

HISTOCHEMICAL STUDIES OF LYSOSOMES AND LYSOSOMAL ENZYMES IN VIRUS-INFECTED CELL CULTURES

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In an earlier communication (1) it was pointed out that multiplication of viruses to high titer does not always lead to cytopathic effects. Hence the changes in nucleic acid and protein metabolism associated with virus replication are not necessarily damaging to host cells. It was suggested that another process sometimes set in motion during virus infection, namely release of lysosomal enzymes into the cytoplasm, makes an important contribution to cytopathic effects. Biochemical evidence that activation of lysosomal enzymes occurs in livers of mice infected with mouse hepatitis virus and in monkey kidney cells infected with vaccinia virus was presented. In experiments described in the accompanying paper (2) activation of lysosomal enzymes was found in chick embryo cells infected with fowl plague virus, which produces marked cytopathic effects, but not in the same cells infected with a related influenza virus which does not have any demonstrable cytopathic effects.

Allison and Burstone (3) followed by histochemical techniques the changes in several enzymes in livers of mice infected with mouse hepatitis virus. A marked increase in acid phosphatase activity was found in foci of infection, preceding histologically demonstrable cell degeneration. The experiments described in this paper were undertaken to show in more detail by histochemical methods the changes in lysosomes, and in the distribution of lysosomal enzymes, occurring in cultured cells infected with viruses.

Material and Methods

Cell Types.—

Monkey kidney cells: Primary cultures of *Macaca* monkey kidney cells were grown on coverslips in Parker's medium 199 (Glaxo Laboratories Ltd., Greenford, England) plus 10 per cent calf serum inactivated by heating at 56°C for ½ hour. The medium was changed after 5 days and the cells infected after a further 2 to 5 days' growth before they had formed a confluent sheet.

Chick embryo cells: These were obtained from 10-day-old chicks and grown in a tris(hydroxymethyl)aminomethane-buffered Gey's solution with peptone and lactalbumin hydrolysate (4). They were infected after 2 days' growth on coverslips or in 6 mm diameter Petri dishes.

HeLa cells: These were propagated in medium 199 plus 5 per cent inactivated calf serum and were infected 3 to 6 days after subculture by trypsinization.

Mouse macrophage cultures: The term "macrophage" in this context refers to mononuclear cells obtained from peritoneal washings without prior stimulation, which become attached to the glass substrate in culture. Nearly all the cells take up large amounts of carbon or particulate dyes immediately after the cultures are set up, so that they can be considered as belonging to the rather miscellaneous group of cells of macrophage type. The cells were obtained by inoculating 3 ml of culture medium (solution 199 plus 20 per cent inactivated calf serum) containing 10 i.u. of heparin per ml into mice of strain VSBS/NIMR weighing 18 to 20 gm. After brief massage the cells were harvested, diluted, and dispensed in 1 ml volumes containing 2×10^6 cells in tubes containing small coverslips. They were infected after 1 to 2 days.

Viruses.—

Influenza viruses: The Dutch strain of fowl plague virus and the Melbourne strain of influenza A virus were cultivated in the allantois of 11-day-old chick embryos and titrated by hemagglutination, egg infectivity, and, in the case of fowl plague virus, by plaque counts in chick embryo cell cultures. Virus suspensions were partially purified by adsorption to and elution from chick red cells and differential centrifugation (2).

Newcastle disease virus (NDV): The standard white-plaque strain and red-plaque mutant of NDV previously described (5) were kindly provided by Dr. L. Thiry, and propagated and titrated by plaque counts in cultures of chick embryo cells.

Mouse hepatitis virus MHV-3 (6) was propagated and titrated by intraperitoneal inoculation into weanling VSBS/NIMR mice. Comparative titrations were also carried out by the microplaque technique in macrophage cultures described by Mallucci (7) and below.

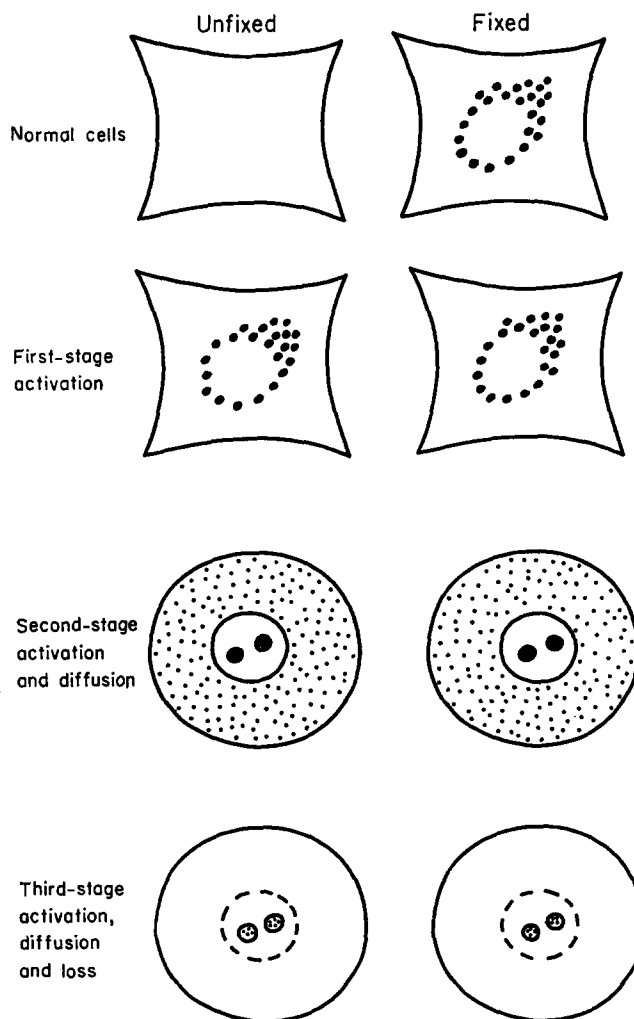
Adenovirus type 5: This was propagated and titrated in HeLa cells as described by Pereira (8). Supernatant fluids containing the B antigen (early cytopathic factor) were obtained after centrifugation at 20,000 g for 1 hour at 4°C. Suspensions of virus were incubated for 1 hour at 37°C with 0.1 per cent crystalline trypsin (Worthington Biochemical Corporation, Freehold, New Jersey) in bicarbonate buffer at pH 7.4, and an equivalent amount of trypsin inhibitor then added, in order to obtain infective material without the early cytopathic factor.

Vaccinia virus: The Lister Institute egg-adapted strain of vaccinia virus was propagated in chick embryo chorioallantoic membranes and partially purified by fluorocarbon extraction and differential centrifugation (9).

Histochemical Methods.—

Acid phosphatase: Cells were stained either without prior fixation or after fixation in 2 per cent glutaraldehyde in 0.2 M cacodylate buffer made isotonic with Gey's saline solution at 4°C for 2 to 14 hours, followed by repeated washing with Gey's saline solution. A modification of the Gomori technique was used. (a) Cells on coverslips were washed briefly in isotonic sodium chloride and incubated for 15 to 20 minutes in a medium consisting of 0.01 M sodium β -glycerophosphate (British Drug Houses Ltd., Poole, Dorset, England) in 0.05 M acetate buffer, pH 5.0, containing 0.004 M lead nitrate, and 0.05 M sodium chloride. The medium was prepared the day before, incubated at 37°C overnight, and filtered before use. (b) Coverslips were rinsed for 10 seconds in 1 per cent (w/v) acetic acid. (c) Coverslips were transferred to distilled water saturated with hydrogen sulphide for 5 minutes. (d) Coverslips were washed briefly in distilled water and mounted in buffered glycerol or Farrant's medium.

