by heat had been achieved before treatment with the enzyme mixture as shown by dermal inoculation in rabbits. No appreciable hydrolysis was observed. It is clear, therefore, that resistance of elementary bodies of vaccinia to pancreatic enzymes is not to be accounted for by the native state of the virus.

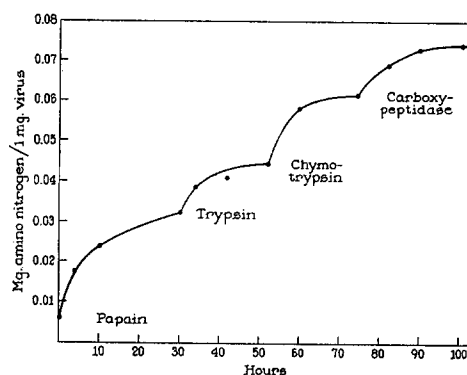
*Effect of Crystalline Pepsin.*—Lohmann and Cheng (19) found no evidence of the hydrolysis of vaccine virus by pepsin. However, the incubation was carried out at pH 4.5 which is well outside the range for optimum peptic activity. Moreover, the virus preparations used were admittedly crude. The low pH necessary for optimum activity of pepsin makes studies of the effect of this enzyme on infectivity of vaccine virus impossible, since pH values lower than 4 result in rapid and irreversible inactivation of the virus (17). The effect of pepsin on the inactivated virus, however, was deemed of sufficient chemical interest to include it in a study of the action of proteolytic enzymes on vaccine virus.

5 mg. of vaccine virus were emulsified as described above, and the pH lowered to 2 with 0.2 N HCl. 1 mg. of crystalline pepsin was added, and the mixture was incubated at 38°C. Samples were taken at regular intervals for amino nitrogen determinations.

Hydrolysis, measurable as amino nitrogen, began within one hour. Maximum hydrolysis, with equilibrium, was reached within 10 hours as indicated in Text-fig. 1. No further hydrolysis occurred after this time, although incubation was continued with addition of more enzyme for 90 hours. The staining reaction of the virus was lost almost immediately, due in part no doubt to the low pH of the mixture. Within 2 hours all



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIG. 1. Enzymatic hydrolysis of vaccine virus followed by appearance of amino nitrogen.

TEXT-FIG. 2. The hydrolysis of vaccine virus initiated by papain and continued by consecutive treatment with trypsin, chymotrypsin, and carboxypeptidase.

that remained of the suspension of elementary bodies was a slight turbidity which disappeared instantly when the pH was raised to 8.5 with sodium hydroxide. This turbidity is due to nucleic acid released by peptic activity (18). Although a powerful proteolytic enzyme, pepsin carries protein substrates only to the peptide stage, hence the amino nitrogen values as recorded indicate the rate, rather than the completeness of hydrolysis. The curve of this rate has been plotted in Text-fig. 1.

*Effect of Crystalline Ribonuclease.*—Ribonuclease, an enzyme from pancreas, was crystallized by Kunitz in 1939 (20). It is concerned with the depolymerization of ribonucleic acid (21). Although the nucleic acid in vaccine virus is principally of the thymus type, there is some indication that a small amount of ribonucleic acid may also be present; the evidence for this, however, is not conclusive (18).

5 mg. of vaccine virus were suspended in phosphate buffer at pH 7.0 and incubated with 1 mg. of crystalline ribonuclease. Samples were removed at regular intervals for chemical study, tests for infectivity, and for changes in staining reaction.

No nucleotide phosphorus was released in the supernatant fluid after 24 hours' incubation, as determined by the technique of MacFadyen (22), indicating that no measurable depolymerization of nucleic acid had occurred. No changes in staining reaction were observed, and the virus was still highly infectious after prolonged incubation.

*Effect of Purified Papain.*—No record of comprehensive studies of the effect of vegetable enzymes on vaccine virus has been found. Bawden and Pirie (23) showed that papain destroyed both the activity of latent mosaic virus of potato and its power of reacting with antiserum. Weineck (19), however, found no effect of papain, activated both with H<sub>2</sub>S and with cystein, on crude preparations of vaccine virus. In view of the interest aroused recently in the observation that certain vegetable enzymes may attack living cells (24–26), it was thought advisable to repeat the studies of purified papain with vaccine virus.

