Hydrolytic Enzymes in KB Cells Infected with Poliovirus and Herpes Simplex Virus

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ABSTRACT

FLANAGAN, JOHN F. (Duke University School of Medicine, Durham, N.C.). Hydrolytic enzymes in KB cells infected with poliovirus and herpes simplex virus. J. Bacteriol. 91:789–797. 1966.—The effect of poliovirus and herpes simplex virus infection on the activity of five hydrolytic enzymes was studied in tissue culture cells of KB type. During the course of poliovirus infection, the activity of β-glucuronidase, acid protease, acid ribonuclease, acid deoxyribonuclease, and acid phosphatase in the cytoplasm rose to levels two- to fourfold greater than the activity present in the cytoplasm of uninfected cells. The rise in cytoplasmic activity was accompanied by a concomitant decrease in enzymatic activity bound to cell particles. Shift of enzymatic activity from the particulate to soluble state was first detected at 6 hr after poliovirus infection, coinciding with the appearance of new infectious particles and virus cytopathic effect. No net synthesis of these enzymes after poliovirus infection was found. Hydrocortisone added to the culture medium failed to affect either the titer of virus produced in the cells or the release of hydrolytic enzymes from the particulate state. Herpes simplex infection produced minimal alterations in the state of these enzymes in KB cells. It is hypothesized that the breakdown of lysosomes and release of hydrolytic enzymes accompanying poliovirus infection is produced by alterations in cell membrane permeability during the course of virus replication and by the consequent change in the ionic content of the cell sap.

Despite the collection of much information on the biosynthetic events induced by virus infection of susceptible mammalian cells (11, 14, 15, 26, 27), the mechanisms of host injury by viruses are to a large extent unknown. Evidence has recently been presented that poliovirus (16), pseudorabies (4), and adenovirus (Levine and Ginsberg, Federation Proc., 24:597, 1965) infection of tissue culture cells suppresses host messenger ribonucleic acid (RNA) synthesis and expression. The data suggest that this suppression is produced by accumulation of virus-specific inhibitory proteins in the parasitized cell. Diversion of substrate from the synthesis of cellular components into virus-directed synthesis has been proposed as a mechanism of virus injury in T-even bacteriophage infection (5) and in adenovirus infection (12) of appropriate host cells. Direct evidence that cell starvation by such a mechanism is an important factor in injury by viruses has not been obtained yet. The demonstration that infection of Escherichia coli by T2, T5, and lambda bacteriophages is accompanied by severe degradation of deoxyribo nucleic acid (DNA) and marked increases in nuclease activity within infected cells (17, 29, 30) has raised the possibility that destruction of host macromolecules may be an important mechanism of virus damage to host cells.

The work described in this report was undertaken to examine the possible role of hydrolytic enzymes in virus injury to mammalian cells. As shown by deDuve (7, 8) and others (22, 23) in numerous studies during the past 10 years, the hydrolytic enzymes of mammalian cells are concentrated in single membrane-enclosed particles within the cytoplasm, which deDuve termed "lysosomes." In virtually all of the cells examined, these particles sedimented with a centrifugal force slightly greater than that necessary to sediment mitochondria. By cytochemical study, these particles appeared to be exclusively in the cytoplasm (23), whereas cell fractionation procedures consistently yielded a portion of the hydrolytic enzyme activity in the nuclear fraction. Release of bound enzymes from these structures was shown to be an accompaniment of cell injury from anoxia.
(2), vitamin A toxicity (21), and disturbance in osmotic or ionic balance (18). Increased fragility of these particles has been demonstrated in ascites tumor cells exposed to antibody and in certain autoimmune disease states of man (31).

This report describes studies on alterations in activity of five lysosomal enzymes during virus replication in KB cells. The enzymes assayed in these experiments were: acid ribonuclease, acid deoxyribonuclease, acid phosphatase, acid protease, and β-glucuronidase. Two viruses were employed in these studies for purposes of comparison: poliovirus, which produces rapid and profound morphological and chemical changes in the infected cells, and herpes simplex virus, which shows less dramatic effect on the host cell as a result of replication.

**MATERIALS AND METHODS**

*Tissue culture.* These experiments were performed with monolayer cultures of KB epithelial cells maintained in Eagle's minimal essential medium supplemented with 7.5% calf serum. The control and infected cells for enzyme assay were grown in 32-oz Duraglas prescription bottles containing at maturity 35 × 10^6 to 45 × 10^6 cells per bottle. Repetitive culturing of these cells revealed no pleuropneumonia-like organisms.