To check the specificity of the method, controls were run with 10^{-3} M sodium fluoride in the incubation medium (a); this inhibits lysosomal phosphatase. As Bitensky (10) has pointed out, staining cells for acid phosphatase under carefully controlled conditions without fixation provides useful information about the state of the lysosomes. If the lysosomes are intact, no staining is observed, presumably because the lysosomal membranes are impermeable to the



TEXT-FIG. 1. Diagrammatic representation of the appearance of unfixed and fixed cells stained for acid phosphatase. In normal unfixed cells there is no staining because the membranes of lysosomes are impermeable to the β -glycerophosphate substrate, but fixed cells show enzyme in lysosomes. In first-stage activation the membranes of lysosomes show increased permeability, so that there is staining of lysosomes in unfixed cells. In second-stage activation enzyme has diffused out of the lysosomes into the cytoplasm, often with secondary adsorption to the nucleus. The cells are rounded up. In third-stage activation enzyme has diffused out of or been inactivated in the cytoplasm, so that there is very little staining in fixed or unfixed cells.

phosphatase substrate (Text-fig. 1, Figs. 1, 5 and 11); de Duve (11) has shown that isolated lysosomes are impermeable to glycerophosphate. If the same cells are fixed, enzyme is readily demonstrable in the lysosomes (Text-fig. 1, Figs. 2 and 6). Minor degrees of change in lysosomes result in their membranes becoming permeable to the phosphatase substrate so that enzyme is stained within the lysosomes in unfixed cells (see diagram in Text-fig. 1, and Figs. 7 and 12). The cells are still fully extended at this stage, which we call *first-stage activation*: evidence will be presented below that this stage is reversible. There should be no nuclear staining in normal cells during the first stage of activation; any nuclear staining is referable to incorrect technique, usually overprolonged incubation in the staining solution. In *second-stage activation* enzyme has leaked out of the lysosomes and is stained more or less diffusely in the cytoplasm in fixed or unfixed cells; often the nuclei show staining at this stage, presumably owing to secondary adsorption of enzyme. The cells have become rounded, and the associated damage appears to be irreversible (diagram in Text-fig. 1 and Fig. 8). At this stage many cells leave the glass support on which they have been cultivated so that *third-stage activation* is seldom seen in preparations of this type. In the third stage the enzyme has diffused out of, or been inactivated in, the cells, so that little enzyme is seen in fixed or unfixed cells, although some residual nuclear staining may be present.

Although it cannot be supposed that the cells remain viable after incubation at pH 5.0 in a medium containing lead, the test is empirically useful in revealing preexisting changes in lysosomal membranes. The results are regularly reproducible in monolayers of cultured cells, but in our hands it has been much more difficult to obtain reproducible results with frozen sections in which varying degrees of damage due to freezing occur.

A second lysosomal enzyme which can be conveniently demonstrated by histochemical techniques is the esterase-splitting indoxyl acetate derivatives (12, 13). We have carried out experiments with 5-bromo-4-chloroindoxyl acetate (Sigma Chemical Company, St. Louis) as substrate. The distribution of this enzyme in normal and infected cells was found to parallel that of acid phosphatase, so it seems that the behaviour of either one can be taken as a useful indicator of the integrity of lysosomes. Nevertheless, the possibility cannot be excluded that other lysosomal enzymes for which no good histochemical methods are available may behave somewhat differently.

Uptake of Vital Dyes and Fluorochromes.—

Neutral red: Evidence is accumulating (see references 14, 15) that neutral red in non-toxic quantities is taken up and concentrated in lysosomes. We have confirmed this for the cells used in this investigation by incubating them in culture medium containing neutral red (G. T. Gurr, London, vital and fluorochrome; 1 in 100,000 w/v) overnight in the dark, fixing in glutaraldehyde, washing, and staining for acid phosphatase. The neutral red is retained in organelles having the distribution of lysosomes (Fig. 3) and in many cases containing lead sulphide deposit.

Aminoacridines: We have undertaken a systematic study of vital staining with aminoacridines, the results of which are summarized elsewhere (16). Many aminoacridines, including proflavine, acridine orange and euchrysin, are taken up both by the nuclei and the lysosomes of living cells, and can be used as vital fluorochromes for studying lysosomal changes after virus infection (Figs. 13, 15, and 16). The staining of lysosomes by acridine orange has recently been observed independently by Koenig (17) and Robbins *et al.* (18). It should be recognised that this type of vital staining is quite different from the use of aminoacridine fluorescence as a histochemical technique for nucleic acids in fixed cells (19, 20).

In our experiments euchrysin (2 GNX, CI number 46040, supplied by T. Gurr, London) was added to culture media to give final concentrations of 1 in 5×10^5 to 1 in 10^6 . The cells were incubated in culture medium for 2 hours at 37°C in the dark before being mounted in

fresh medium for fluorescence microscopy. Blue-violet light (wavelength 400 m μ to 500 m μ) from a 250 watt high-pressure mercury vapour lamp was used to activate fluorescence. The lysosomes appeared as green or orange granules in the cytoplasm, depending on the amount of acridine taken up, and nuclear chromatin, nucleoli or viral inclusions fluoresced green.

OBSERVATIONS

Chick Embryo Cells Infected with Fowl Plague Virus.—When a high multiplicity of partially purified infecting virus was used (10 p.f.u. per cell), the majority of cells showed histochemical changes within 4 to 5 hours after infection. The earliest detectable change corresponded to first-stage activation of the lysosomes, that is, stainability by the acid phosphatase technique in unfixed cells (Fig. 7). The lysosomes appeared as discrete organelles widely distributed in the cytoplasm of the cells, but there was little or no diffuse enzyme staining. At this stage the cells were still fully extended and by ordinary or phase-contrast microscopy appeared normal. Neutral red or euchrysin were taken up in relatively large amounts, and the lysosomes were larger and more conspicuous than in uninfected cells. After 6 to 8 hours infection many cells were rounded and beginning to leave the coverslips. At this stage a high proportion of infected cells, whether fixed or unfixed, showed diffuse acid phosphatase and indoxyl acetate esterase staining, indicating second-stage activation—that is, widespread release of lysosomal enzymes into the cytoplasm (Fig. 8). Reduced uptake of neutral red or euchrysin into lysosomes was observed at this stage, infected cells showing diffuse green cytoplasmic fluorescence with euchrysin. These changes spread progressively through the monolayer until by 12 hours few normal cells remained.

Chick Embryo Cells Infected with Influenza Virus.—No cytopathic effect was visible in chick embryo cells infected with the Melbourne strain of influenza A virus, and only slight first-stage activation of lysosomal enzymes in a few cells was detected. Neutral red and euchrysin uptake were as in uninfected cells.

