SOME ASPECTS OF THE ULTRASTRUCTURE AND ENZYME CYTOCHEMISTRY OF NORMAL AND VIRUS-TRANSFORMED CELLS

by

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PUBLICATIONS

Paper a) contains some material from this thesis.

Paper b) is taken wholly from this thesis.

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CONTENTS OF VOLUME I

SUMMARY

Morphological and hydrolytic enzyme cytochemical investigations have been applied to three normal and transformed cell systems in order to relate changes in hydrolytic enzyme activity to changes in ultrastructural organisation occurring when normal cells *in vitro* are transformed by tumour virus.

On transformation changes in cell shape, growth and social behaviour are detected with the light microscope. At the ultrastructural level the major changes are in the extent of the GERL (Golgi, endoplasmic reticulum, lysosome) systems and are likely to be in response to altered rates of entry of metabolites through the cell membrane caused by transformation-dependent modifications of the cell surface.

Five hydrolytic enzymes are demonstrated by light microscopic enzyme cytochemistry, mainly by azo-dye methods. Differences in levels of activity are difficult to assess visually but microdensitometry shows that transformed 3T3 cells have higher acid phosphatase activity than normal cells. This is confirmed by biochemical assays which also show that, unlike acid phosphatase, glucosaminidase has higher activity in normal cells. Biochemistry additionally provides a means of studying the effect of glutaraldehyde fixation on enzyme activity.

Azo-dye techniques are found to be unsuitable for use in electron microscopic enzyme cytochemistry since dye is lost from cultured cells during processing and end product-like deposits appear in secondary lysosomes of control material. This latter phenomenon is investigated in other tissues. Metal-salt methods are, therefore, utilised for the demonstration of three enzymes, activity being largely confined to Golgi elements and lysosomes. Some activity is localised at the cell surface

and this lends support to the possibility of surface modification through a process of sublethal autolysis by lysosomal enzymes. Examination of GERL morphology and enzyme activity gives an insight into the functioning of the system and provides evidence of possible mechanisms of cellular autophagy.

INTRODUCTION

Modern cancer research and cell culture are inextricably linked. The relationship between the two was established in the early days of cell/tissue culture when Carrel (1913) demonstrated the artificial activation of growth and cell division by means of saline extracts from embryo tissues. This work showed that cell/tissue culture was an ideal medium for the study of growth and hence for the study of cancer which was recognised as a growth disorder. Cancer may be described as a disease of multicellular organisms which is characterised by the seemingly uncontrolled multiplication and spread within the organism of apparently abnormal forms of the organisms' own cells. Cancers or malignant growths were known and documented in antiquity but the history of cancer research goes back for only about 200 years and only in the first decade of the present century did this research begin to prosper. This period coincided with the advent of cell/tissue culture. The advantages of being able to study cells and tissues in isolation and away from the influences of other tissues in the body are so great that it is surprising that the culture of animal cells and tissues was not seriously attempted until the very end of the nineteenth century and only successfully launched in 1907 when Harrison (1907) reported that pieces of frog embryonic nervous tissue grew and differentiated when cultured in vitro in clotted lymph from an adult frog. Such cultures could be maintained for about four weeks. Subsequently Carrel (1912) demonstrated that chick connective tissue could be grown in culture for many months and he put forward the view that, given the right conditions, tissues in vitro should have permanent life.

Sanford et al. (1948) made a significant step forward when they succeeded in producing the first pure cell clones, strain L mouse fibroblasts, derived from single cells. This work stimulated the production of further mammalian cell clones including some originating from tumours, the most well known of which is the Hela strain of human cervical carcinoma cells (Gey et al., 1952). Concomitant with this work were the advances made in the production of defined synthetic media by Eagle (1955), Puck and Marcus (1955) and others. Meanwhile, Abercrombie and Heaysman (1954) examined the social behaviour of normal cells growing out from explants of chick embryo heart and observed that where cells encountered each other they ceased both movement and division and remained as a monolayer. They called this phenomenon contact inhibition and it was later shown to be a characteristic of the established normal cell lines such as BHK21C13, a hamster fibroblast line isolated by Macpherson and Stoker (1962), and 3T3, a mouse fibroblast line introduced by Todaro and Green (1963). Loss of contact inhibition of division was used as a parameter of the transformation of normal cells, usually fibroblasts, by tumour virus. Transformed cells are obtained when normal fibroblasts, freshly isolated or cell lines, are infected but not killed by tumour virus. With some tumour viruses such as the avian and murine sarcoma viruses which contain RNA, virus replication does not kill the infected cell. DNA - containing viruses such as polyoma and SV40, however, normally kill the cells in which they replicate so that transformation can only occur after incomplete infection with virus which is defective in the genes causing cell death, or in non-permissive cells which restrict the function of these viral genes. BHK21 cells, which are non-permissive for polyoma virus

and 3T3 cells, which are non-permissive for SV40 virus, are readily transformed and have been extensively used for transformation studies. There are two types of transformation. Abortive transformation occurs at high frequency, but the changes are temporary and only last for a few cell divisions. Stable transformation is less frequent but the abnormalities are perpetuated indefinitely in successive cell generations by stable association of the viral genome with a functional part of the cell genome. Thus established transformed cell lines are produced. Temin and Rubin (1958) demonstrated the transformation of chick fibroblasts by Rous sarcoma virus, an RNA tumour virus. Stoker and Macpherson (1961) used polyoma virus for the transformation of BHK21 cells whilst Todaro *et al.* (1964) utilised SV40 (simian virus 40) for the transformation of 3T3 cells. Just as tumours *in vivo* can occur spontaneously or be induced by chemical carcinogens as well as by tumour viruses, so also can normal cells *in vitro* be transformed spontaneously or by certain chemicals as well as viruses.

Transformed cells *in vitro* are useful experimental models of cancer cells and their use offers considerable advantages over *in vivo* systems, chiefly due to the ease of control, manipulation and reproduction of experimental conditions and variables. During the last fifteen years there has been a huge proliferation in the volume of cancer research, much of which has been devoted to the comparison of the properties and behaviour of normal and transformed cells in culture. Stoker (1972) has stressed the importance of the fact that normal cells in culture are not completely normal because they grow freely and may spontaneously become tumourigenic. They do, however, retain important normal characteristics which are altered in transformation by tumour viruses. 'Normal', therefore, simply means

not transformed. Likewise, transformed cells are not exactly synonymous with cancer cells since they are not always transplantable and transplantability is the common standard index of malignancy. Recently transformed cells, in particular, are rarely transplantable and only after many cell generations does transplantability occur. This suggests that the changes which appear after *in vitro* transformation are not sufficient to produce real cancer and indicates that transformation represents an initial event only which cannot give rise to cancer without further progression by mutation and selection.

Many of the aspects of research into the transformed cell state have been aided by the use of temperature-sensitive (ts) mutant viruses (for review, see Wyke, 1975). Cells infected by these ts mutants become themselves temperature sensitive transformants. Cultures maintained at the permissive temperature, when the viral genes for transformation are functional, exhibit transformed characteristics but if the cultures are shifted to the non-permissive temperature, when the viral genes for transformation are non-functional, the cells revert to the normal state.

A number of phenotypic changes occur when normal cells are transformed. These changes have been recently reviewed by Stoker (1972) and Benjamin (1974). The most immediately obvious differences between normal cells and their transformed derivatives are in morphology and manner of growth. Normal BHK21 cells, for example, are fusiform, bipolar fibroblasts which grow in parallel orientation but when they are transformed by polyoma virus the cells become assymetric with more major cell processes and they grow in a random, crisscross fashion (Stoker and Macpherson, 1961). Transformed cells also undergo loss of

density-dependent inhibition of movement, growth and division. This phenomenon, described by Stoker and Rubin (1967), encompasses the concept of contact inhibition, a more local effect which can also be termed topoinhibition (Dulbecco, 1970). All normal cell lines are sensitive to density-dependent inhibition but there is some variation in the degree of sensitivity. 3T3 cells are highly sensitive and have a low saturation density. The cells cease movement and growth on contact (topoinhibition) and the whole culture passes into the stationary phase on the attainment of a confluent monolayer of cells, exhibiting very little overlap. Normal BHK21 cells have a higher saturation density than 3T3 and may exhibit overlap and grow past the monolayer stage. Transformed cells, not being sensitive to density-dependent inhibition of movement, growth and division, are not paralysed by contact and continue to grow past the saturation density of their parent normal line so that a dense multilayered culture of growing, dividing cells results. This situation parallels that of cancer cells <u>in vivo</u> which are also less responsive to their social environment than normal cells (Stoker, 1972). A further characteristic of transformed cells is their ability to grow in suspension culture in semisolid medium whereas normal cells require a solid surface for movement and division. This loss of anchorage dependence allows the formation of large spherical colonies of transformed cells in suspension culture (Macpherson and Montagnier, 1964). Transformed cells also have a greatly reduced serum requirement for growth (Holley and Kiernan, 1968) and exhibit increased glycolysis and enhanced uptake of amino acids, sugars and some other small molecules (Pardee, 1971). Cyclic AMP has been widely implicated in the regulation of cell growth and there have been many reports that the level of this cyclic

nucleotide is decreased in transformed cells (Sheppard, 1972).