*Virus and virus infection.* The poliovirus was type I, Cox attenuated strain, obtained from Lederle Laboratories, Pearl River, N.Y. The herpes simplex virus was a prototype strain isolated by Sydham Osterhout, and was tested for purity against several reference antisera. Poliovirus pools were prepared by infecting culture bottles with 2 × 10^8 plaque-forming units (PFU) per bottle. The cultures were harvested 48 hr later and clarified by centrifugation for 10 min at 10,000 × g. Titers on the supernatant virus suspension were 10^6 to 10^7 PFU per ml. Herpesvirus was grown under similar conditions with the use of 2 × 10^4 TCID<sub>50</sub> of virus as determined by tube culture assay (13). The cells were harvested 30 to 40 hr after inoculation and were centrifuged for 10 min at 1,500 × g. The infected cells were suspended in one-half the volume of original medium and homogenized at top speed in a Servall homogenizer. Titers of this virus by tube culture assay were 10^6 to 10^7 TCID<sub>50</sub> per ml. Infectivity of the herpes preparations was stable on storage at -70 C.

*Cell fractionation.* Tissue culture cells were infected with either 20 PFU of poliovirus type I or 8 TCID<sub>50</sub> of herpes simplex virus per cell. The virus inoculum was removed 2 hr later by decanting, the cell sheets were washed three times with Hanks solution, and Eagle's medium supplemented with 7.5% calf serum was replaced on the cells. The cultures were incubated at 36 C for the selected time, removed from the glass with a rubber policeman, and submitted to the fractionation procedure described in Fig. 1. Duplicate cultures of control uninfected cells were submitted to the same procedures with each experiment. After preparation of the pelleted fractions from the cells, these fractions were suspended in 2 ml of distilled water, frozen and thawed eight times, diluted with an equal volume of 0.5 M sucrose plus 0.002 M ethylene diaminetetraacetate (EDTA), and centrifuged at 100,000 × g for 1 hr. Enzyme assays were performed on the supernatant fractions from this final centrifugation.

*Enzyme studies.* Enzymatic activity of the uninfected cells was assayed in citrate, acetate, glycine, and tris(hydroxymethyl)aminomethane (Tris)-maleate, or Tris-hydrochloride buffers through a wide pH range. The ionic composition of the buffer had no measurable effect on activity of these enzymes; activity of these enzymes was altered by changes in pH, not by change in buffer composition. After optimal pH was determined, all enzyme studies were performed at 37 C under the standard conditions outlined below.

Acid phosphatase was assayed by incubating a mixture of 0.5 ml of enzyme preparation with 0.5 ml of 0.5 M acetate buffer (pH 4) containing 0.1 M β-glycerophosphate. The reaction was stopped after 1 hr at 37 C by the addition of 0.5 ml of 15% trichloroacetic acid. The inorganic phosphate released by enzymatic action was measured by the method of Fiske and SubbaRow (10). Activity was expressed as micrograms of inorganic phosphate produced per hour per milligram of enzyme protein.

β-Glucuronidase was assayed in a mixture of 0.5 ml of enzyme preparation, 0.4 ml of 0.2 M acetate buffer (pH 4.5), and 0.1 ml of 0.01 M phenolphthalein glucuronide. After incubation for 1 hr at 37 C, 1 ml of 10% trichloroacetic acid was added, the precipitate was removed by centrifugation, and free phenolphthalein in the supernatant fluid was measured by the method of Fishman et al. (9). Activity was expressed as the number of micrograms of phenolphthalein produced per hour per milligram of enzyme protein.

Acid ribonuclease was determined by incubating 0.2 ml of enzyme preparation with 0.7 ml of sodium cacodylate at pH 6.0 and 0.1 ml of 1% yeast RNA (Worthington Biochemical Corp., Freehold, N.J.). The RNA had previously been precipitated twice from phosphate-buffered saline with 2 volumes of absolute ethyl alcohol at -20 C. The reaction was stopped after 30 min of incubation at 37 C with the addition of 1 ml of 6% HClO<sub>4</sub> containing 0.25% uranyl acetate. The precipitate was removed by centrifugation, and optical density of the supernatant fluid was measured at 260 nm in a Beckman DU spectrophotometer. The results were expressed as increments in optical density units in the acid-soluble supernatant fluid per milligram of enzyme protein.