Chick Embryo Cells Infected with Newcastle Disease Virus (NDV).—Cells infected with the white-plaque strain of NDV showed very similar changes to those described above for fowl plague virus. However, cells infected with a high multiplicity of the red-plaque strain of NDV showed after 6 to 8 hours only first-stage activation of lysosomal enzymes; *i.e.*, stainability of the acid phosphatase *in situ* in the lysosomes of unfixed cells. There was increased uptake of neutral red and euchrysin into enlarged lysosomes.

Progression to second-stage activation did not occur and after 12 hours the cells began to revert to the normal stage. No cytopathic effects were visible by ordinary or phase-contrast microscopy. Infected cells in Petri dishes were incubated under agar overlay for 4 days and neutral red added (2 ml of 1 in 2 \times 10⁶ with incubation at 37°C for 2 hours). Both white- and red-plaques produced by the corresponding virus strains were clearly seen (Fig. 4); if petri dishes with red-plaques were exposed to visible light, the background cell coloration was

bleached and the plaques thereby intensified. However, as Thiry (5) had previously noticed, if addition of neutral red is delayed to the 6th day, no plaques are observed, the cell sheet appearing quite normal. This shows that the red-plaque stage represents a reversible cellular change associated with multiplication of certain relatively avirulent viruses.

Mouse Macrophages Infected with MHV-3.—The growth of MHV-3 in mouse macrophages has been described in detail elsewhere (7). The earliest sign of infection is rounding up of individual cells and accumulation in the cytoplasm of viral antigen demonstrable by the fluorescent antibody technique. (Figs. 18 and 19). Within 6 to 12 hours after infection formation of foci of giant cells has occurred and these become progressively larger with passage of time (Fig. 9). The foci are readily visible as areas of increased uptake of neutral red (Fig. 10) and can be counted under the naked eye or dissecting microscope. Up to 18 hours the number is proportional to the virus inoculum even in the absence of an agar overlay. Later the counts are complicated by formation of secondary foci unless an agar overlay has been added. Acid phosphatase staining of unfixed cells shows little or no enzyme activity in the uninfected controls (Fig. 11), but unfixed cells infected with MHV-3 show by 4 to 6 hours marked phosphatase staining. At this stage the staining is mainly particulate (first-stage activation), but later diffuse staining of the syncytia (second-stage activation) is observed (Fig. 12). The foci of infection stand out very prominently against the background of uninfected cells.

Vital euchrysin staining of normal macrophages shows numerous orange fluorescent lysosomes in the cytoplasm (Fig. 17). In MHV-3-infected cells these increase in size and aggregate, then there is generalized green fluorescence of the rounded cells and finally as giant cells are formed there is little uptake of the vital fluorochrome (Figs. 20 to 22). This is further evidence of progressive lysosomal disruption accompanying virus multiplication in this system.

Vaccinia Virus in Macrophage Cultures.—As Nishimi and Bernkopf (21) have described for leukocytes, vaccinia virus has a marked toxic effect on mouse macrophages although there is no detectable yield of virus. When macrophage monolayers are infected with a high multiplicity of vaccinia virus (about 10 p.f.u. per cell), the cytopathic effects are obvious after 3 to 4 hours. Most of the cells are rounded, and there is marked acid phosphatase staining of unfixed cells, which is at first particulate but rapidly becomes diffuse. By 6 hours after infection most of the cells are rounded and stained, and many have already left the glass. These cells fail to take up neutral red or euchrysin into lysosomes.

Vaccinia Virus in Monkey Kidney Cells and HeLa Cells.—In these systems vaccinia virus multiplies to high titer with the production of cytoplasmic inclusions and cytopathic effects. By 4 hours after infection with high multiplicities of virus (about 10 p.f.u. per cell), staining of lysosomes in localized areas of the cytoplasm of unfixed cells was already apparent. By 8 hours after infec-

tion, many acid phosphatase-positive bodies were stainable, with a progressive increase of diffuse staining, and rounding up of infected cells, thereafter. By 8 hours uptake of neutral red and euchrysin into swollen lysosomes, many of which were concentrated in the vicinity of cytoplasmic bodies showing DNA-type fluorescence, was observed (Fig. 15). After 16 hours a few large masses and diffuse cytoplasmic fluorescence were seen in infected cells, suggesting that many of the lysosomes had already been disrupted and were unable to concentrate neutral red or euchrysin (Fig. 16).

Adenovirus Type 5 in HeLa Cells.—Several workers have shown (23) that a non-viral early cytopathic or cell-detaching factor is formed when the adenovirus type 5 multiplies. The early cytopathic factor is the same as the B antigen identified by immunodiffusion (26); it is inactivated by proteases or antiserum. Within 2 to 5 hours of exposure of HeLa cells to the virus-free supernatant, the cells round up and many become detached from the glass. Acid phosphatase staining of unfixed cells shows well defined lysosomal activation, chiefly first stage at this time. The effects are fully reversible, the cells recovering and extending, and their lysosomal enzymes becoming unstainable, after 24 to 48 hours' incubation in fresh medium.

The use of trypsinized virus inocula was introduced (22) for detailed cytological and fluorescent antibody studies of adenovirus-infected cells uncomplicated by early cytopathic effects. The same system was used in the present investigation for studying the lysosomes in HeLa cells infected with adenovirus type 5 lacking the early cytopathic factor. The earliest detectable changes occurred at about 6 hours, considerably later than if the inoculum contained the cytopathic factor. The changes increased progressively both within cells and in the number of cells involved until about the 18th hour. The changes were characterized by phosphatase staining of lysosomes in unfixed cells followed by some diffuse phosphatase staining of rounded cells. Neutral red or aminoacridine uptake was first increased in swollen lysosomes of living cells (6 to 8 hours), the aminoacridine staining also the developing intranuclear virus inclusions. Later many of the lysosomes were aggregated or disrupted and there was diffuse green cytoplasmic fluorescence with aminoacridine in place of the orange pinpoint lysosomal fluorescence of uninfected cells. Observations of similar changes in cells infected with adenovirus and vitally stained with acridine orange were previously mentioned by Mayor (20) but were not interpreted in terms of lysosomal changes.

DISCUSSION

In cells infected with cytopathic viruses two stages of lysosomal enzyme activation were observed. In the first stage of activation increased permeability of the lysosomal membrane could be demonstrated by acid phosphatase or indoxyl-acetate esterase staining within the lysosomes of unfixed cells or by

increased capacity of lysosomes to take up neutral red or euchrysrine. In certain virus infections lysosomal changes do not progress beyond this stage, so that red plaques are formed as a consequence of the increased neutral red uptake. Bitensky (10) has pointed out in another context that this stage of lysosomal activation can be reversible, and our results support this view. Host cells recover completely from infection by the red-plaque strain of NDV. However, in macrophage cultures infected with the MHV-3, red plaques are associated with giant cell formation, which does not appear to be a reversible change.

Red plaques have been observed in other systems, including cells infected with adenovirus type 2 in certain culture conditions (24) and chick embryo cells infected with Rous sarcoma virus (25). Furthermore, Thiry (5) noted that even with the white-plaque strain of NDV there is a transient phase of increased neutral red uptake in infected foci on the 3rd day. We have confirmed her observation and seen the same sequence of events with other viruses, including Chikungunya and tick-borne encephalitis viruses. Red plaques are much more obvious when neutral red is added some days after infection than when it is added to the original overlay, which is a common practice with chick cells. Minor cytopathic effects leading to red-plaque formation may often have been overlooked for this reason.

