Lysosomal Enzyme Activity in Poliovirus-infected HeLa Cells and Vesicular Stomatitis Virus-infected L Cells: biochemical and Histochemical Comparative Analysis with Computer-aided Techniques

By K. KOSCHEL, H. M. AUS AND V. TER MEULEN
Institut für Virologie der Universität Würzburg, Würzburg, Germany, and Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany

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SUMMARY

The behaviour of lysosomal enzymes in poliovirus-infected HeLa S3 cells and vesicular stomatitis virus (VSV)-infected L cells was investigated both biochemically using enzyme assays, and histochemically using acid phosphatase dependent staining. The presence of the enzyme was shown histochemically under the light microscope by its reaction with naphthole-AS-BI-phosphate and a coupling reaction with diazotized pararosaniline. The light absorption of this stain in infected and uninfected cells was measured on a Universal Micro Spectrophotometer (UMSP-1) and recorded on-line as gray value cell images in a PDP-12 computer. These scanned images were analyzed by FORTRAN programs on a UNIVAC 1108. The histochemically obtained distributions of the lysosomal enzyme are comparable to the results of the biochemical analysis. Lysosomes of poliovirus-infected cells displayed a release of lysosomal enzymes into the cytoplasm starting at 3 h after infection; VSV infection did not produce this type of effect. This investigation shows that it is possible to extract and demonstrate specific virus dependent changes using computer-aided cytophotometric techniques.

INTRODUCTION

The role of the lysosomal enzymes in a virus-host relationship in which lysis of the host cell occurs has not been fully determined to date. Biochemical studies of the lysosomal enzymes of a lytic virus show an increasing amount of lysosomal enzymes outside the lysosomes in the cytoplasm during a cycle of virus multiplication (Allison & Sandelin, 1963; Flanagan, 1966; Blackman & Bubel, 1969; Guskey, Smith & Wolff, 1970). This effect has not been observed with non-lytic viruses (Wolff & Bubel, 1964; Flanagan, 1966). These findings supported the hypothesis of Defendi (1962) that the early release of lysosomal enzymes is responsible for the occurrence of the cytopathic effect. These conclusions are, however, based only on biochemical data which do not eliminate the possibility that the results are due to artifacts. The enzymes release could be caused when the cells are mechanically ruptured during preparations for the biochemical tests (Killington et al. 1974). During the infection lysosomes could become more fragile to mechanical treatment without ever releasing the enzymes in vivo. The increased fragility of the lysosomes could be caused by the continually changing and synthesizing membranes which occur during picornavirus infection (Amako & Dales, 1967; Plagemann, Cleveland & Shea, 1970; Mosser, Caliguiri & Tamm,
I972). Such potential uncertainties can be eliminated only if the release of lysosomal enzymes in the sequence of infection is studied on single cells which have not been homogenized or otherwise ruptured. Allison & Malucci (1965) have reported qualitative histochemical studies of lysosomal enzymes for some viruses (influenza, Newcastle disease, fowl plague, vaccinia, mouse hepatitis and adenovirus type 5 viruses) in the appropriate fixed and un-fixed cell systems. A kinetic study of the lysosomal changes, however, was not carried out.

In the present studies, the biochemical events were correlated to the histochemically detectable lysosomal enzyme changes occurring in the cycle of virus multiplication for the lytic poliovirus and the non-lytic VSV. Computer-aided cytophotometric techniques were applied to trace and analyse the histochemically stained lysosomal acid phosphatase in un-infected, poliovirus- and VSV-infected cells.

METHODS

Virus. Type I poliovirus (Mahoney) propagated in HeLa cells and VSV in L 929 cells were used in this study. Virus multiplication proceeded at the same rate in both of these systems.

Cell cultures. HeLa S3 cells (Flow Laboratories, Irvine, Scotland) were kept in Roux bottles grown in minimal essential medium (MEM) supplemented with 10 % calf serum. Twenty-four hours prior to the start of the experiment, HeLa cells were trypsinized and stirred in MEM Spinner Medium supplemented with 5 % calf serum. The suspension culture contained 2 to 5 × 10^5 cells/ml.

L 929 cells were treated in the same way but the medium was supplemented with 8 % calf serum.

Biochemical assay for lysosomal enzymes. 3 × 10^8 cells were infected at an input multiplicity of 100 for poliovirus or VSV. Forty-five min after infection the cells were washed with complete medium and resuspended in 250 ml medium. Samples were taken at hourly intervals and analysed for lysosomal enzyme activity as described below. The uninfected control cells were treated identically.

The preparation of the cell samples for the analysis of lysosomal enzymes was carried out according to Guskey et al. (1970). A sample from the total Dounce homogenate was stored to determine the intracellular infectious virus material. The enzyme characterization was carried out in the Dounce supernatant fluid as well as in the pellet obtained from each sample harvested. The pellet fractions were solubilized with 0·2 % Triton X-100 in water.