Acid deoxyribonuclease was assayed by mixing 0.5 ml of enzyme preparation with 0.4 ml of 0.1 M sodium cacodylate (pH 5.5) and 0.1 ml of 0.5% thymus DNA (Worthington Biochemical Corp.) suspended in 0.01 M sodium chloride. After 1 hr at 37 C, the reaction was stopped by addition of 1 ml of 6% perchloric acid containing 0.25% uranyl acetate. The precipitate was discarded after centrifugation, and optical density in the supernatant fluid was measured at 260 nm. Activity was expressed as the increase in optical
Infected or control cells scraped from monolayer cultures

Cells and medium centrifuged for 10 min at 1,000 × g

Cell pellet washed twice in cold phosphate-buffered saline

Sample removed for cell counting

Cell pellet suspended in 2.5 ml of 0.25 M sucrose + 0.001 M EDTA (pH 7)

Cells submitted to gentle homogenization in motor-driven glass grinder for 2 min at 4 C

Centrifuged at 1,000 × g for 10 min

 Supernatant fluid

Pellet resuspended in 0.25 M sucrose + 0.001 M EDTA at pH 7

Homogenization cycle repeated for total of three times

Centrifuged at 2,500 × g for 10 min

Pellet: nucleic and unbroken cells

Supernatant fractions pooled

Centrifuged at 15,000 × g for 30 min

Supernatant fraction

Centrifuged 100,000 × g for 1 hr

Supernatant fluid: cell sap

Pellet: lysosomes + mitochondria

Pellet: microsomes

Fig. 1. Method for preparing lysosome-rich fraction from KB cells.

density in the supernatant fluid per milligram of enzyme protein.

Protease determinations were performed by mixing 0.5 ml of enzyme preparation with 0.5 ml of 6% dialyzed hemoglobin and 0.5 ml of 0.2 M glycine buffer (pH 3.0). The mixture was incubated at 37 C for 1 hr. The reaction was stopped with 1.5 ml of 10% trichloroacetic acid. The amount of acid-soluble tyrosine liberated from hemoglobin into the acid-soluble supernatant per milligram of protein was determined with a tyrosine standard for comparison.

Suitable zero-time controls and incubated blanks were prepared with all the enzyme studies described above by precipitating complete mixtures of enzyme, buffer, and substrate prior to incubation. Enzymatic activity was computed by measuring the differences in colorimetric or spectrophotometric readings between the zero-time control and the incubated specimens.

Substrate concentrations for all enzymatic determinations were at a level providing linear enzyme activity for at least 2 hr of total incubation time under these experimental conditions.

RESULTS

Determination of pH optimum for the five hydrolytic enzymes in uninfected cells. As shown in
Fig. 2. Determination of pH optimum for five lysosomal enzymes. The pH range used for testing ribonuclease activity was the same as that used for deoxyribonuclease. The range used for testing activity of acid phosphatase was identical with that used for testing β-glucuronidase.

TABLE 1. Distribution of acid hydrolases in uninfected KB cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity of enzymes*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Phosphatase</td>
</tr>
<tr>
<td>Cell sap</td>
<td>8.39</td>
</tr>
<tr>
<td>Lysosomes + mitochondria</td>
<td>31.1</td>
</tr>
<tr>
<td>Microsomes</td>
<td>8.59</td>
</tr>
<tr>
<td>Nuclei</td>
<td>5.24</td>
</tr>
</tbody>
</table>

* Activity per milligram of protein. Phosphatase activity expressed as micrograms of inorganic phosphate released per hour per milligram of protein; ribonuclease activity as increase in optical density at 260 μm of acid-soluble supernatant fluid per 0.5 hr per milligram of protein; β-glucuronidase activity as micrograms of phenolphthalein released per hour per milligram of protein; protease activity as micrograms of tyrosine released in acid-soluble supernatant fluid per hour per milligram of protein; deoxyribonuclease activity as increase in optical density at 260 μm of acid-soluble supernatant fluid per hour per milligram of protein.

Fig. 2, the pH optimum for all enzymes was decidedly in the acid range. Despite pH optima ranging from 2.5 to 6, some of these enzymes, notably ribonuclease and deoxyribonuclease, showed appreciable activity at neutral pH. Ribonuclease obtained from the various particulate fractions was compared with unbound ribonuclease of the cell supernatant fraction in respect to heat stability, pH, and temperature optimum. Enzyme preparations from all the particulate and supernatant fractions showed identical reactions to these variations in experimental conditions, suggesting that only one species of this enzyme was present in KB cells.