The cell surface has been the focus of a great deal of recent research into the differences between normal and transformed cells. Reviews by Burger (1974), Noonan and Burger (1974), Pollack and Hough (1974) and Pardee (1975) cover most of this field. The structure of the cell surface is changed by the appearance of a thicker coat (Poste, 1973), new antigens (O'Neill, 1968), shorter glycolipid chains (Hakomori and Murakami, 1968) a new fucose-containing glycopeptide (Buck et al., 1970) and alterations in the receptor sites for lectins, plant glycoproteins which differentially agglutinate normal and transformed cells. Wheat germ agglutinin (Burger and Goldberg, 1967), concanavalin A (Inbar and Sachs, 1969) and soybean agglutinin (Sharon and Lis, 1972) are all lectins which have been utilised for the investigation of changes in the surface architecture occurring on transformation, changes which are thought to have significance in growth control. It is believed by a number of workers that alterations in the surface architecture are brought about by enzyme activity. The levels of activity of certain glycosyl transferases are reported to change on transformation (Den et al., 1971) whilst Bosmann (1969, 1972) and Bosmann and Pike (1970) have demonstrated increased activity of certain glycosidases in transformed cells. Both these types of enzymes could be responsible for bringing about alterations in the glycoproteins and glycolipids of the cell surface. Several lines of evidence suggest that proteases play an important role in the control of cell proliferation and in effecting cell surface changes. Unkeless et al. (1973) and Ossowski et al. (1973) have shown that transformed cells exhibit fibrinolytic activity whilst their normal counterparts do not and that the appearance of this

activity precedes the morphological changes which occur on transformation. If normal cells are subjected to mild protease treatment (Sefton and Rubin, 1970) a sequence of changes characteristic of transformation is set in motion. Changes in agglutinability by concanavalin A, decreased intracellular cyclic AMP, increased transport of uridine and increased RNA synthesis are followed by protein and DNA synthesis and finally cell division. Schnebli and Burger (1972) demonstrated that administration of protease inhibitors to transformed cells caused growth inhibition but similar treatment of normal cells had no effect. These and other studies have led some workers to believe that elevated levels of proteases and other degradative enzymes are responsible for the maintenance of the transformed or malignant state. This process whereby the degradative enzymes modify the cell surface without causing cell death has been termed sublethal autolysis (Bosmann, 1974). The mechanism of such a process has not been elucidated but it is known that lysosomal degradative enzymes can be released from the cell and may then act at the surface (Poole et al., 1974).

It should be noted that many of the characteristics of transformed cells are encountered, to some extent at least, in subconfluent normal cells which are actively dividing. This suggests that these features may be consequences of unrestricted growth. Cell shape, movement, membrane glycolipids and transport characteristics of isolated growing normal cells resemble those of transformed cells and lectin agglutinability and the fucose containing glycopeptide are reported in normal cells in mitosis.

A great deal of the research into the differences between normal and transformed cells, particularly that concerned with the cell

surface and the possible role of degradative enzymes, has been monopolised by biochemists. One of the major drawbacks of the biochemical approach is that information on enzyme activity levels, for instance, cannot be directly related to the organisational state of the cells. If biochemical studies such as those described above are accompanied by enzyme cytochemical investigations then it should be possible to correlate enzyme activities with the state of ultrastructural organisation of the cells. Cytochemical localisation of degradative enzyme activity at the cell surface would lend great weight to the concept of sublethal autolysis, a concept which by no means enjoys universal support (see, for example, McIlhinney and Hogan, 1974).

Techniques for the cytochemical demonstration of a wide range of enzymes are now available (Burstone, 1962; Pearse, 1968; 1972) and a number of these are applicable at the ultrastructural level, including some for lysosomal hydrolases. In addition, the procedures for the preparation and processing of enzyme cytochemical material for electron microscopy are well established (for a recent review see Hayat, 1973), the introduction of glutaraldehyde fixation (Sabatini et al., 1963) being perhaps the most significant single advance. The use of this dialdehyde fixative facilitates enzyme preservation whilst at the same time preserving ultrastructural integrity during incubation, after which osmium tetroxide fixation may be employed. The adaptation by Sheldon et al. (1955) of the Gomori lead-salt method for the demonstration of acid phosphatase (Gomori, 1952) was the first application of an enzyme cytochemical technique to electron microscopy. Most of the methods currently available for hydrolytic enzymes are based upon the production of an azo-dye, the use of

azo-dye techniques in electron microscopy being developed by Barrnett (1959). A wide range of synthetic substrates are commercially available, the naphthol AS derivatives introduced into histochemistry by Burstone (see Burstone, 1962 for review) being most important and most widely utilised. When the enzyme has hydrolysed the substrate the released naphthol AS is coupled with a diazonium salt to produce an insoluble, substantive azo-dye. As the azo-dye must be electron dense in order to be detectable in the electron microscope, the diazonium salt which is employed must contain a heavy metal or have a molecular arrangement which renders it electron opaque (for recent review see Bowen, 1973). Some of these techniques are applicable to a range of enzymes provided suitable substrates are available. There is, however, dissatisfaction with certain methods (Smith and Fishman, 1969; Bowen, 1973) and the fact that the comparatively old Gomori and related procedures are still so widely used indicates that progress in electron microscopic enzyme cytochemistry has been difficult.

In view of the great amount of research that has been carried out on certain normal and transformed cell lines, such as BHK21 and 3T3, it is surprising that there has been no comprehensive investigation of the ultrastructural morphology of these cells. This is, perhaps, a consequence of the biochemical domination of the field. It is ironic, then, that some of the very earliest uses of the electron microscope for the examination of biological material involved the study of cultured cells. Claude and his associates (Porter et al., 1945; Claude et al., 1947), who wanted to examine specimens which could be processed for electron microscopy without the need for embedding and sectioning, investigated the periphery of thinly spread fibroblasts grown in vitro from chick embryo explants. These cells, which were

flat and thin enough for most of the electron beam to pass through, were grown on glass coverslips covered with thin plastic film. After osmium tetroxide fixation the preparations were transferred to metal grids for examination in the electron microscope. The nucleus was visible but appeared blurred due to electron scattering caused by the thickness in the nuclear region. In the thinly spread periphery, however, the results were excellent and amongst the fine structure described were a lace-like reticulum (the first description of endoplasmic reticulum) and virus particles in chicken tumour cells. According to Palade (1971) this work marked the beginning of the electron microscope era in cell biology. Cellular ultrastructure has been exhaustively studied during the past thirty years and amongst the major landmarks in this history was the elucidation of the GERL system (Golgi, endoplasmic reticulum, lysosome) by Novikoff et al. (1964). Although aspects of cultured cell ultrastructure have been incidental to a number of studies and certain cellular systems have been thoroughly examined in isolation, there has been no attempt to present a comprehensive, unifying investigation of cultured cell fine structural morphology.

The present investigation is an attempt to fill some of the gaps in the present state of knowledge of normal and transformed cells by combining a morphological survey of important cell lines with a cytochemical examination of a number of hydrolytic enzymes in the hope of relating changes in enzyme activity to changes in ultrastructural organisation. Such information may, in turn, throw light on the role of enzyme and ultrastructural changes which occur when a normal cell in vivo becomes a cancer cell.

Chapter 2

CELL MORPHOLOGY

2.1. Cell lines studied

The cell lines utilised in this investigation have been widely used in research, especially in the field of cancer research. Two established normal cell lines and two established transformed lines derived from them have been investigated in addition to a primary normal line and its virus infected derivatives.

The baby hamster kidney line, BHK21C13, and its polyoma virus transformed derivative BHK21J1 were the first pair of established cell lines to be examined. BHK21C13 cells were originally described by Macpherson and Stoker (1962). The method of preparation of baby hamster kidney cultures was described by Stoker and Macpherson (1961) who subsequently outlined the origin of the cells, listed viruses to which they were susceptible and summarised the main characteristics of some derivatives (Stoker and Macpherson, 1964). BHK21 cells arose from cultures of kidneys from one day old Syrian hamsters. They are described as being elongated and growing in parallel orientation similar to fibroblasts. They have a mean doubling time of twelve hours in logarithmic growth and even after the cell sheet is confluent growth is maintained. If the cells are stored at $-70^{o}C$ or $-196^{o}C$ in medium containing glycerol or dimethyl sulphoxide they can be resuscitated with high viability after long periods. The karyotype of BHK21C13 cells is that of diploid male Syrian hamster cells. The cells have low transplantability in adult hamsters. Neoplastic transformation of BHK21 cells is rapidly produced by exposure to polyoma virus and after transformation the cells lose all trace of the infective virus. Compared with the original, normal cells, virus transformed cells such as BHK21J1 are randomly orientated in culture. They have a high

transplantability in adult hamsters and possess the other altered characteristics previously described (pp. 4, 5).