β-glucuronidase (E.C. 3.2.1.31). This enzyme test was carried out according to Allison & Sandelin (1963) with 4-nitrophenyl-glucopyranoside uronic acid (Merck, Darmstadt, Germany).

β-galactosidase (E.C. 3.2.1.23) was determined with 2-nitrophenyl-β-D-galactopyranoside (Merck) as substrate according to Guskey et al. (1970).

Acid DNase II (E.C. 3.1.4.6). This enzyme was assayed as follows: 0·5 ml of 0·175 M-sodium acetate buffer, pH 4·7, and 0·05 ml [3H]-thymidine labelled HeLa cell DNA (56000 ct/min/100 μl) and 0·45 ml of cell extract were incubated for 3 h at 37 °C. This mixture was subsequently precipitated with 1 ml of 10 % trichloroacetic acid (TCA). The precipitates were pelleted in a Sorvall centrifuge at 34800 g. 0·5 ml of the supernate was measured in a scintillation counter in 10 ml of Bray's solution (Bray, 1960).

Calculation of enzyme release into the cytoplasm. The enzyme release was calculated as:

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\text{% release} = \frac{\text{enzyme units in the cytoplasmic supernatant fluid}}{\text{enzyme units in the cytoplasmic supernatant fluid + pellet}}
\]
The linearity of the enzyme reactions with increasing amounts of enzyme extract and the region of linear kinetics of the reaction were determined in separate experiments.

Protein determination. The protein determination was carried out by the Folin method described by Lowry et al. (1951).

Virus titration. Infectious poliovirus was titrated in HeLa monolayers (Dulbecco, 1952) and VSV in L cell monolayers. The infectivity was expressed as p.f.u.

Cell preparation for cytophotometry. Approx. $5 \times 10^4$ HeLa S3 cells or L cells were seeded on round cover slips (17 mm) and stored at 37 °C. Twenty-four hours later a given number of coverslips were infected every hour over a period of 7 h in order to achieve a uniform harvest and staining start. All the infected coverslips were, therefore, harvested at the same time. A control coverslip was included for each hour of infection.

Cytochemical staining of acid phosphatase (E.C. 3.1.3.2). Coverslips were harvested, washed in medium without serum and air dried. Air-dried cover slips were fixed in 0.5\% glutaraldehyde in phosphate buffered saline (PBS) for 10 min. The preparation was subsequently stained according to the procedure of Barka & Anderson (1962). The substrate for the staining reaction was naphthole-AS-BI-phosphate (6-bromo-2-phospho-hydroxy-3-naphthoyl-o-anisidine) (Serva, Heidelberg, Germany). The product is coupled simultaneously by fresh diazotized pararosaniline (Merck, Darmstadt) during the enzymatic reaction and is therefore a stoichiometric staining procedure. The preparations were subsequently washed with H2O, fixed in formaldehyde/calcium chloride solution, dehydrated in an alcohol/water series and embedded in Entellan (Merck, Darmstadt). The absorption maximum of the stained complex was at 510 nm.

Cytophotometry. A recording microspectrophotometer (UMSP-1, Carl Zeiss, Oberkochen, Germany) with a fast scanning stage interfaced to a mini computer (PDP 12, Digital Equipment Corporation, Maynard, Mass., U.S.A.) was used in this study (Aus et al. 1974). User interaction with the PDP 12/UMSP-1 system is possible by typing commands on a teletype or by manipulating a joy-stick. The Apamos computer programs, available from Zeiss, control the movement of the microscope scanning table, record and store the measured light transmission at each point, and print the measured recorded values on the teletype. The stored cell images are transferred via magnetic tape to a UNIVAC 1108 where the data is analyzed by FORTRAN computer programs.

The monochromatic light transmitted through an area of a cell was measured and recorded using a photodensitometer (Casparsson et al. 1955). The diam. of the measured area was 0.5 μm. These measurements were repeated over the entire cell by moving the cell past the light beam in fixed step sizes of 0.5 μm using a scanning table. The recorded measurements at each step were digitalized and stored on magnetic tape to form a scanned image of the measured cells. This scanning method permitted the quantitative measurement of minute optical cellular variations within a biological sample. These stored images were then analysed by computer programs. From each infected and control coverslip, prepared as described in the cell preparation for cytophotometry, 30 cells were randomly selected and scanned. The measurements were carried out at 510 nm.