The second stage of activation involves the release of lysosomal enzymes into the surrounding cytoplasm. This stage is associated with rounding up of the cells and diminished adhesion to the glass support, so that many cells accumulate in the culture media. When the cells are trapped under an agar overlay, microscopical examination shows rounding and other degenerative changes. At this stage some or all of the lysosomes in the cell are disrupted, and neutral red or aminoacridines are not concentrated in lysosomes in the usual way. Hence white plaques are formed and cells show after exposure to aminoacridines varying degrees of diffuse green cytoplasmic fluorescence instead of having all, or nearly all, of the fluorochrome concentrated in lysosomes and nuclei.

The role of lysosomal enzymes in virus infection can now be considered briefly. It is widely accepted that, after attachment to the host cell surface, viruses are taken up into pinocytotic vesicles (phagosomes) and within these show progressive morphological changes which suggest that they are being broken down to release the nucleic acid which initiates the infection (26). Lysosomal enzymes, which are discharged into phagosomes, may well contribute to the "uncoating" of viruses. One of the pox virus-uncoating enzymes described by Abel (27) is present in normal cells and appears to be a lysosomal protease (28). Another pox virus-uncoating enzyme may be specifically induced in infected cells (27, 29). This localized release of lysosomal enzymes into the phagosome, although possibly important in virus uncoating, is as a rule well tolerated by host cells.

However, in some situations, for instance with mouse macrophages and vaccinia virus, exposure to virus rapidly leads to widespread liberation of lysosomal

enzymes, which is damaging to host cells. The toxic effects produced in animals by intravenous injections of relatively large amounts of influenza and other viruses may well be due, at least in part, to lysosomal enzyme release. It is also possible, as discussed in the accompanying paper (2), that lysosomal enzymes in phagosomes will sometimes inactivate infecting viruses. Nevertheless, in many cases virus multiplication follows. This process is sometimes, but not always, accompanied by detectable changes in lysosomes; first, increased permeability of lysosomal membranes, and second, release of lysosomal enzymes into the cytoplasm. A number of questions are raised by the phenomenon, of which the most important is whether the lysosomal changes precede or follow other damaging effects produced by viruses in the cells they infect. It is difficult to answer this question with certainty, but lysosomal changes are seen before other histological or histochemical changes are manifest. In HeLa cells infected with herpes simplex virus, breakdown of host cell DNA is observed during the 3rd to the 6th hour after infection (30); this corresponds with increase in activity of lysosomal DNAase in the supernatant fraction (28). Furthermore, Vainio and his colleagues (31) have reported that in the presence of antihistamines multiplication of mouse hepatitis virus is not inhibited but cell degeneration is markedly reduced.

The second problem is why virus infection should sometimes result in increased permeability of lysosomal membranes. Again, no definite answer can yet be given. One factor could be a fall in intracellular pH, which is known to activate lysosomes (11). Perhaps more important is the fact that certain products of virus multiplication, like certain bacterial toxins (32), have an affinity for lysosomes and increase the permeability of their membranes. The action of the B antigen of adenovirus type 5 described above would be a case in point. Electron micrographic studies show that some viruses multiplying in the nucleus secondarily become associated with lysosomal membrane systems; *e.g.*, polyoma and rabbit vacuolating viruses (33).

Massive activation of lysosomal enzymes would bring about rapid and complete degeneration of host cells, but less complete activation might produce more subtle changes not resulting in death of host cells. These include the rounding up and altered adhesiveness of cells leading to polykaryocytosis, as described above for macrophages infected with MHV-3. Rounding up and morphological alterations in cell membranes are observed when leucocytes are exposed to streptolysin S, which activates lysosomal membranes (32). We are investigating further the possible role of lysosomal enzymes in giant cell formation in other systems.

Another point of interest is the possible involvement of lysosomal enzymes in carcinogenesis and in transformation *in vitro* of cell cultures. This approach acquires further significance from the recent finding that carcinogenic hydrocarbons are concentrated in lysosomes (34). Preliminary observations made in

collaboration with Dr. A. Kisch indicate that in the BHK line of hamster kidney cells exposed to large doses of polyoma virus activation of lysosomal enzymes occurs; this is more marked under slightly alkaline conditions which favour transformation than under slightly acidic conditions which favour virus multiplication rather than transformation. Thus lysosomes represent one group of cell constituents at which the actions of viruses and chemical carcinogens converge; how this might lead to malignancy is discussed elsewhere (34). The presence of red plaques in cells infected with Rous sarcoma virus (25) indicates that lysosomes are changed, and further study of this problem should be rewarding.

Several examples are now known in which infection by oncogenic or other viruses leads to breaks and other abnormalities in chromosomes (35, 36). We are investigating the possibility that lysosomal enzymes are responsible for some or all of these breaks. Another effect in which lysosomes are involved is in the photodynamic effects of neutral red and acridine orange (37). Less photoinactivation of virus was observed when the dyes were added to cells shortly after infection than when they were added later. This is consistent with the interpretation that shortly after infection the dyes are taken up by lysosomes which are still intact; later much of the dye is taken up by newly formed virus particles, rendering them susceptible to photoinactivation.

SUMMARY

The appearance of lysosomes and the distribution of lysosomal enzymes have been studied in a number of cell cultures exposed to viruses. Lysosomes were shown by fluorescence microscopy after vital staining with aminoacridines and light microscopy after vital staining with neutral red. The lysosomal enzymes studied histochemically in unfixed and fixed cells were acid phosphatase and 5-bromo-4-chloro-indoxyl acetate esterase.

Activation of lysosomal enzymes was found to take place in three stages. The first is characterized by permeability of lysosomal membranes without release of enzymes. This is demonstrable by staining of lysosomal enzymes in unfixed cells and by increased uptake of aminoacridine fluorochromes and neutral red into lysosomes. In cell sheets initially stained with neutral red this gives rise to red plaques. This stage can be fully reversible; cells infected with, and yielding, the red-plaque strain of NDV, recover fully afterwards.

In the second stage lysosomal enzymes are released into the cytoplasm, the cells round up and there is decreased uptake of aminoacridines and neutral red into lysosomes. In cell monolayers this results in the formation of white plaques. In the third stage, not usually seen in cell cultures, lysosomal enzymes are released from or inactivated in the cells and are not seen in either fixed or unfixed preparations.

The possible roles of lysosomal enzymes in production of cytopathic effects, polykaryocytosis and malignant cell transformation are discussed.