Bergmann and Fruton (27) have been able to achieve a high degree of purification of papain by fractional precipitation of the enzyme with 0.5 to 0.7 per cent ammonium sulfate saturation from extracts of crude papain. Purified papain prepared in this manner, and activated by HCN was highly active in the hydrolysis of casein and gelatin.

125 mg. of papain purified by precipitation with ammonium sulfate were extracted with 15 cc. of distilled water. The extract was filtered into a 50 cc. volumetric flask and the residue was washed with 10 cc. of 0.1 M citrate buffer at pH 5.5. 10 cc. of HCN solution, made by treating 0.3 gm. of KCN with N HCl to pH 5.0 (methyl red), were added and made up to 50 cc. volume with additional 0.1 M citrate buffer. The material was then incubated 2 hours at 38°C. for activation of the papain, after which it was stored at 4°C. until used.

To 10 mg. of vaccine virus suspended in 8 cc. of 0.1 M citrate buffer, pH 5.5, were added 2 cc. of activated papain solution. The mixture was incubated at 38°C. and samples were removed at regular intervals for amino nitrogen determination, infectivity studies, and reaction to Morosow's stain.

A measurable release of amino nitrogen occurred in 2 hours, and increased steadily for the next 18 hours of incubation. Infectivity, as determined by intradermal inoculation into the skin of rabbits, had disappeared entirely by the 3rd hour of incubation. A control lot of virus, incubated with 2 cc. of heat-inactivated enzyme with an identical HCN concentration and at the same pH, maintained its infectivity over a period of 24 hours. At this time a Morosow's stain revealed definite histological changes in the

virus preparation. Complete aggregation of the elementary bodies into large clumps had occurred, with a change in staining reaction from the characteristic deep black of the individual bodies to a deep brown of the clumped aggregates. After 8 hours' incubation, beginning dissolution of the larger aggregates was clearly evident, with particle outlines indistinct and "moth-eaten" in appearance. At this stage, the particles took on a light brown, uneven stain. By 24 hours nothing that could be identified with certainty as virus could be made out. These changes are shown in Figs. 1 to 4. A Morosow's stain made on the control elementary bodies, incubated for 24 hours with heat-inactivated papain, revealed no important changes in their size, contour, or staining properties.

While the virucidal effect of at least one proteolytic enzyme was shown by Stanley (9) to be due to an effect of the enzyme on the cells of the host instead of the virus, the evidence in this case for a direct effect on the virus is clear. Virus which had been in contact with papain for a period of less than 3 hours produced definite vaccinal lesions in the skin of rabbits in spite of the fact that the host cells were subjected to the same concentration of papain as in the later instances when no lesions occurred, due, we believe, to enzymatic decomposition of the virus.

*Effect of Papain Followed by Trypsin, Chymotrypsin, and Carboxypeptidase.*—Experiments with trypsin, chymotrypsin, and carboxypeptidase indicate that neither singly nor with a mixture of these is it possible to effect appreciable hydrolysis of elementary bodies of vaccinia. These enzymes are also without effect in the hydrolysis of heat-inactivated virus. Papain, on the other hand, initiates prompt hydrolysis of both active and heat-inactivated virus. It became of interest accordingly to learn whether, if once degradation of the virus were initiated by papain, it could be carried to completion by a mixture of crystalline pancreatic enzymes or whether the split products released by papain from the virus protein were, like the original virus, resistant to these agents.

10 mg. of vaccine virus were treated with 2 cc. of the activated papain solution described above, made up to 20 cc. with citrate buffer, and incubated at 38°C. Samples were removed at regular intervals for amino nitrogen determinations. At the end of 24 hours, the pH was adjusted to 7.8 and 1 mg. of trypsin was added. Samples for amino nitrogen determinations were again taken at regular intervals until no further release of amino nitrogen was observed, denoting maximum hydrolysis by this particular enzyme. The action of trypsin was followed in turn by that of chymotrypsin, and, finally, by that of carboxypeptidase; in each case incubation was allowed to proceed until no further hydrolysis was detected.