The 3T3 cell line, first described by Todaro and Green (1963), was derived from 17 to 19 day old Swiss mouse embryos. 3T3 cells are extremely sensitive to contact inhibition and do not grow after the attainment of a confluent monolayer. They are fibroblastic in sparse culture but grow considerably flatter. They are finely granular and difficult to trypsinise. In confluent cultures cell borders are obscured and a thin syncytium-like sheet forms with no tendency toward multilayering. The Simian virus 40 transformed derivative of 3T3 cells, SV40 3T3, was first described by Todaro et al. (1964). SV40 3T3 cells are spindle-shaped rather than epithelioid and lose susceptibility to contact inhibition, becoming able to grow to a high saturation density. They are able to synthesise collagen and display other characteristics of transformed cells.

All four of these cell lines were grown at 37°C without antibiotics in the Glasgow modification of Minimum Essential Medium supplemented with 10% calf serum and in an atmosphere of 10% carbon dioxide in air.

It is possible to produce a cell line which is temperature sensitive for transformation. This is achieved by infecting normal cells with temperature sensitive (ts) mutant virus. Such ts virus mutants will transform cells at a permissive temperature but if these cells are then shifted to the non-permissive temperature they will revert to the normal state because the viral genes for transformation are not expressed at the non-permissive temperature. Such a system is valuable since it means that the normal and transformed cell states can be investigated in the same cell culture merely by shifting from one temperature to another. This gives us an interesting control which

can be compared with results obtained from comparative studies of the established normal and transformed cell lines.

The cells to be infected with ts virus mutants were primary cultures of chick embryo fibroblasts which were derived from chick embryos removed from the egg on the tenth day of incubation. The cells were maintained in Ham's F10 medium supplemented with 10% tryptose phosphate broth and 5% calf serum and in an atmosphere of 5% carbon dioxide in air. Cells were infected with temperature sensitive mutant ts29 of the Prague strain of Rous sarcoma virus (PR-RSV) which induced transformation when cultures were maintained at the permissive temperature of 35°C. At the non-permissive temperature of 41°C, however, infected cells retained the characteristics of normal, uninfected cells. Uninfected cells and infected cells at the non-permissive temperature are elongated, typically fibroblastic and often grow in parallel orientation whilst the infected cells at the permissive temperature (the transformed cells) were rounded and refractile. Production of chick embryo fibroblast cultures and virus infection are described by Vogt (1969), the use of temperature sensitive virus mutants is described by Martin (1970) and the means by which they are isolated are described by Wyke (1973). RNA tumour viruses such as Rous sarcoma virus, unlike the DNA tumour viruses (e.g. polyoma or SV40), are not cytocidal so that in infected cells viruses can be produced and released into the medium while the cells continue to grow and proliferate.

2.2. Light microscopy

A light microscopic morphological study of cultured cells is relatively simple to perform. The cells are grown on glass coverslips and can be photographed alive or fixed using phase contrast microscopy. Alternatively they can be fixed, stained and then photographed through

transmission microscopy. In the present investigation cells were fixed and studied by phase contrast microscopy. Cells were grown on glass coverslips and on the attainment of a confluent state the culture medium was removed. The cells were given a brief rinse in cacodylate buffer (0.1M, pH 7.4) containing 4.5% sucrose and then fixed for 30 minutes in a 2.5% solution of cacodylate-buffered glutaraldehyde (Taab Laboratories) at room temperature. After fixation the cells were washed overnight in cacodylate buffer containing 4.5% sucrose and mounted in Farrant's medium on glass slides. Cells were examined and photographed through phase contrast optics on a Carl Zeiss Standard RA microscope fitted with photomicrography attachments.

BHK21 C13 (Fig. 1)

These were typical fibroblasts, being extremely elongated and growing in close-packed parallel orientation with little overlapping. Individual cells were generally spindle-shaped with two major cell processes which could be very long and very fine towards their terminal ends. The length of the cells was difficult to determine owing to much interdigitation but measurements of 100 µm were not unusual. Much of this length was, however, taken up by the fine extensions of the cell processes, the main body of a cell rarely exceeding 50 µm in length. Cells were widest at the nuclear region with a mean width of around 6 µm. Nuclei were ovoid, up to 12 µm long and 5 µm wide and had a maximum of four nucleoli. The cell surface manifested very short protruberances and the cytoplasm appeared uniform and granular. Cells which had rounded up for division were rarely observed.

BHK21 J1 (Fig. 2)

The appearance and manner of growth of these fibroblasts differed markedly from those of the normal (C13) cells. They were randomly

orientated and tended to pile up and overlap each other, especially in a crowded culture. The proportion of cells which were rounded up for division was noticeably greater than in the C13 culture. Some cells were spindle-shaped with two major cell processes whilst many more were rather angular or irregularly shaped with three or more major cell processes which often had very long, fine extensions. Cell length could be in excess of 100 μm whilst the width at the nuclear region varied from about 3 μm in some of the spindle-shaped cells to about 9 μm in the more irregular ones. Nuclei were rather smaller and more irregular than in the C13 cells and had two or three nucleoli. Short protruberances occurred on the cell surface and the cytoplasm was finely granular.

3T3 (Fig. 3)

3T3 cells in confluent culture were epithelioid in appearance. They were extremely thin, flat and roughly polygonal, sometimes with short protruberances emerging from the cell edges. There was little or no tendency toward overlapping. Size was variable, diameters of 60 μm not being unusual. Nuclei were large, circular or ovoid in outline and up to 16 μm long and 13 μm wide. There were up to thirteen nucleoli. The cytoplasm was very granular especially in the perinuclear region.

SV40 3T3 (Fig. 4)

These cells, unlike the 3T3, were generally fibroblastic although a few large flattened cells also occurred. Not being subject to density-dependent inhibition of growth and division, SV40 3T3 cells could overlap and pile up to form multilayered cultures. The fibroblastic cells were similar in shape to the transformed BHK21 cells but rather larger and often with more major cell processes. Cells approaching 200 μm in length were quite commonplace and the width at the nuclear region varied from about 5 μm up to 20 μm. Nuclei were variable

in size, usually circular or ovoid in outline, from 7 to 20 μm long and 6 to 12 μm wide. Some cells appeared to be binucleate, especially the large flattened ones. There was a maximum of seven nucleoli. The cytoplasm was granular and vacuolated, particularly in the perinuclear region.

Chick embryo fibroblasts (Fig. 5)

These cells, whether grown at 35°C or 41°C (or shifted from one temperature to the other), were typically fibroblastic, having spindle-shaped or angular cell bodies with two or more radiating major cell processes. In some parts of the culture the cells grew in parallel orientation whilst in other parts the growth was more random with some overlapping but with little tendency toward piling up. Many of the cells were elongated though rarely exceeding 100 μm in length. Width varied between 5 and 15 μm. Nuclei appeared circular or ovoid and were between 3 and 7 μm wide and 6 and 10 μm long. There were up to four nucleoli. The cytoplasm was finely granular and often vacuolated, some cells exhibiting a very high degree of vacuolation.

ts virus-infected chick embryo fibroblasts (Figs. 6, 7)

The appearance of cultures of chick embryo fibroblasts infected with the temperature sensitive mutant virus depended upon whether they were maintained at the permissive temperature of 35°C or the non-permissive temperature of 41°C. A shift from one temperature to the other resulted in the complete change of appearance after about 24 hours although within as little as 2 hours visible changes had started to occur.

Cultures maintained at 35°C (or set up at 41°C and then shifted to 35°C) had a distinctive appearance and contained cells of two different types. One type consisted of small dense fibroblasts between

3 and 10 μm wide and generally up to about 40 μm in length. Most had two or three major cell processes and short protruberances from the cell surface. The cytoplasm was often vacuolated and nuclei were small and indistinct. The other type of cell was small, dense, rounded and refractile with a diameter of between 5 and 10 μm. These rounded cells were often clumped together.

Cultures maintained at 41°C (or set up at 35°C and then shifted to 41°C)were indistinguishable from the uninfected cells.

2.3. Electron microscopy

Since cultured cells *in vitro* usually grow in layers only one cell, or perhaps a few cells, thick they cannot be prepared for electron microscopic examination with the same ease as a solid piece or section of tissue. The simplest solution to this problem is to detach the cells from their substrate and centrifuge them to a solid pellet which can then be handled like a piece of tissue. This approach was adopted by Soto and Castejon (1969) in a study of BHK21 C13 cells. A similar procedure was utilised in the present investigation to study this cell line.