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EXPLANATION OF PLATES

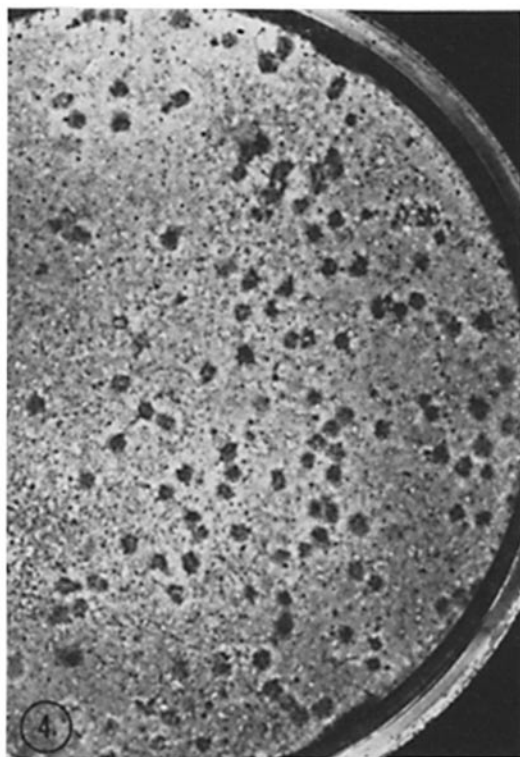
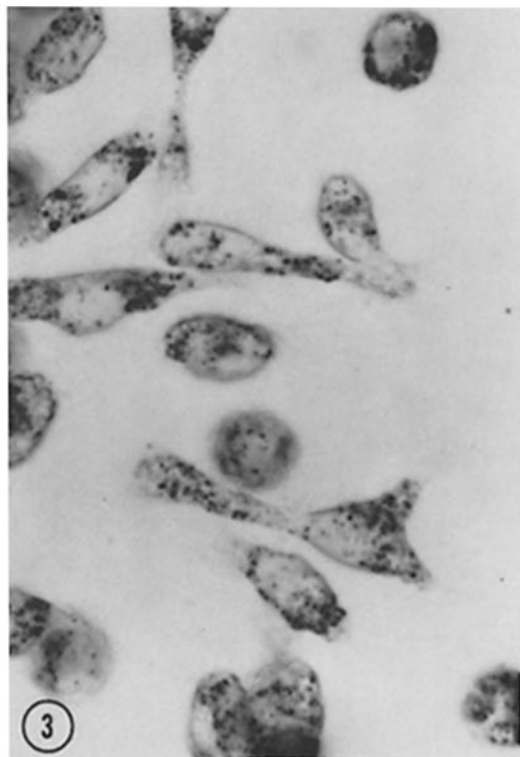
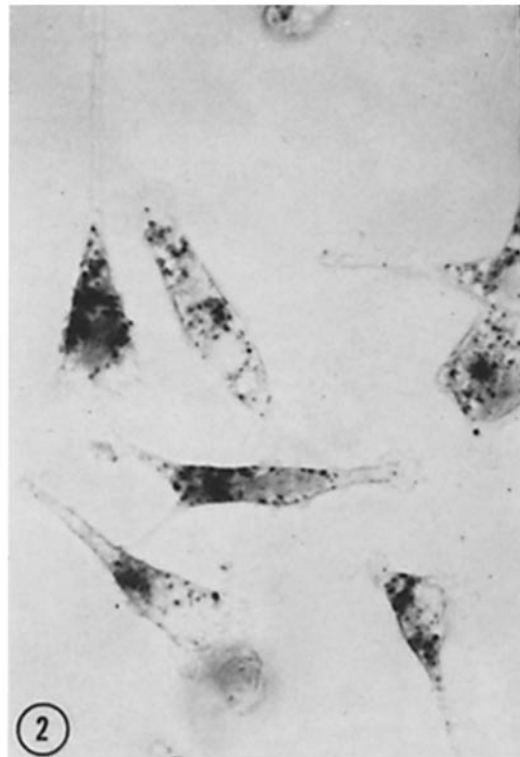
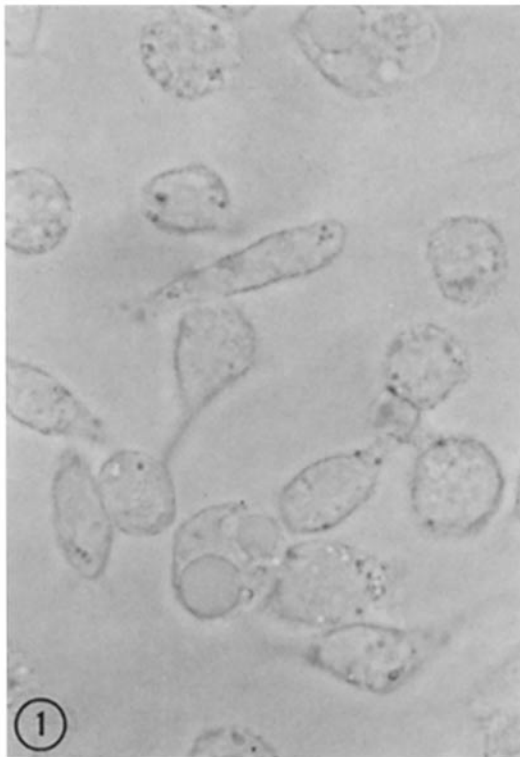
PLATE 40

FIG. 1. Unfixed macrophages, Gomori acid phosphatase, no staining. \times 1130.

FIG. 2. Fixed macrophages, acid phosphatase. Numerous lysosomes are discretely stained in the cytoplasm. \times 1130.

FIG. 3. Living macrophages vitally stained with neutral red, showing uptake of the dye into the lysosomes. \times 1140.

FIG. 4. Petri dish culture of chick embryo cells vitally stained with neutral red 4 days after infection with the red-plaque strain of Newcastle disease virus, showing increased uptake of neutral red in foci of infection. \times 1.5



(Allison and Mallucci: Lysosomes and lysosomal enzymes)