The results of this experiment are plotted in Text-fig. 2. Although the virus is initially resistant to pancreatic enzymes, once hydrolysis is begun



by papain, continued degradation of the virus protein by trypsin, chymotrypsin, and carboxypeptidase is seen to proceed in an orderly fashion. This appears to indicate that resistance of the virus to pancreatic enzymes is a function of the structure of the elementary body as a whole, rather than to any deep seated peculiarities in the structure of the finer units which make up the virus protein.

*Effect of Crystalline Ficin.*—Ficin, obtained from the latex of certain fig trees, is a potent proteolytic enzyme, and belongs to the general class of vegetable papains. Its crystallization was reported by Walti (29) in 1938. Interest in this substance was aroused in 1930, when Robbins (24) reported that it would digest living ascarids *in vitro*. Heretofore, proteolytic enzymes had not been considered capable of attacking living tissue (13).

10 mg. of vaccine virus were suspended in phosphate buffer at pH 7.0, treated with 1 mg. of crystalline ficin, which had been activated with HCN after the manner described for papain, and incubated at 38°C. Samples were removed at regular intervals for amino nitrogen determinations, studies of staining reaction and of infectivity. A portion of the same enzyme was very active in the hydrolysis of casein.

No effect of crystalline ficin on vaccine virus was demonstrable. There was no change in staining reaction, and no appreciable release of amino nitrogen. The virus was fully active following 24 hours incubation with the enzyme.

*Effect of Purified Cathepsin on Vaccine Virus.*—Cathepsin is a proteinase occurring in animal cells and in many unicellular organisms. So far it is the only intracellular animal proteinase known (30). In many respects this enzyme, or group of closely related enzymes, is analogous to the plant papains in that it is activated by cystein and HCN, and functions optimally at a pH lower than neutrality. Anson (31) and Fruton and Bergmann (32) have described the properties of cathepsin in detail. Anson (30) has recently been able to achieve a fair degree of purification of this enzyme.

The possible action of cathepsin on vaccine virus was of particular interest because of its presence within the animal cell, and because papain, a counterpart of cathepsin but occurring in plant cells, had already been shown by us to attack the active virus.

1 mg. of cathepsin, purified by a technique similar to that employed by Anson, was activated by HCN in the manner described for papain and added to 10 mg. of purified vaccine virus. The mixture was incubated at 38°C. and samples were taken at intervals for amino nitrogen determination, studies of staining reaction and of infectivity.

No appreciable effect was noted on infectivity or staining reaction of the virus after incubation for 24 hours. There was considerable amino nitro-

gen released but not more than that released by a preparation of the enzyme without virus. This was believed to be due to the action of the activated enzyme on protein material contaminating the cathepsin preparation.

#### DISCUSSION

The susceptibility of elementary bodies of vaccinia to hydrolysis by a proteolytic enzyme, papain, furnishes indisputable proof that they contain large amounts of protein. The fact that coincident with the loss of the structural integrity of the elementary body, as demonstrated by loss of its ability to stain, there is a loss of infectivity, is additional evidence that the elementary body represents the infectious unit of vaccinia. Amino nitrogen determinations indicate that only partial hydrolysis of the virus by papain is required to bring about complete inactivation. It is hoped that further studies on the hydrolysis of vaccine virus by selected enzymes may, in view of the known close relationship existing between the structure of proteins and reactivity with enzymes, throw some light on the chemically active groups in the elementary body of vaccinia.

#### SUMMARY

The effects of a number of crystalline and highly purified enzymes on elementary bodies of vaccinia are reported. These effects have been followed by determination of amino nitrogen, staining reaction, and studies of infectivity.

Pepsin, at a pH which inactivates the virus, results in its solution and rapid release of amino nitrogen.

Crystalline trypsin, chymotrypsin, carboxypeptidase, and ribonuclease are without appreciable effect on the virus.

Papain within a short time produces profound alteration in the staining reaction of the elementary body with release of amino nitrogen accompanied by complete inactivation of the virus. This reaction is not shared by crystalline ficin, another plant papain, or by cathepsin, an intracellular proteinase analogous to plant papains but of animal origin.