2.3.1. Cells centrifuged to a pellet

Cells were grown on the bottom of a Petri dish and when they had reached confluence the culture medium was removed, the cells rinsed in phosphate-buffered saline (PBS) and then detached by scraping with a silicon rubber policeman. The cell suspension was transferred to a centrifuge tube and the cells spun at low g into a pellet. This was removed and fixed for 3 hours at room temperature in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4) followed by overnight washing at 4°C in the same buffer containing 4.5% sucrose. The pellet was then post-fixed for 2 hours in 1% osmium tetroxide in cacodylate buffer at

4^{o}C followed by dehydration through a graded ethanol series and infiltration and embedding in Araldite epoxy resin (Glauert, 1965) according to the following scheme:

30% ethanol	4^{o}C	20 mins. each stage.
50% "		
70% " containing 1% uranyl acetate		
90% " , 4^{o}C rising to room temperature (r.t.)		
100% "		
100% "		
Equal parts 100% ethanol and propylene oxide	r.t.	
Propylene oxide		
Equal parts propylene oxide and Araldite I (see below)		1 hour
Araldite I		16 hours
Araldite II (see below)		6 hours

Araldite I was prepared as follows:

Araldite A (epoxy resin)	5 ml
Araldite B (hardener)	5 ml
Araldite D (plasticiser)	0.25 ml

Araldite II was prepared by the addition of 0.2 ml of Araldite C (accelerator) to an Araldite I mixture, made up as above.

After 6 hours in Araldite II the pellet was cut into smaller pieces and each piece placed in a Taab embedding capsule which was then filled with fresh Araldite II. The capsules were transferred to a 60^{o}C oven and left for 48 hours by which time the Araldite had polymerised. The block was trimmed and silver ultrathin sections cut, using a glass knife, on an LKB Ultrotome III. These sections were mounted on Parlodion-coated copper grids and examined with an AEI EM 6B electron microscope operating at 60 kv and an objective aperture

of 50 µm.

Most cells were oval or pear-shaped although they were often more angular or bent when in very close contact with each other. This may have been due to being forced together during centrifugation. There were no extensive major cell processes such as those observed in the light microscope study. The mean size of the cells was 13 µm long and 8 µm wide. Extracellular debris was widespread and most of it appeared to be disrupted cellular material. A fuzzy material could be seen on or near the surface of some cells (Figs. 9, 10) and perhaps represented part of a cell coat. Some cells had smooth surfaces, especially those which were in close contact with other cells (Fig. 8), whilst others had a number of small processes or microvilli up to 1 µm in length on their surfaces (Fig. 9). Soto and Castejon (1969) studied typsinised cells and found them to be smooth surfaced but Franks and Wilson (1970) indicated that cells prepared in this way tended to appear smoother surfaced than cells prepared by scraping. Specialised cell junctions, such as tight and gap junctions (Pinto da Silva and Gilula, 1972), were not positively identified but possible examples were occasionally seen (Fig. 10).

Nuclei were ovoid, pear-shaped, kidney-shaped or very irregular (Figs.9, 12) with a mean size of 7 µm long and 4 µm wide. A layer of heterochromatin of varying thickness lined the inner nuclear membrane whilst the main part of the nucleus contained euchromatin except where small patches of condensed chromatin and one or two nucleoli occurred. Light patches in the lining heterochromatin probably indicated the sites of nuclear pores (Figs. 11, 12). The outer nuclear membrane, sometimes uniformly parallel to the inner membrane and sometimes irregular, had ribosomes on its outer surface. There was

evidence of endocytosis at the cell surface. Micropinocytic vesicles of about 0.1 μm in diameter were present (Fig. 10) as also were larger pinocytic vesicles (Fig. 9). An example of the formation of a pinocytic vesicle by coalescence of surface folds was also observed (Fig. 9).

The cytoplasm contained numerous ribosomes which were for the most part aggregated into rosettes. Ribosomes were also found on the surface of the rough endoplasmic reticulum (ER), the cisternae of which were markedly distended. This rough ER was present throughout the cytoplasm although no very extended profiles were seen. Skeletal elements, in the form of very fine, unbranched microfilaments, occurred throughout the cytoplasm but were particularly noticeable in the vicinity of the Golgi complex (Figs. 11, 13). A few microtubules, also unbranched, were observed (Fig. 12). Golgi complexes were not well developed and, like the ER, their cisternae, vesicles and vacuoles were swollen.

Components of the lysosomal system were in evidence in virtually all the cells. Secondary lysosomes were most obvious and usually circular in profile with diameters of between 0.5 μm and 1.0 μm (Figs. 10, 11). Some of these were autolysosomes since they contained identifiable fragments of cellular material in varying degrees of breakdown by lysosomal hydrolytic enzymes (Fig. 14). Certain of the autolysosomes exhibited sections of double membrane around their perimeters. Many of the small, single-membrane-bound bodies found throughout the cytoplasm, but particularly in the region of the Golgi complex, were probably primary lysosomes (Fig. 13). Identification was difficult on purely morphological grounds but could be confirmed by demonstrating the presence of acid phosphatase (Fig. 65). This

enzyme cytochemical test also endorsed the identification of the other categories of lysosomes (see for example Figs. 65, 66). A possible example of sequestration by membrane formation around an area of cytoplasm containing several organelles was observed (Fig. 13). This process would lead to the production of an autophagosome (or cytosegresome), a pre-lysosomal stage which becomes a true lysosome when it receives its complement of hydrolytic enzymes.

Mitochondria were small with a dense, granular matrix and were of two main types. Some had a circular profile and a diameter of between 0.2 μm and 0.5 μm whilst others were more extended, having a length of between 1 μm and 1.5 μm and a width of about 0.2 μm. Cristae ran transversely or longitudinally through the matrix, sometimes crossing it completely and joining opposite sides of the mitochondrial profile. Also present in the cytoplasm were numerous vesicles, usually with a circular profile and a diameter of between 0.05 μm and 1.5 μm. Most were empty but several contained small amounts of amorphous material. Their origin was uncertain.

It was clear from the results of this ultrastructural examination of a pellet of cells that this method of preparing cultured cells for electron microscopic investigation was unsatisfactory since spatial relationships between cells were altered or destroyed, cell shape was distorted and organelles were disturbed. It is also conceivable that enzyme distribution and activity could be affected. Some improvement is possible if cells are fixed before they are scraped from the culture dish. Fixed cells tend to stick together so that spatial relationships are retained to a greater extent than is possible with unfixed cells. This method has been advocated by Goldman (1972) who fixed cells in 1% glutaraldehyde, post-fixed in 1% osmium tetroxide and scraped them

from the plastic Petri-dish during ethanol dehydration. They were then centrifuged at 600 rpm and the pellet embedded in Epon. Cells treated in this manner appeared to be identical in morphology to cells which had been flat embedded. A very similar scheme was followed by Kisch et al. (1973). When a similar method was employed during the present investigation it was found that, although individual cells retained undisturbed morphology, there was still some distortion of cell shape and spatial relationships were not preserved to any marked degree (Fig. 90). To ensure that cell structure and spatial relationships are not affected by the preparative procedures for electron microscopy, a technique for in situ embedding of cells, whereby they do not need to be removed from their substrate, is required.

2.3.2. In situ embedded cells

A number of in situ embedding techniques have been developed. The earliest methods utilised cells grown on glass coverslips. Howatson and Almeida (1958) embedded cells in methacrylate whilst Gorycki (1966) used epoxy resin. Detachment of the glass from methacrylate was facilitated by cooling with solid CO_2 but it was found that separation of glass from epoxy resins was both difficult and unsatisfactory. To assist coverslip detachment, materials less adherent to epoxy resin than glass, such as silicone (Rosen, 1962), collagen gel (Heyner, 1963), carbon (Robbins and Gonotas, 1964), gelatin (Speirs and Turner, 1966) or Teflon (Chang, 1971) have been coated onto the glass coverslips prior to culturing. Many of the methods of flat embedding on glass have been reviewed by Flickinger, (1966).

Another approach, which obviated the problem of detachment, was to grow cells on slips of polymerised embedding resin so that, after processing, the cells were left embedded in a resin "sandwich". Such

sandwich-embedding methods have utilised methacrylate (Kjellen et al., 1955), Epon (Egeberg, 1965), Araldite (Smith et al., 1969) and Spurr (Sabbath et al., 1973). A variant of the sandwich-embedding method involved growing the cells on a material which was amenable to ultrathin sectioning. McCombs et al. (1968) and Dalen and Nevalainen (1968) employed Millipore filters whilst Cornell (1969) used nucleopore polycarbonate filters. Nelson and Flaxman (1972) and Douglas and Elser (1972) used conventional plastic culture vessels, Papadimitriou (1972) used cellophane and Richters and Valentin (1973) grew their cells on polystyrene cover slips. All of these sandwich-embedding procedures are most useful when vertical sections of cells are required, especially when studies are being made of the cell-substratum interface.