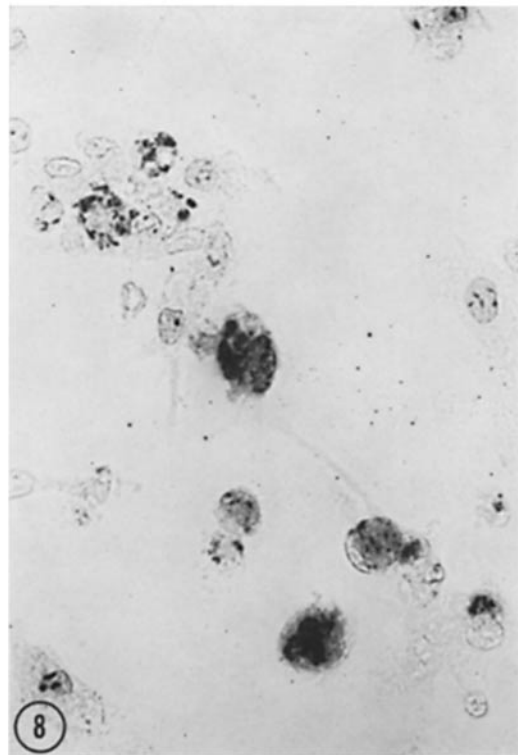
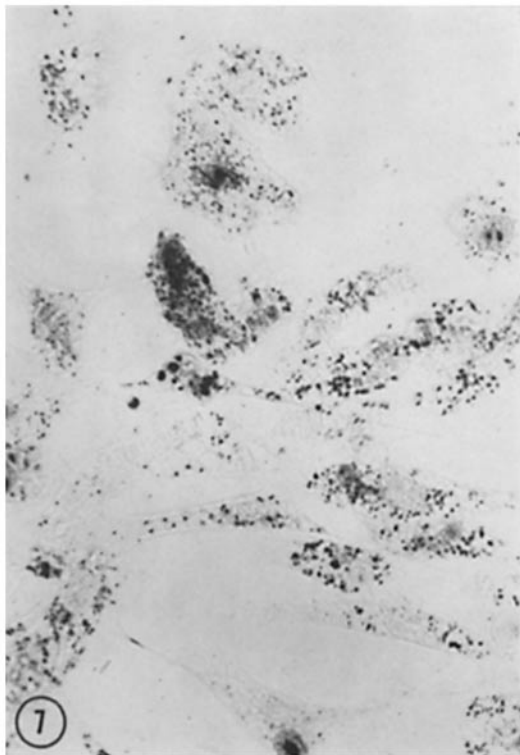
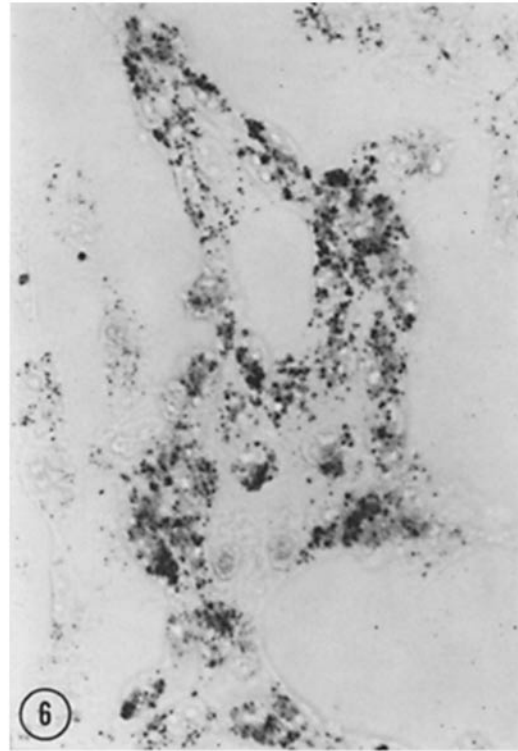
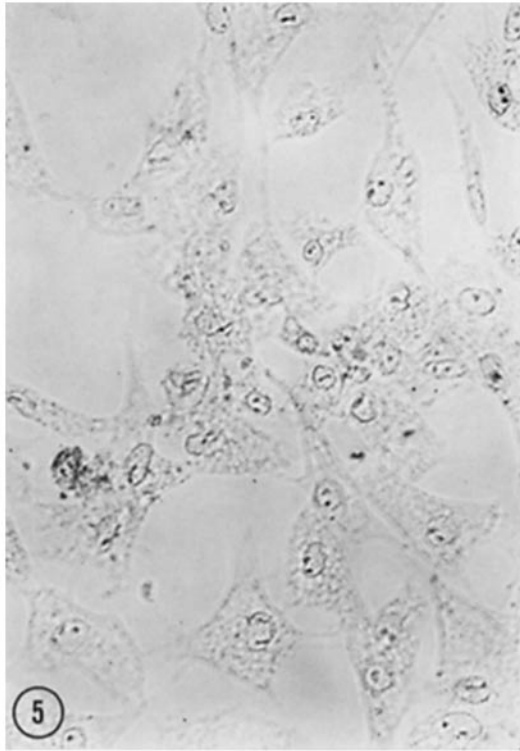
PLATE 41

FIG. 5. Normal chick embryo cell culture unfixed, stained for acid phosphatase, no staining. $\times 600$.

FIG. 6. Normal chick embryo cell culture, fixed and stained for acid phosphatase. Numerous discrete lysosomes are visible in the cytoplasm. $\times 600$.

FIG. 7. Chick embryo cell culture 5 hours after infection with fowl plague virus, unfixed and stained for acid phosphatase. Discrete staining of enzyme in lysosomes shows that first-stage activation has occurred. $\times 600$.

FIG. 8. Chick embryo cell culture 8 hours after infection with fowl plague virus, unfixed, and stained for acid phosphatase. There is diffuse cytoplasmic staining of some of the rounded cells that remain, many cells having already been shed into the medium. $\times 600$.



(Allison and Mallucci: Lysosomes and lysosomal enzymes)

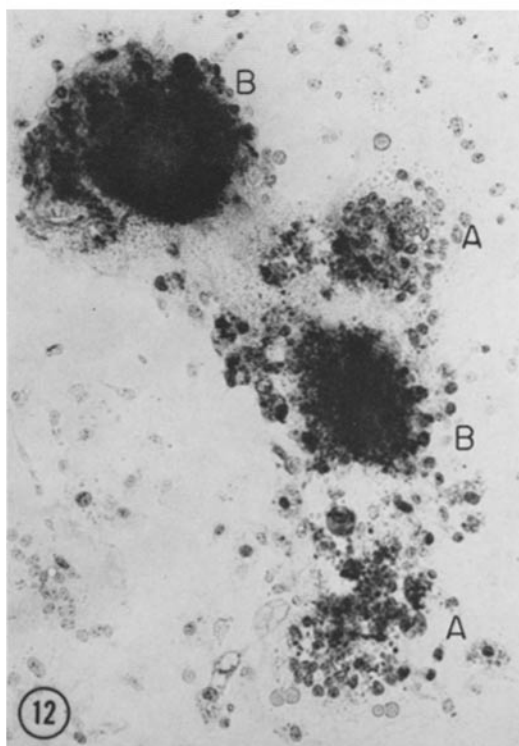
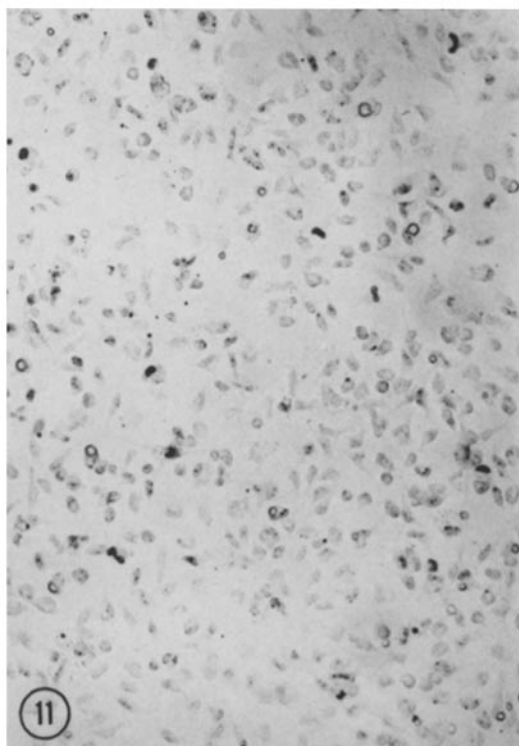
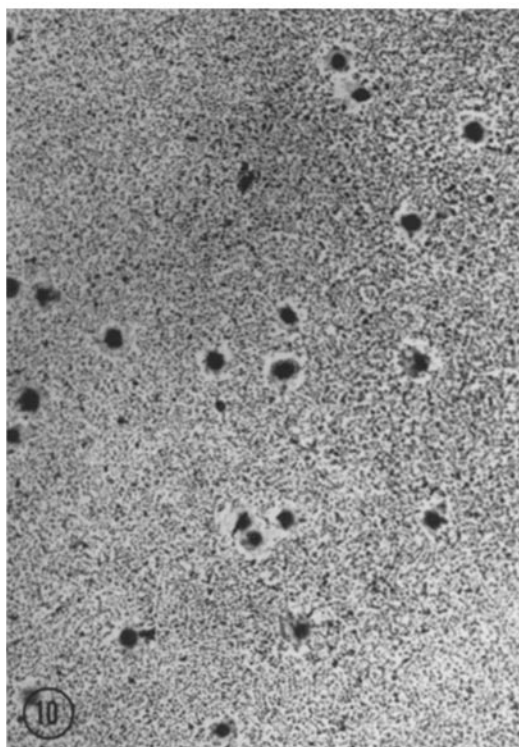
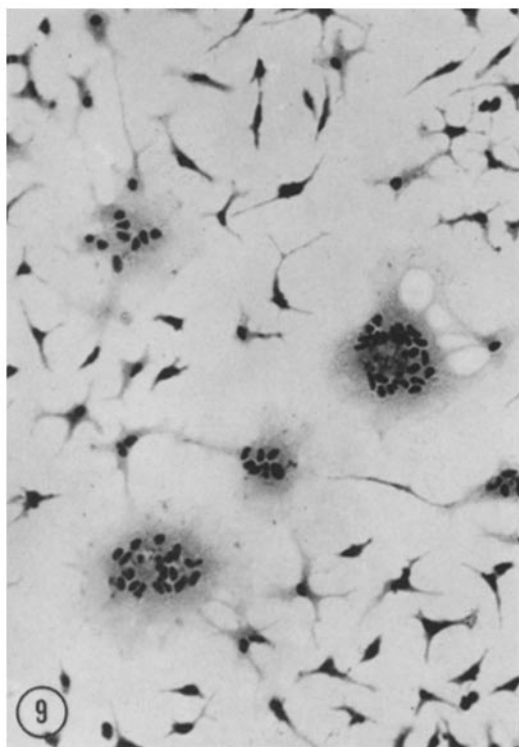
PLATE 42