Distribution of hydrolytic enzymes in uninfected cells. Tables 1 and 2 show the geometric mean results of seven separate studies on the amount of enzyme activity associated with the various cell fractions. It is evident that for all five enzymes the activity is concentrated in the fraction sedimenting at 15,000 × g for 30 min (the "lysosomal" fraction). On the basis of activity per milligram of
Table 2. Total activity of acid hydrolases per million uninfected KB cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity* per 10^6 cells</th>
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<tbody>
<tr>
<td></td>
<td>Phosphatase</td>
</tr>
<tr>
<td>Cell sap</td>
<td>1.12</td>
</tr>
<tr>
<td>Lysosomes + mitochondria</td>
<td>1.62</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.24</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.65</td>
</tr>
</tbody>
</table>

* Units of activity as in Table 2, except activity expressed per 10^6 cells rather than per milligram of protein.

Fig. 3. Alterations in the amount of hydrolytic enzyme activity associated with the lysosomal particles and cell sap during poliovirus I replication.

Fig. 4. Alterations in total cell content of lysosomal enzyme activities during poliovirus I replication.
protein, the enzymatic activity of this fraction was found to be 4 to 10 times greater than that present in the cell supernatant fluid. Table 2 shows the total activity in each fraction per million uninfected cells. These studies demonstrated that 10 to 30% of the enzymatic activity was present in the cell supernatant fraction, whereas the remainder was present in a bound form, principally in the lysosomal and nuclear fractions. The finding of significant amounts of enzyme activity in the nuclear fraction was somewhat unexpected. It is possible that this nuclear enzyme activity was a result of contamination of the nuclear fraction with cytoplasmic tags and unbroken cells. Previous cytochemical studies have strongly suggested that lysosomes are constituents of the cytoplasm only, and it is believed by some workers that the activity invariably present in the nuclear fraction on physical separation of the cell components reflects contamination by cytoplasmic material (Ciba Symposium, Lysosomes, p. 306, 1963). At present, this question is not resolved. Attempts were made to separate the mitochondrial and lysosomal fractions by brief centrifugation at 10,000 × g, followed by more prolonged centrifugation at 15,000 × g (3). These attempts produced no further enrichment of enzymatic activity in the postmitochondrial fraction on comparison with the specific activity of the mixed mitochondrial-lysosomal preparation obtained by a single-step centrifugation at 15,000 × g for 30 min. In all subsequent experiments, the mitochondrial and lysosomal fraction was centrifuged with the one-step procedure.

**Experimental results with poliovirus infection.** A summary of the results from experiments performed at selected time intervals in the replication cycle of poliovirus is shown in Fig. 3 to 6. The studies showed that activity for all enzymes increased in the cytoplasm after 6 hr after infection (Fig. 3). Activity per milligram of protein rose rather rapidly in this fraction to reach a maximal level 12 hr after infection. As protein leaked from the cells, these levels declined to a somewhat lower point 16 hr after inoculation. The levels of activity at the time of maximal accumulation in the cytoplasm were two- to fourfold greater than the activity of these enzymes in the uninfected cells. This increase of cytoplasmic activity was accompanied by a simultaneous decrease in bound enzyme activity. No net synthesis of any of these enzymes occurred after infection, and total activity within the cells declined steadily from 6 hr after infection to the time of last measurement, 16 hr past inoculation (Fig. 4). Comparison of the release of these enzymes with production of infectious particles and cytoplasmic effect by microscopy showed that all of the parameters changed concomitantly. As shown in Fig. 5, the first measurable increase in infectious poliovirus occurred 6 hr after infection, accompanying the earlier shift in hydrolytic enzymes from the bound to unbound state and the earliest appearance of cytoplasmic effect. The protein content of all cell fractions decreased rapidly (Fig. 6). This decline began 6 hr after infection. The total cell content of protein at that time was 48% of the content in control cells.

Since reports have appeared suggesting that
adrenocorticosteroids increased the stability of lysosomes in other cells (32), experiments were performed to determine the effect of hydrocortisone on the release of bound hydrolytic enzymes accompanying polio infection. No discernible effect of hydrocortisone hemisuccinate on virus-induced release of these enzymes could be demonstrated when the compound was maintained on the cells at a concentration of 100 μg/ml from 24 hr before virus inoculation until the termination of the experimental period.

Experiments with herpes simplex virus. Studies with herpes simplex virus infection of KB cells were performed to compare enzyme alterations produced by poliovirus with the effect of a virus which initiates less dramatic changes in host cell function. As shown in Fig. 7 and 8, the enzyme alterations during the selected times of observation in the replication cycle of herpes simplex virus (from the 4 hr after infection until 24 hr after inoculation) were decidedly less pronounced than was the case with poliovirus. During the course of infection with this virus, the activity of β-glucuronidase rose to a maximal level 35% above the enzyme cytoplasmic activity of control cells, despite the development of frank cytopathic effect by 12 hr after inoculation. The other hydrolytic enzymes studied in these experiments showed less significant alterations. A 10% loss of cell protein from the cells occurred during the experimental period with herpes simplex virus infection. In addition to measurements of the three enzymes described in Fig. 7, assay of ribonuclease and protease activity in the cytoplasm of herpes-infected cells was performed. These enzymes showed approximately 20% increase in cytoplasmic enzyme activity of infected cells. The maximal activity for both enzymes was recorded 16 hr after infection.