A different approach was developed by Flaxman et al. (1968). They coated coverslips with polymerised nitrocellulose on which the cells grew readily. During fixation the nitrocellulose and cells were floated off the glass. After dehydration the preparation was transferred to propylene oxide which dissolved the nitrocellulose. The cells remained undisturbed and were embedded in Epon.

Yet another technique involved growing cells on a material which separated readily from epoxy resin after flat-face embedding. Yardley and Brown (1965) used coverslips made of mica whilst Anderson and Doane (1967) grew cells in vinyl cups which were easily stripped from the polymerised epoxy block. Brinckley et al. (1967) and Chang (1972) found that the polystyrene of Falcon T-30 flasks (routinely used for growing cells) could be easily separated from Epon. Firket (1966) and Sykes and Basrur (1971) found that cells grew well on Melinex, a transparent polyester plastic film, and that this material separated extremely easily from epoxy resin.

After an initial trial of Araldite embedding of cells grown on

glass and polystyrene, detachment of the resin block from the glass or polystyrene proving very difficult, the use of Melinex was adopted for the present investigation. Some workers (see for example Smith et al., 1969 or Chang, 1971) regarded Melinex as unsuitable owing to its flexibility and tendency to float in the culture medium. They also found that the hydrophobic surface of the plastic made cell attachment difficult. However, these problems have not been encountered and Melinex has successfully fulfilled all the following requirements, listed by Firket (1966), of a substitute for glass: it should (a) be easily sterilised, (b) provide an adequate support for cells and have no deleterious effect on the culture, (c) be transparent for good observation by light microscopy, (d) be unattacked by all processing fluids and (e) be easily separated from the polymerised resin.

Melinex of Type O, 100 μm thick, was obtained from Boyden Data Papers, Parkhouse Street, London SE17 in the form of a roll of width 9.5 cm. Pieces, approximately 20 mm square, were cut from the roll and placed in carbon tetrachloride where they were stirred for 10 minutes. After removal from the carbon tetrachloride they were dried in air and finally sterilised by autoclaving. The clean, sterile Melinex slips were then placed on the bottom of tissue culture Petri-dishes (Nunc Ltd.) prior to the addition of cell suspension and culture medium. Culture conditions were as previously described. It was found that when culturing chick embryo fibroblasts the cells grew satisfactorily on Melinex that had simply been rinsed in alcohol. If a number of Melinex slips were placed in the same Petri-dish they were often held on the bottom of the dish by very small pieces of sterile, waterproof adhesive tape.

When cells had grown to the required density the Melinex slips

were removed from the dish, rinsed in buffer and fixed for 30 minutes in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4). They were then washed overnight in buffer, post-fixed in 1% osmium tetroxide for 1 hour, dehydrated through a graded ethanol series, with uranyl acetate staining in 70% ethanol, and infiltrated with Araldite. After the preparations had been in Araldite II for 6 hours, small round polythene pots of 14 mm internal diameter and 8 mm depth were filled with fresh Araldite II. The Melinex slips were then placed cells-down onto the Araldite in the pots, after first wiping off excess Araldite from the clear side of the Melinex slips. Polymerisation of the Araldite was achieved by transferring the pots to a 60^{o}C oven where they were left for 48 hours. After this time the Melinex was stripped from the surface of the block and the polythene pot removed from around it. If the surface of the block was examined through the light microscope the embedded cells could be studied and areas selected for sectioning. The block was then cut into segments, usually eight, with a small hacksaw. After trimming, the desired cells were sectioned parallel to the surface of the block, mounted on grids, stained with lead citrate and examined in the electron microscope.

BHK21 C13

The appearance of these cells was very different from that of similar cells which had been spun to a pellet (compare Figs. 8 and 15). They were very elongated and spindle-shaped, being widest around the nuclear region where the mean width was 6 µm. Sections were cut from confluent monolayer regions where the cells were largely unbranched and lay in close, parallel arrangements with frequent interdigitations (Fig. 15). Apart from an occasional microvillus and small process cells appeared smooth-surfaced, especially where they lay closest to

each other (Figs. 15, 16). The intercellular distance between such close-packed cells was 0.02-0.05 μm. Zones of intercellular contact or specialised junctions occurred but it was difficult to classify them as gap or tight junctions (Figs. 18, 19). Also observed was a bridge-like connection made by two entwined microvilli (Fig. 20). Some amorphous material was found between the cells and occasionally evidence of a cell coat (Figs. 17, 21).

Nuclei were ovoid with few irregularities and a mean size of 11 μm long and 4.5 μm wide. A layer of heterochromatin of varying thickness lined the inner nuclear membrane and euchromatin formed the major part of the nucleus. Up to three nucleoli and small patches of condensed chromatin were also present. The outer nuclear membrane, parallel to the inner at a distance of approximately 0.03 μm, had ribosomes on its outer surface. There was evidence of continuity between nuclear envelope and rough ER (Fig. 16) and nuclear pores were common.

At the cell surface endocytosis was evident in the form of micropinocytic coated vesicles (Figs. 18, 21) and smooth micropinocytic vesicles (Figs. 16, 18). Larger vesicles in the cytoplasm (Figs. 15, 16) were probably of pinocytic origin. The cytoplasm was rich in ribosomes, both free and grouped into rosettes, in addition to those which were found on the surface of the rough ER. Elements of the rough ER had a diameter of approximately 0.08 μm and were represented by short lengths throughout the cytoplasm. They were sometimes in close association with mitochondria (Figs. 18, 19) and in the cell processes mainly followed the long axis.

Skeletal elements were quite prominent. Filaments were usually orientated along the main axis of the cell and found throughout the

cytoplasm, often aggregated into bundles near the surface or along the centre of a cell process (Figs. 18, 21). Microtubules, less frequent than the filaments, followed similar paths (Fig. 18) or occurred separately (Figs. 21, 22). Microfilaments were abundant and found generally throughout the cytoplasm, in bundles below the cell surface (Fig. 18) or in dense masses around the Golgi/centriolar region (Figs. 17, 20). Unlike the filaments, they did not appear to have any particular orientation. None of these skeletal elements exhibited branching. Golgi complexes were not particularly extensive (Figs. 17, 22). They consisted of small stacks of cisternae, the ends of which were expanded into rounded profiles. Small vesicles of Golgi origin were seen in the cytoplasm adjacent to the stacks of cisternae and also farther away, often in close proximity to much larger vesicles and lysosomes (Figs. 17, 21). A few Golgi vacuoles were present and in certain cells (Figs. 17, 22) a pair of centrioles was present in the Golgi region. One centriole was seen to serve as the basal body of a cilium which penetrated the cytoplasm of a neighbouring cell (Fig. 22).

Lysosomes were common. Secondary lysosomes were most obvious, usually being circular in profile and up to 1.1 μm in diameter although some were ovoid or irregular. There was evidence of cytoplasmic material in certain of them (Fig. 22), indicating that a process of autophagy was in progress. Some autolysosomes (or autophagosomes) exhibited traces of double membrane around them (Fig. 22). Other lysosomes were of the multivesicular body type (Figs. 17, 20) and these contained a number of small vesicles. Similar vesicles were present in the surrounding cytoplasm. Primary lysosomes were synonymous with small dense Golgi vesicles.

Mitochondria were common and variable in shape. Some had small

circular profiles of about 0.25 μm in diameter and some were large ovoid structures, 1.6 μm longand 1.1 μm wide. Others were long and thin, being 0.2 μm wide and up to about 3 μm in length. The mitochondrial matrix was dense and granular. Cristae were quite well developed and arranged transversely, some of them appearing completely septate. Open spaces in the cytoplasm were seen in many cells (Figs. 15, 16) and were up to 2 μm across. They were of varying shape, not membrane bound and of uncertain function. It is possible, though doubtful, that these spaces were fixation artifacts.

BHK21 J1.

The shape and orientation of these cells were different from those of the C13 cells, although at the electron microscope level these differences were not as pronounced as they were at the level of the light microscope. The size and shape of J1 cells were variable, some being rounded, some ovoid and others more angular or spindle-shaped. The mean width at the nuclear region was greater than that of C13 cells, about 7.5 μm.

The cell surface, unlike that of the normal cells, had many cytoplasmic extensions and microvilli up to 1 μm in length (Figs. 25, 26). The ends of some major cell processes had a number of finger-like microvilli which appeared to probe the environment immediately in front of them (Fig. 25). Specialised cell junctions were scarce but intercellular contact seemed to be made, in certain areas, by complexes between microvilli of neighbouring cells (Fig. 27). Extracellular material was observed and often appeared to be of cellular origin but evidence of a cell coat was only rarely seen (Fig. 31).