FIG. 9. Mouse macrophage culture 12 hours after infection with MHV-3, Bouin-fixed, and stained with haematoxylin and eosin, showing giant cell formation. \times 200.

FIG. 10. Mouse macrophage culture 10 hours after infection, vitally stained with neutral red (1:30,000 for 10 hours), showing microfoci of infection. \times 27.

FIG. 11. Uninfected macrophage culture, unfixed and stained for acid phosphatase, very little staining. \times 165.

FIG. 12. Macrophage culture parallel to that shown in Fig. 11, infected with MHV-3 for 18 hours, unfixed, and stained for acid phosphatase. Foci of infection show marked enzyme activity, which is at first discrete in individual lysosomes (foci marked *A*) and is later diffuse (foci marked *B*). \times 165.



(Allison and Mallucci: Lysosomes and lysosomal enzymes)

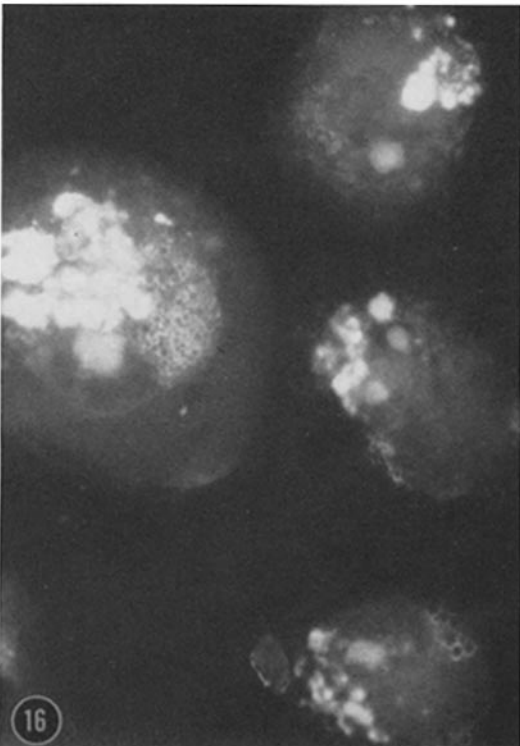
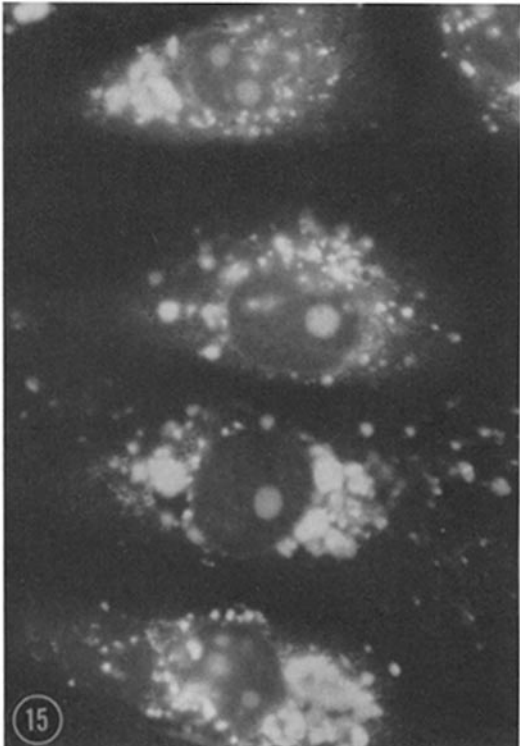
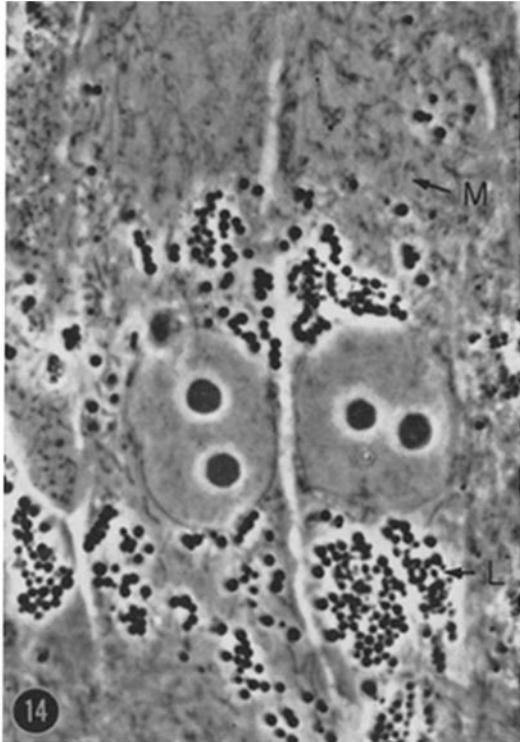
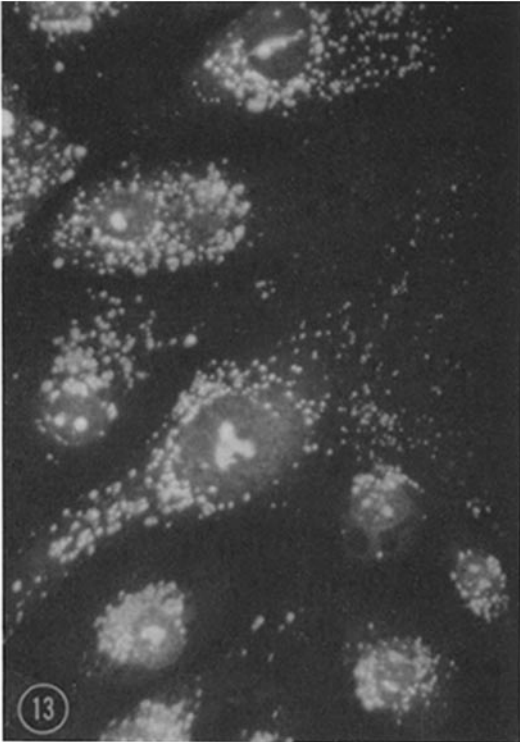
PLATE 43

FIG. 13. Uninfected monkey kidney cell culture, vitally stained with euchrysin, showing pinpoint fluorescence of lysosomes in cytoplasm (orange in the original) and fluorescence of nuclei and nucleoli (green in the original). $\times 650$.