The scanned images consist of 1500 to 4000 recorded light transmission values for each cell measured at each hour of infection and control. Thirty cells were measured at each hour. These large amounts of data were then reduced to a smaller, more comprehensible representation of the original data. One such commonly used method for this reduction is the formation of histograms of the values found in the recorded data of each cell. The histogram of a cell image is calculated by determining how many times (dn) a value or a range of values occur in the cell image as a fraction of the total number of measured points (n). The relative
frequency of occurrence of a given value or range of values is therefore $dn/n$. In this study, the scanned data was converted to absorption values and divided into ten equal intervals of absorption values from 0 to 100% absorption. These individual cell histograms were then averaged over all the cells measured from the same coverslip to produce the population histograms shown in our figures. The population histograms were calculated in order to statistically check the validity of the results.

RESULTS

Biochemistry

In order to correlate the kinetics of virus multiplication and virus release in our cell systems to the changes of lysosomal enzyme during the events of virus multiplication, the specific lysosomal enzymes β-glucuronidase, β-galactosidase and acid DNase II were measured in poliovirus infected and uninfected HeLa cells (Fig. 1) and VSV-infected and uninfected L cells (Fig. 2). Starting at 4 h after poliovirus infection (Fig. 1d), the virus multiplication increased perceptibly and reached a maximum 4 h later. Extracellular infectious poliovirus increased in greater quantities after 6 h post-infection (p.i.). The released
virus resulted in a decrease of intracellular infectious virus. The lysosome-free cell extract changes in the relative amounts of enzymes measured in the cytoplasm occurred, beginning at 2 to 4 h p.i. for \( \beta \)-glucuronidase (Fig. 1a), beginning at 4 to 6 h p.i. for \( \beta \)-galactosidase (Fig. 1c), and beginning at 4 to 6 h p.i. for acid DNase II (Fig. 1b). Each enzyme reached its maximum value approx. 8 to 10 h after infection. Fig. 3 shows the total activity of the enzymes during the period of infection. The total amount of enzyme activity decreased perceptibly approx. 6 h after infection in the infected cells and remained relatively unchanged in the control cell cultures. Similar trends were also observed with the total protein content and, as shown in Fig. 1d, with the virus progeny which was released at 6 to 7 h p.i. These measured changes reflect the lysis of the cells due to virus infection as demonstrated by Blackman & Bubel (1969). Similar results were obtained using the release of \( ^{31} \text{Cr} \)-chromate as indicator for cell lysis (Koschel, 1971). The VSV-infected L cells, in contrast to the poliovirus-infected cells, did not show any lysosomal activity changes during the period of infection (Fig. 2). Infectious extracellular virus was measurable in larger quantities starting after 4 h p.i. and reached its maximum at 9 to 10 h. In the VSV-L cell system there was no difference in the rate of synthesis and release of virus (Fig. 2d). The release of infectious VSV did not influence the total activity of the measured lysosomal enzymes in the cells. Total protein content of the VSV-infected cells versus uninfected cells showed no difference (Fig. 4). The release of
Fig. 3. Total enzyme activity and protein content of poliovirus-infected and uninfected HeLa \( S_3 \) cells during the infectious cycle (in percentage of the value at 4 h p.i.). Protein: \( \nabla \quad \nabla \), uninfected cells; \( \nabla \quad \nabla \), infected cells. \( \beta \)-galactosidase: \( \square \quad \square \), uninfected cells; \( \square \quad \square \), infected cells. \( \beta \)-glucuronidase: \( \bullet \quad \bullet \), uninfected cells; \( \bigcirc \quad \bigcirc \), infected cells.

\([^{35}\text{Cr} \text{O}_4^-]\) ions from pre-labelled cells exhibited no difference between infected and uninfected \( L \) cells because the virus induced no lysis (Horak, Jungwith & Bodo, 1971).

**Histochemistry**

The histochemical staining of the acid phosphatase observed in the light microscope appears in the uninfected cells mostly as perinuclear red granules in the cytoplasm. These granules are quite pronounced, of different shape, and randomly oriented. They are also visible in the poliovirus-infected HeLa cells in the earlier hours of infection but become less pronounced as c.p.e. develops. The c.p.e. is microscopically recognizable at 5 to 6 h p.i. These changes are not observed in the \( L \) cells infected with VSV.

The distribution of absorption values of stained acid phosphatase in uninfected HeLa cells is represented by the histogram in Fig. 5. Samples were taken at hourly intervals over a period of 7 h after seeding HeLa cells on cover slips. From each of these samples (0 to 7 h) 30 cells were randomly selected and scanned. The resulting histogram of all these cells (a total of 240 cells) shows that most of the recorded absorption values occurred in the range
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Fig. 4. Total protein content of VSV-infected L cells during the infectious cycle.

- - - - O, infected cells; • --- •, uninfected cells.