Nuclei were generally smaller than those of the normal cells and their shapes were variable, some being very irregular (Fig. 28). As the

transformed cultures were not subject to density-dependent inhibition of division there was a likelihood of sectioning cells in mitosis. Several such cells were observed and one exhibited chromosomes arranged in a circular configuration (Fig. 30). Interphase nuclei had a thin layer of condensed heterochromatin lining the inner nuclear membrane and this layer was interrupted opposite the nuclear pores. Nucleoli, up to three in number, were very granular with dense patches (Figs. 23, 24). The euchromatin which formed the bulk of the nucleus occasionally contained bodies of an uncertain nature (Figs. 26, 28). The nuclear envelope was, like that of the normal cells, about 0.3 μm wide and had ribosomes on its surface.

Endocytosis at the cell surface was represented mainly by the formation of micropinocytic coated vesicles (Figs. 29, 32). Smooth micropinocytic vesicles and larger pinocytic vesicles were scarce. Possible examples of phagocytosis were observed in some cells (Fig. 31) and exocytosis was in evidence with residual bodies or telolysosomes being egested or defaecated from the cell (Fig. 28). The apparent fusion of micropinocytic coated vesicles with larger bodies was occasionally seen (Fig. 29). The cytoplasm was rich in ribosomes which were mainly grouped into rosettes. Ribosomes were also present on the outer surface of the rough ER which in a number of cells was extensive and represented by short lengths throughout the cytoplasm or by extended sections with frequent branching and expansions of the cisternae (Figs. 27, 33), the interior of which contained a flocculent, opaque material. The unexpanded rough ER elements had a diameter of 0.08 - 0.1 μm. The tendency, observed in the C13 cells, for ER to be closely associated with mitochondria was more marked in the transformed cells. Some mitochondria were almost surrounded by a complete loop of ER or by a

number of short sections (Fig. 23). At certain sites (Figs. 27, 33) mitochondria which were closely surrounded by ER appeared to be undergoing a process of breakdown. Mitochondria were more plentiful than in the normal cells and were of a variety of shapes and sizes. There were rounded ones from 0.3 - 1.0 μm in diameter and similar sized ovoid ones in addition to some that were long and thin, 0.3 - 0.4 μm wide and up to 3.5 μm long. Cristae were well developed and mainly transverse, some being completely septate, although a few were longitudinal, diagonal or even, in the case of certain round mitochondria, radial. The matrix was granular or flocculent.

Skeletal elements were similar to those of the C13 cells. Filaments ran parallel to the long axes of cell processes, often in dense bundles just below the surface (Figs. 33, 34), whilst microtubules were prominent in the middle of the mitotic cell (Fig. 35), presumably in the role of spindle fibres. Microfilaments occurred randomly throughout the cytoplasm and in dense masses in the Golgi region (Figs. 27, 29). All three types were unbranched. Golgi complexes were more extensive than those of the normal cells. Centrioles were found in the Golgi region (Fig. 36) and in the centre of the circle of chromosomes in the mitotic cell (Fig. 35).

Lysosomes were numerous and usually rounded or ovoid in profile, the largest being 1.7 μm long and 1.2 μm wide. The small, dense vesicles which were particularly prominent in the Golgi zone were identified as primary lysosomes. Secondary lysosomes often contained identifiable cellular components (Figs. 26, 34), suggesting that they were autolysosomes and certain of these (Figs. 31, 34) displayed sections of double membrane. Multivesicular bodies were also in evidence (Figs. 27, 31). A large proportion of the lysosomes of some cells were residual bodies

or telolysosomes which contained myelin figures, dense material and amorphous fragments and were often less regular in outline than the secondary lysosomes. Residual bodies could sometimes be seen to fuse with each other (Fig. 26) and some were exocytosed from the cell (Fig. 28).

Clear spaces in the cytoplasm were not infrequent and were of various shapes and sizes and were not membrane-bound, although some were associated with membranous material (Figs. 33, 34). Some contained condensed masses of cytoplasm (Fig. 32). There were also regular, opaque regions in some cells (Figs. 29, 32) and these were also not membrane-bound.

3T3

3T3 cells were epithelioid and angular and wider in the nuclear region than the fibroblastic BHK cells but not usually as long, major cell processes being rare. The cell surface had many small convolutions and folds and there were numerous microvilli and short cytoplasmic extensions. Although some microvilli appeared to make contact with neighbouring cells (Fig. 37) specialised cell junctions were rare and not easily identified (Fig. 38). Membranous and fibrous extracellular material was present but evidence of a cell coat was only rarely observed (Fig. 38).

Nuclei were quite large and ovoid, up to 12.5 μm long and 9 μm wide, with occasional small indentations of the surface (Fig. 37). A thin layer of heterochromatin lined the inner nuclear membrane whilst the bulk of the nucleus was taken up by euchromatin in which were found small patches of dense chromatin and up to seven nucleoli. Areas of pars amorpha and pars granulosa could be distinguished in the nucleoli (Fig. 37). Cytoplasmic invaginations into the nucleus were occasionally

observed (Fig. 38). Nuclear pores were commonly seen and had a diameter of 0.06 μm. The distance between the two membranes of the nuclear envelope was 0.02 - 0.03 μm, the outer membrane having ribosomes on its surface.

Cells were engaged in active endocytosis in the form of pinocytosis and micropinocytosis. Pinocytosis was achieved by coalescence of surface folds or microvilli (Fig. 37) whilst micropinocytosis consisted of the formation of vesicles of the coated (Figs. 38, 40) or smooth (Fig. 39) variety. All three types of endocytic vesicle were found deeper in the cytoplasm, having migrated from the surface. The cytoplasm was densely populated with ribosomes, a large proportion of which were grouped into rosette-like arrangements. Ribosomes were also found on the outer surface of the nuclear envelope and on the surface of the rough ER which extended throughout the cytoplasm in the form of short, undulating profiles between 0.04 μm and 0.06 μm in width and up to 2 μm in length. Some of the rough ER elements were branched and some were slightly expanded, the cisternae containing a very fine, flocculent, opaque material. Rough ER and mitochondria were often in close association and it was noticeable in certain cases that where the two components came closest together the ER was devoid of ribosomeson its surface (Figs. 38, 41). In addition to the rough ER there was also a system of smooth ER and at a number of sites the two systems were seen to be connected (Figs. 41, 43). The smooth ER was particularly prominent in and around the Golgi region (Figs. 41-44) and its elements varied from extended branching profiles up to 1.4 μm in length and 0.05-0.075 μm wide to small, almost circular profiles of about 0.05 μm in diameter. They presented a rather irregular appearance and had a very fine, granular, opaque matrix which was denser than that of the rough ER.

Some elements were distended into quite large sac-like profiles up to 0.6 μm in diameter containing less dense flocculent material (Figs. 42, 43). In Golgi regions where the smooth ER was closely involved it was sometimes difficult to distinguish between certain elements of these two organelles (Figs. 42-44).

Golgi complexes were extensive, perinuclear and multicentric and the most conspicuous components were the characteristic stacks of three to seven saccules or cisternae, either flat or curved. These saccules were of varying length, up to 1.4 μm, and width, up to 0.05 μm, and were separated by a distance of about 0.02 μm. Material of variable density was found within the saccules, around the ends of which and also in the surrounding cytoplasm were large numbers of small, fairly dense vesicles up to 0.06 μm in diameter. A large flattened saccule was observed (Fig. 42) which had dense vesicles forming and being released from its convoluted surface. This saccule was either a Golgi or smooth ER component. Golgi vacuoles appeared like irregularly distended saccules. Centrioles were also found in the Golgi region (Fig. 42).

A great many microtubules, filaments and microfilaments occurred in the Golgi region (Figs. 42, 43). These skeletal elements appeared to have no preferred orientation and frequently crossed each other and also other organelles. They were not confined to the Golgi region and all three types were found at other sites. Filaments and microfilaments were prominent near the cell surface (Figs. 38-40). Microtubules were straight or slightly curved whilst filaments were variable, some being serpentine (Fig. 40). Structures which were probably connective tissue fibres were occasionally observed (Fig. 42). These were 0.5 μm long and 0.035 μm wide with regular transverse striations at intervals of 0.07 μm.

Mitochondria were common and small with a dense granular matrix.

Some were rounded with a diameter of 0.25 μm whilst others were slightly larger and ovoid. Longer mitochondria were rare but some, up to 1.3 μm in length, did occur and had a width of 0.1 - 0.3 μm. Cristae were transverse or longitudinal.