FIG. 14. Phase-contrast photomicrograph of living monkey kidney cells in culture, showing dense lysosomes (*L*) in a perinuclear position and less dense, filamentous mitochondria (*M*) in peripheral cytoplasm. $\times 1300$.

FIG. 15. Monkey kidney cells 6 hours after infection with vaccinia virus, vital euchrysin staining, showing intense fluorescence of swollen lysosomes, many of which have aggregated into perinuclear masses which show green fluorescence. $\times 1200$.

FIG. 16. Monkey kidney cells 16 hours after infection with vaccinia virus, vitally stained with euchrysin, showing a few large green fluorescent masses weak green cytoplasmic fluorescence and rounding of cells. $\times 1300$.



(Allison and Mallucci: Lysosomes and lysosomal enzymes)

PLATE 44

FIG. 17. Uninfected macrophages, vitally stained with euchrysrine, showing brilliant orange fluorescence of lysosomes in cytoplasm. $\times 900$.

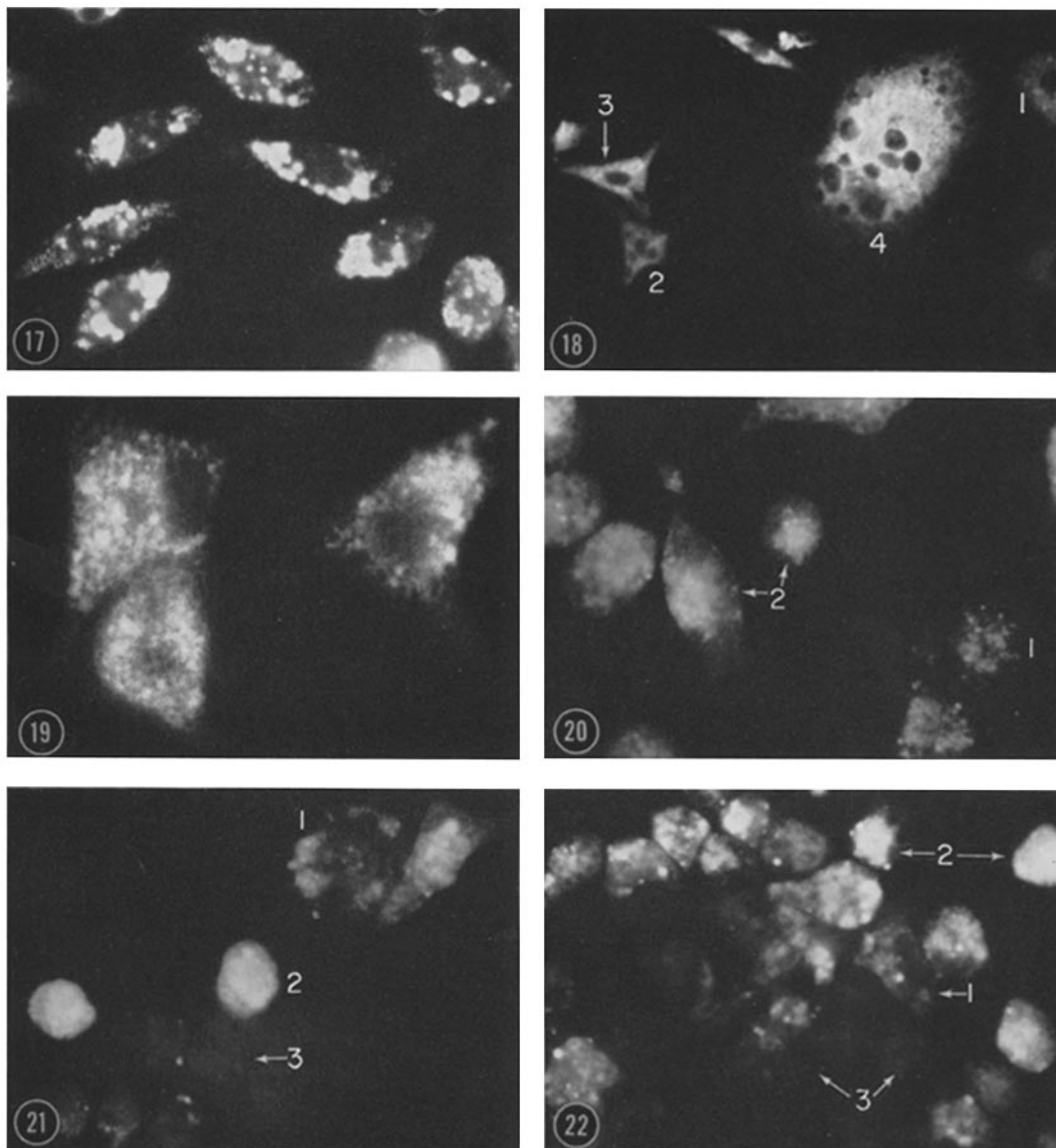
FIG. 18. Macrophages 24 hours after infection with MHV-3, stained by the fluorescent antibody technique with homologous antiviral serum. Fixation in ethanol at -68°C . Progressive stages of accumulation of viral antigen in the cytoplasm are shown in single cells (1 to 3) and in the syncytial formation (4). The dark areas are nuclei. $\times 610$.

FIG. 19. High magnification of macrophages infected with MHV-3 and stained by the fluorescent antibody technique as in Fig. 18, with antiviral serum. $\times 1500$.

FIG. 20. Macrophages 5 hours after infection with MHV-3 vitally stained with euchrysrine. There is some clumping of lysosomes in cells marked (1) and diffuse fluorescence in cells marked (2). Fluorescence microscopy. $\times 900$.

FIG. 21. Macrophages 10 hours after infection with MHV-3, vitally stained with euchrysrine. Cells with aggregated lysosomes are marked (1), rounded cells with uniform, diffuse fluorescence marked (2) approaching a clump of cells (early syncytial formation) in which there is very little residual fluorescence (3). $\times 900$.

FIG. 22. Macrophages 10 hours after infection with MHV-3, vitally stained with euchrysrine. Cells in region 3 are fused together, with very little residual fluorescence. They are being joined by cells in stages 1 (aggregated lysosomes) and 2 (showing intense, uniform fluorescence). $\times 900$.



(Allison and Mallucci: Lysosomes and lysosomal enzymes)