Fig. 5. Distribution of average absorption values measured in uninfected control HeLa cells at 510 nm after 0 to 7 h of incubation. 30 cells were measured at each hour for a total of 240 cells. For Fig. 5, 6, 7 and 8, $dn$ is the number of values within an absorption interval (shown on the abscissa as a fraction of the total number of measured points $n$). The bar graphs at each interval of absorption show the maximum variations of the measured values. The curves are drawn through the average value at each interval.
Fig. 6. Distribution of average absorption values measured (510 nm) in poliovirus-infected HeLa cells incubated 1 to 7 h after infection. - - - - , 1 to 3 h; ....... , 4 h; -- , 5 h; ° -- ° , 6 h. •--• , 7 h. 30 cells were measured at each hour.

Fig. 7. Definite separation of the recorded (510 nm) absorption values after 5 h of incubation of poliovirus-infected and uninfected HeLa cells. - - - - , uninfected; - - - - , infected. 30 cells each were measured.
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Fig. 8. Distribution of average absorption values measured (510 nm) in the non-lytic VSV-infected and uninfected L cells incubated 1 to 7 h. 30 cells were measured at each hour. ———, uninfected cells; ———, infected cells.

of 20 to 40% absorption indicating that relatively few areas of dark stain have been measured in the uninfected cells. The analysis of absorption data from poliovirus-infected HeLa cells is demonstrated in Fig. 6. In the first 3 h p.i. the distribution of population absorption data of the infected cells shows no significant variation in comparison to the control population histograms. The absorption data in the subsequent hours of infection reveal increasing differences relative to the first 3 h of infection and to the control. More of the recorded values occurred in the range of 50 to 90% of absorption for the 4 to 7 h infected cells. This indicates that larger areas of lighter stain have been measured starting at 4 h after infection. In comparison to the control population there appears a statistically significant separation in the measured absorption values beginning at 5 h after infection in the range of 50 to 70% absorption (Fig. 7).

In contrast to these findings, histograms of absorption data from the VSV-infected L cells showed no significant differences in the sequence of infection relative to the control L cell population (Fig. 8).

DISCUSSION

The biochemical analysis of the lysosomal enzyme activities during the event of poliovirus multiplication in HeLa cells demonstrates the release of different lysosomal enzymes into the cytoplasm before marked visible c.p.e. occurs and before poliovirus is released by the lysis of the cell. These data are confirmed by the applied histogram investigation in combination with computer-aided analysis. The stoichiometrically-stained reaction product of the lysosomal acid phosphatase was recorded as absorption values using the UMSP-1. This investigation demonstrates that this enzyme, detectable in the cytoplasm as distinctly stained granules, diffuses further into the cytoplasm starting at 3 to 4 h after infection. This is shown in the variation of absorption values in the population histograms; the percentage of
absorption values from 40 to 90% increases as the acid phosphatase occupies a greater portion of the scanned images. These results support the model and scheme published by Allison & Malucci (1965).

In the VSV-infected L cells no changes in the lysosomal enzyme distribution could be observed or recorded during the virus multiplication as seen from the biochemical and cytophotometric data.

The release of a lysosomal enzyme during the sequence of poliovirus multiplication in correlation with the biochemical analysis can only be documented with the aid of computer analysis. Macieira-Coelho, Fernandes & Mellman (1965) investigated the release of acid phosphatase in poliovirus-infected cells only at the stage of visible c.p.e. The changes of enzyme distribution were only qualitatively described as they were observed by light microscopy. The correlation of the biochemical events to the kinetics of the histochemical lysosomal enzyme changes was not performed.

In contrast to our findings, Killington et al. (1974) did not observe a release of lysosomal acid phosphatase at the early stages of infection during the sequence of poliovirus type I and rhinovirus multiplication in L 132 cells. The authors suggested that the lysosomal enzyme release is an artifact resulting from the method to homogenize the cells. It is conceivable that these differences are either due to the different cell systems applied or to the enzyme method employed. The amount of free phosphate is determined by the enzymatic hydrolysis of β-glycerophosphate. Phosphate is, however, present as background in the cells and cannot be eliminated completely by washing with phosphate-free buffers. Therefore, a small increase in the amount of phosphate after the enzyme test is difficult to detect if the amount of enzyme released from the lysosomes is minute. As Fig. 1 shows, there is only 10% or less, lysosomal enzyme release of total activity at 4 h p.i.

The correspondence of cytophotometric and biochemical results obtained in our experiments with the two different viruses indicate that automated cytophotometry can be applied to detect specific biochemical changes occurring in virus infections. The sensitivity of the applied technique is a function of the staining for a specific biochemical marker, the measurement of the staining reaction in monochromatic light, the scanning steps size which records 1500 to 4000 gray values per cell and the appropriate data reduction analysis programs.

The sequence of enzyme release measured by computer-aided cytophotometry confirms the biochemical enzyme analysis. The lysosomal enzymes had been released already during the earlier stages of poliovirus multiplication and represented a true change of the state of the host as a result of the infection rather than an artifact.

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