Lysosomes were abundant and probably the most conspicuous feature of the 3T3 cells, apart from nuclei. Primary lysosomes were most prominent in and around the Golgi region and several were apparently in the process of fusing with larger bodies (Figs. 42, 43). Secondary lysosomes, up to 0.75 μm in diameter, were of several types. There were autolysosomes containing identifiable cytoplasmic material (Figs. 38, 40), multivesicular bodies (Figs. 43, 44) and others with a light, granular, largely homogeneous content (Figs. 39, 41). "Signet-ring-like" bodies were seen (Figs. 42, 43) which may have been secondary lysosomes or pre-lysosomal stages containing recently sequestered material. Also observed was a large body, 0.9 μm in diameter, which appeared to represent a late stage in the sequestration of a portion of cytoplasm (Fig. 44). The contents were not degraded, suggesting that enzyme activity was not present. By far the most obvious members of the lysosomal system were the numerous residual bodies or telo-lysosomes which varied in size from small rounded bodies, 0.2 μm in diameter, to large structures with a diameter of 3 μm. Shapes varied and though some were irregular, most were rounded or ovoid. They were very dense and heterogeneous, many containing myelin figures and dark particulate matter. There was no evidence of exocytosis of residual bodies.

Lipid droplets occurred in most cells and were rounded with a diameter of up to 1.2 μm. They were uniform and opaque, although some had a darker perimeter, and were found singly or in clusters of up to

twelve droplets. Clear spaces in the cytoplasm were also encountered. These were up to 2 μm across and were not membrane-bound. Some of them had organelles intruding into their interior (Fig. 37).

SV40 3T3

The differences in size, shape and manner of growth between normal and transformed 3T3 cells were not as marked at the electron microscope level as they were at the light level.

The cell surface had smooth sections, especially where cells were in close alignment to each other (Figs. 47-49), sections with microvilli and sections with folds and convolutions (Figs. 45, 46). Microvilli occasionally appeared to form contacts between cells (Fig. 49) and specialised tight junctions were made between cells in a number of places (Figs. 45, 47). There was a certain amount of extracellular material, often fibrous or membranous (Figs. 45-47), and evidence of a cell coat at several sites (Figs. 47, 51).

Most nuclei were smaller than those of 3T3 cells although some were large. Shape was varied with some smooth-outlined ovoid, kidney-shaped or approximately square ones and others which were irregular with indentations of the surface (Figs. 45-47). Those which in section were ring-shaped probably appeared thus as a result of having large indentations of their upper or lower surfaces. A layer of hetero-chromatin of varying thickness lined the inner nuclear membrane which was separated from its outer partner by a space of up to 0.06 μm. The nuclear envelope was often uneven (Fig. 48) and was seen to be in continuity with the rough ER (Fig. 51). The outer nuclear membrane had ribosomes on its surface. Euchromatin formedthe bulk of the nucleus and amongst it were found patches of condensed chromatin and the nucleoli which appeared to be as numerous as those of the 3T3 cells.

Zones of pars amorpha and pars granulosa were distinguished within the nucleoli. Nuclear pores were frequent and had a diameter of 0.05-0.06 μm.

Endocytosis was in evidence at the cell surface. Coalescence of surface folds and microvilli was responsible for the formation of pinocytic vesicles (Figs. 45-47) whilst micropinocytosis was brought about by the formation of smooth (Figs. 48, 51) and coated (Figs. 49, 51) micropinocytic vesicles. The cytoplasm contained abundant ribosomes which occurred free or clustered into rosette formations in addition to being present on the surfaces of the nuclear membrane and the rough ER. The elements of the rough ER were present throughout the cytoplasm and were straight or undulating and occasionally branched. The cisternae, which contained an opaque granular material, were up to 0.08 μm wide but usually narrower. The longest section observed measured 3.5 μm. When in close association with mitochondria, the rough ER was sometimes devoid of its surface ribosomes (Fig. 48). A system of smooth ER was also present, largely in the Golgi region, but was not as extensive as that of 3T3 cells.

Golgi complexes were not as extensive as those of the normal cells although they were in most respects similar. In one cell a cilium arose in the Golgi region (Fig. 50), a centriole acting as its basal body. All three types of skeletal elements occurred in the SV40 3T3 cells but they were not as prominent as in the normal cells. Microtubules ran longitudinally along cell processes (Fig. 49), near the periphery of other parts of the cell (Fig. 51) or within the Golgi complex (Fig. 50). Filaments accompanied the microtubules (Fig. 51) or occurred separately (Figs. 49, 51) whilst microfilaments were found throughout the cytoplasm.

Mitochondria were more numerous in the transformed cells than they were in the normal. Their matrix was much less dense although it still appeared finely granular. The smallest mitochondria were rounded with a diameter of 0.25 μm whilst the larger ovoid mitochondria, which formed the bulk of those present, measured up to 1.2 μm long and 0.7 μm wide. In addition there were elongated mitochondria up to 2.2 μm long and 0.4 μm wide.Cristae were transverse, longitudinal, diagonal or even circular (Fig. 48) and in some mitochondria, dense bodies occurred (Fig. 48).

Lysosomes were not such a dominant feature in SV40 3T3 cells as they were in normal cells, owing to the scarcity of residual bodies. The residual bodies which did occur (Fig. 51) were irregular in shape and tended to be large, up to 2.4 μm in diameter. Their contents were mainly dense, consisting of myelin figures, very dark granular material, membranous fragments and amorphous matter with occasional opaque patches. Primary lysosomes were most obvious in and around the Golgi region but could also be identified at other sites (Figs. 49, 51). Secondary lysosomes were mainly rounded bodies up to 0.75 μm in diameter and were multivesicular bodies (Figs. 47, 51), autolysosomes (Fig. 47) or dense bodies (Figs. 47, 48). "Signet-ring-like" bodies (Figs. 49, 51) were either young secondary lysosomes or pre-lysosomal autophagosomes (cytosegresomes).

Lipid droplets, rounded and 0.2-1.2 μm in diameter with a homogeneous, opaque matrix, occurred in most cells. The smaller droplets were often found in clusters of over twenty (Fig. 50) whilst the larger ones remained isolated or formed small groups. Clear spaces in the cytoplasm, of the type previously described (p. 28) were not present in the transformed cells. However, one cell (Fig. 45) had a large

circular vacuole, 6.5 μm in diameter. This vacuole was membrane-bound for about two thirdsof its circumference and contained a small amount of cytoplasm and a number of pieces of membranous material.

Chick embryo fibroblasts

During the preparation of these cultures for electron microscopy the cells were dehydrated by taking them through a water-soluble Durcupan series (Staubli, 1963) rather than through a graded series of ethanols. Infiltration with Araldite was by means of Durcupan/Araldite mixtures (Staubli, 1963). All procedures were carried out at room temperature. The scheme, after post-fixation in osmium tetroxide, was as follows:

Distilled water	30 minutes each stage
70% water : 30% Durcupan	
50% " : 50% "	
30% " : 70% "	
100% Durcupan	
70% Durcupan : 30% Araldite I	
50% Durcupan : 50% "	
30% " : 70% "	
100% Araldite I	16 hours
Araldite II	6 hours

Embedding in fresh Araldite II was as previously described (p. 25).

Three categories of cells were examined viz uninfected at 35°C, ts 29 infected at 35°C and ts 29 infected at 41°C.

Uninfected chick embryo fibroblasts at 35°C.

Sections were taken from a confluent monolayer culture and the cells were broad but spindle-shaped, often with long major cell processes. Cell surfaces were mainly smooth with some undulations, short cytoplasmic

extensions and microvilli. At some sites cell connections appeared to be made by microvilli (Figs. 52, 54) whilst specialised tight junctions were also observed (Figs. 53, 43). Extracellular material of a fibrous or membranous nature occurred (Figs. 52, 54) in addition to exocytosed material (Fig. 54). There was no obvious cell coat.

Most nuclei were rounded or ovoid, up to 10 μm long and 7 μm wide, although some were more irregular (Fig. 52). There was no lining of heterochromatin and only a few patches of condensed chromatin were present in the euchromatin along with the one or two nucleoli, in some of which could be distinguished nucleonemata (Fig. 53). Nuclear pores were not easily identified. The nuclear envelope was 0.02 - 0.03 μm wide and had ribosomes on its outer surface.

Endocytosis did not appear to be particularly prevalent in these cells. Pinocytosis by coalescence of surface folds or microvilli occurred (Fig. 53) but micropinocytosis was rare, few vesicles being observed (Fig. 53). The cytoplasm had a dense ribosome population with less frequent incidence of the rosette arrangement of ribosomes. Some quite large areas of cytoplasm were devoid of all organelles except for ribosomes (Fig. 54). Rough ER was found throughout most of the cytoplasm and was represented by narrow profiles, 0.04–0.06 μm wide and up to about 4 μm in length, or profiles which were branched and expanded with cisternae up to 0.5 μm wide. These expanded cisternae contained an opaque matrix. Rough ER was often associated with mitochondria and secondary lysosomes. Smooth ER was only identified in the Golgi region of one cell (Fig. 55). This Golgi region was neither extensive nor well developed, the profiles of the saccules being difficult to distinguish from the elements of the smooth ER. Golgi vesicles were abundant in and around this region and a few Golgi vacuoles, ovoid and

up to 0.4 μm in diameter, were also observed. Centrioles were present in the Golgi region.