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## EXPLANATION OF PLATE 23

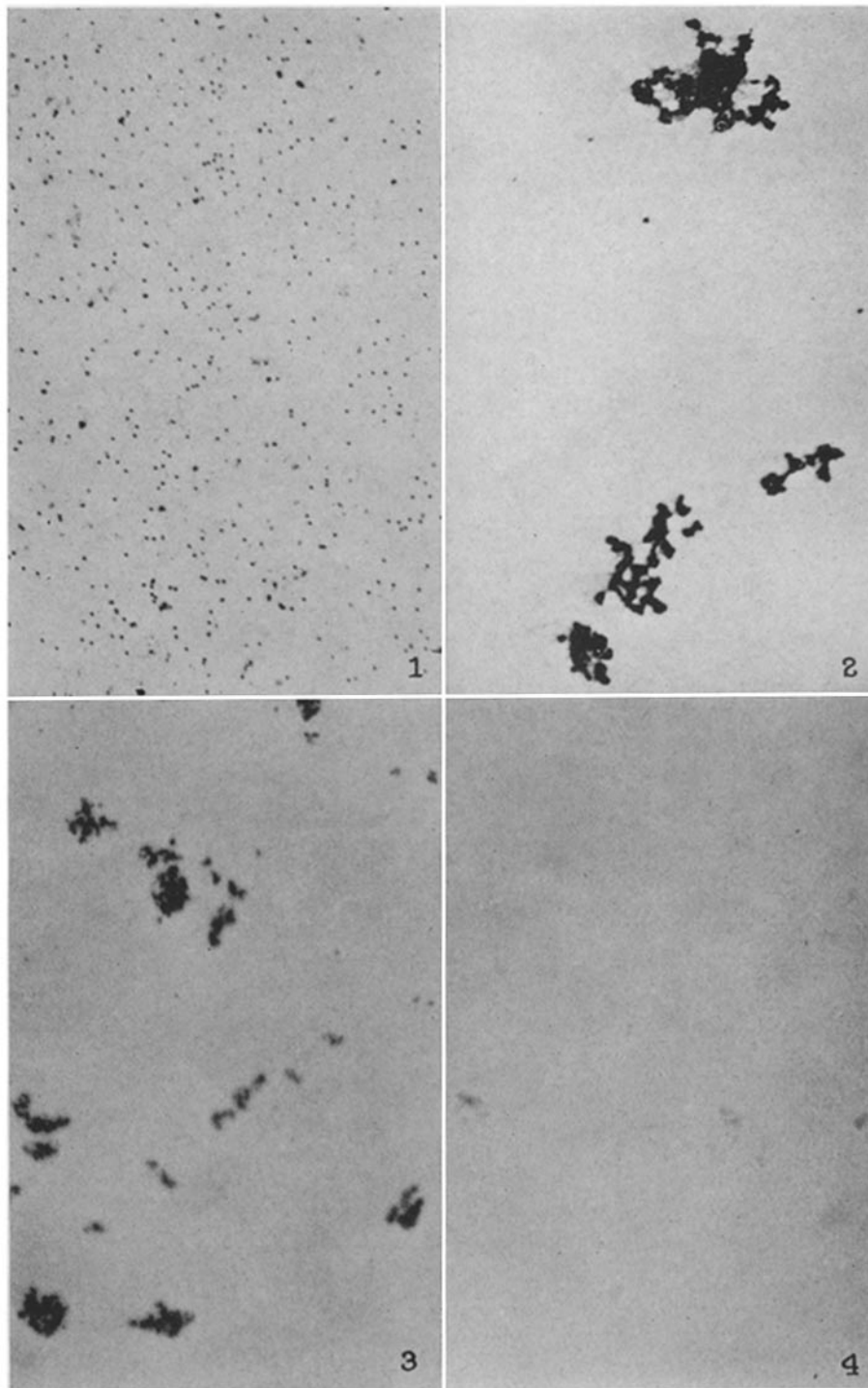
Microphotographs of elementary bodies of vaccinia during successive stages of digestion by purified papain. Morosow's stain.  $\times 1000$ .

FIG. 1. Elementary body preparation before incubation with papain.

FIG. 2. Elementary body preparation after 4 hours incubation with papain.

FIG. 3. Elementary body preparation after 8 hours incubation with papain.

FIG. 4. Elementary body preparation after 24 hours incubation with papain.



Photographed by Joseph B. Haulenbeck

(Hoagland *et al.*: Effect of enzymes on vaccine virus)