**Fig. 7.** Effect of herpes simplex virus infection on cellular distribution of three lysosomal enzymes.

**Fig. 8.** Multiplication cycle of herpes simplex virus in KB cells.

**Discussion**

It is apparent from the data reported in this paper, and from two other studies on virus-induced lysosomal enzyme changes reported while this work was in progress (1, 33), that hydrolytic enzymes are released from the bound state in cells during the course of infection with certain mammalian viruses. Considerable increases in cytoplasmic content of these enzymes were found in liver cells of mice infected with mouse hepatitis virus (1), and during infection of tissue culture cells with poliovirus (33 and our observation). The increase in cytoplasmic activity of lysosomal

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enzymes reported during vaccinia infection of monkey kidney cells by Allison and Sandelin (1) was not observed in the experiments reported by Wolff and Bubel (33) using vaccinia in KB and guinea pig spleen cells. Our studies with herpes simplex infection of KB cells showed little release of hydrolytic enzymes during virus replication. These observations with herpesvirus paralleled the results of the work by Wolff and Bubel (33) on lysosomal enzymes in vaccinia-infected cells.

The precise cause of lysosomal destruction during virus replication remains to be determined. The present work suggests that deterioration of membrane function may be the initiating factor in hydrolytic enzyme release from these particles. Poliovirus infection, which is accompanied by pronounced release of lysosomal enzymes from the bound state, produces obvious alterations in cytoplasmic membrane function. Leakage of protein from the polio-infected cell begins 6 hr after infection, and by 12 hr after inoculation only one-third of the soluble protein originally present in the cell remains with the cell body. In herpes simplex infection, loss of protein from the cell is negligible, and, likewise, alterations in lysosomal enzyme activity within the infected cell are of limited extent.

Allison and Sandelin (1) pointed out that lysosomal enzymes may participate in the uncoating process of virus nucleic acid after the intact particle has entered the cell. Myxoviruses have been shown to enter cells by a process akin to pinocytosis (6, 28), and concentration of lysosomes about pinocytosis vacuoles has been demonstrated by Novikoff (23). Our failure to demonstrate quantitative increases in the activity of unbound hydrolyases within polio-infected cells during the virus latent period does not rule out such a role for lysosomal enzymes. The local release of enzymes around a few infecting particles might not be measurable by the techniques used in this study. Experiments are presently being performed with purified virus and lysosomal enzymes to measure directly the ability of these hydrolyases to render virus nucleic acid susceptible to endonuclease action.

In addition to a possible role in the uncoating process of virus particles, lysosomal enzymes may contribute significantly to virus injury of the infected cell by degrading host macromolecules. In the case of bacterial viruses, marked degradation of DNA occurs with T2 and T5 infection of E. coli (30). The importance of virus-induced hydrolyases in parasitism by these agents remains an open question, since most of the disruption of host cell DNA precedes appearance of virus-specific nucleases (20). Infection of L-strain mouse cells with equine abortion virus has been reported to be associated with extensive degradation of cell DNA (25). This effect was initially thought to be virus-induced, but a recent study indicates that the host nucleic acid degradation found in these studies was produced by enzymes from contaminating pleuropneumonia-like organisms (24).

The data reported in this paper and in two previous publications (1, 33) indicate that lysosomal enzyme release from the particulate to the free state occurs with virus infections that produce a rapidly cytocidal effect, whereas other viruses which show a slower evolution of cellular damage and minimal leakage of constituents from the cell produce little or no change in the state of these enzymes. The close time relationship between measurable release of lysosomal enzymes, maturation of virus particles, leakage of virus particles and protein from the cell, and virus cytopathic effect in infection of tissue culture cells by poliovirus makes it difficult as yet to ascribe any major role in virus pathogenesis to these enzymes. More knowledge about the factors producing changes in membrane permeability and the alterations in ionic composition of the cytoplasm of infected cells at the onset of lysosomal disruption may be useful in defining the cause of disruption of these particles and the role of these hydrolytic enzymes in cellular damage accompanying virus infection.

ACKNOWLEDGMENTS

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LITERATURE CITED