Filaments were found in bundles below the cell surface in certain cell processes (Fig. 54) and also in the cytoplasm near the Golgi region (Fig. 55) where microfilaments were most noticeable. Mitochondria were numerous, the smallest having a rounded profile, 0.25 μm in diameter, whilst the largest was 6 μm long with a width varying between 0.2 and 0.4 μm. A high proportion of the mitochondria were elongated. Some were serpentine and others were branched (Fig. 53). Cristae were transverse, longitudinal or diagonal. A feature common to many of the mitochondria were the dense bodies or granules, up to 0.02 μm in diameter, found in the matrix. These mitochondrial dense bodies are preserved when Durcupan dehydration is utilised but are not seen when cells are dehydrated through ethanol.

The uninfected chick embryo fibroblasts had an extensive lysosomal system. Primary lysosomes were most abundant in and around the Golgi region. Secondary lysosomes and residual bodies (telolysosomes) were numerous and conspicuous. In addition probable autophagosomes (cytosegresomes) were identified. Secondary lysosomes were of two main types, no obvious multivesicular bodies being seen. Both types had rounded or ovoid profiles up to 2.5 μm in diameter. The first type had a homogeneous, light, finely granular, opaque matrix and often displayed the so-called "halo" which Daems et al. (1969) claimed was a criterion in the identification of lysosomes (Figs. 53, 43). There were occasionally small clear spaces in the matrix (Fig. 54). The second type, the autolysosomes, contained endogenous cytoplasmic material (cytoplasmic ground substance, ribosomes, mitochondria etc.) in varying degrees of breakdown (Figs. 52, 53). This classification is complicated by the

fact that in many cases there was fusion of the two types to produce hybrids (Figs. 52-54). There was also fusion of like species (Fig. 54). Residual bodies contained myelin figures and dense amorphous material and were often very large owing to fusion of several bodies. One large residual body (Fig. 54) was 6 μm long and 2.5 μm wide and appeared to have been formed by fusion of at least four smaller residual bodies. The presence of myelin figures outside the cell (Fig. 54) indicated that residual bodies might be exocytosed (defaecated) from the cell. Several probable autophagosomes were observed (Fig. 55) which had two membranes around them for all or part of their circumference. One of them was connected to a short length (0.2 μm) of a tubular structure, possibly smooth ER or a Golgi element. Structures which were interpreted as autophagosomes in the process of formation were also seen. Golgi cisternae and/or smooth ER appeared to be surrounding portions of cytoplasm to create bodies with a double membrane and a content of cytoplasmic material.

Lipid droplets occurred in certain cells (Fig. 52) and were usually clustered together. They measured up to 2.5 μm in diameter and had a discontinuous dark edge. Some were in the process of fusing with other droplets.

ts 29 infected chick embryo fibroblasts.

(1) Cells maintained at 35°C

There were a number of differences in ultrastructural organisation between these cells and the uninfected normal cells but the marked changes in cell size, shape and orientation which were observed at the light microscope level were not as conspicuous at the level of the electron microscope.

Microvilli and surface extensions were much more numerous and cell

junctions were more commonly observed (Figs. 56, 57, 82). Masses of small vacuoles which formed in the cytoplasm were expelled at the cell surface and gave the appearance of clusters of bubbles (Fig. 120). Rough ER was found throughout the cytoplasm but the expanded cisternae which were so prevalent in the uninfected cells were very rare. The Golgi complex was better differentiated (Figs. 85, 120, 122) but mitochondria were fewer with far less elongated individuals. Autolysosomes and residual bodies were much less numerous (Figs. 56, 57). Virus particles were present and were 0.04 - 0.08 μm in diameter. They were dark with a dense core and were found both outside the cells and inside, where they were confined to vesicles (Figs. 79-84).

(2) Cells maintained at 41°C

It was observed at the light microscope level that when infected cells were shifted from 35°C to the non-permissive temperature of 41°C and maintained there, they reverted to the size, shape and orientations of the normal uninfected cells. When infected cells maintained at 41°C were examined with the electron microscope it was found that, apart from the continued presence of virus particles, the persistence of the "bubbling" effect and a slight reduction in numbers of autolysosomes and residual bodies, the reversion to the same ultrastructural morphology as the uninfected normal cells was complete (Figs. 86, 123-126).

2.4 Discussion

Normal and virus-transformed cultured cells were found to be very distinct when compared at the light microscope level by phase contrast microscopy. Differences in cell size, shape and manner of growth were readily observed and it was also possible to compare the size and shape of nuclei and nucleoli and to examine the granularity and degree of vacuolation of the cytoplasm. Light microscopical studies of this type have appeared frequently in the literature and demonstrated similar features to those observed in the present investigation (see, for example, Benjamin, 1974).

In the study of the ultrastructural morphology of cultured cells it was demonstrated that the method of preparation for electron microscopy whereby cells were centrifuged to a pellet prior to processing was unsatisfactory since spatial relationships between cells were destroyed, cell and nuclear shape were distorted and rough ER and Golgi elements became swollen. A technique for *in situ* embedding of cultured cells for electron microscopy, similar to that of Firket (1966) and involving the growth of cells on Melinex polyester film, was developed and utilised for this work. A variety of alternative *in situ* embedding techniques have been described (see review on p. 22) but, nevertheless, comparatively few studies of the ultrastructure of cultured cells have been carried out and there are even less in which normal and transformed cells are compared. A recent study of cultured human skin fibroblasts by Lucky *et al.* (1975) compared the ultrastructure of *in situ* embedded cells with that of cells centrifuged to a pellet after being scraped from the culture dish. It was found, as it was in the present study, that cells which were removed from culture and centrifuged exhibited much distortion whereas the *in situ* embedded cells were undisturbed.

None of the studies dedicated to the cell lines being utilised in the current project have been comprehensive. Instead, the authors have concentrated on a limited number of ultrastructural features. However, the results have largely endorsed the findings of the present investigation. McNutt et al. (1971) looked at normal and SV40-transformed 3T3 cells but restricted their observations to nucleus, ER, cell periphery and skeletal components. Kisch et al. (1973) examined normal and polyoma virus-transformed BHK21 fibroblasts, concentrating on nucleus, ER, cell periphery, skeletal components, Golgi and ribosomes. Neither of these two studies gave even cursory treatment of lysosomes, endocytic vesicles or mitochondria, organelles to which great importance is attached in the present investigation. No comparative ultrastructural studies of normal and ts virus infected chick embryo fibroblasts have been made. There is, however, an exhaustive study of the lysosomes of normal chick embryo fibroblasts (Buckley, 1973).

With all the cell lines examined it was found that overall cell shape, manner of growth and, to a certain extent, relationship with other cells could not be satisfactorily appreciated from ultrastructural morphological appearance alone and that a complementary light microscopical examination was essential. There were a number of differences in the ultrastructural organisation of normal and transformed cells and these, along with the differences in growth and cell shape, are summarised in Table 1.

Table 1

Summary of major differences in growth and morphology of normal and transformed cultured cells.

Criterion	Cell Line						
	BHK21 C13	BHK21 J1	3T3	SV40 3T3	CEF	ts infected CEF at 35°C (transformed)	ts infected CEF at 41°C (not transformed)
Cell shape	Fibroblastic. Spindle-shaped usually with two major cell processes.	Fibroblastic. Most cells irregular with three or more processes.	Epithelioid	Fibroblastic with two or more major cell processes	Fibroblastic with two or more major cell processes.	Culture a mixture of small dense fibroblasts and small, rounded, dense, highly refractile cells.	Same as CEF
Manner of growth	Close-packed parallel orientation. Subject to density dependent inhibition of growth and division.	Random with tendency to overlap and pile up.	Forms confluent monolayer of very flat cells. Extremely sensitive to density dependent inhibition of growth and division.	Random. Forms dense, multi-layered cultures.	Some parallel orientation of cells. Little tendency to overlap or pile up.	Random with tendency to overlap and pile up. Rounded cells often clumped together.	Same as CEF.
Cell surface	Mainly smooth.	Many microvilli and cytoplasmic extensions.	Numerous small folds, convolutions, microvilli and short cytoplasmic extensions.	Similar to 3T3 but with more smooth sections.	Mainly smooth but with some microvilli and cytoplasmic extensions.	Many more microvilli and cytoplasmic extensions. More cell junctions.	Same as CEF
Nucleus	Ovoid and smooth surfaced.	Smaller with variable shape, irregular surface and intranuclear bodies.	Large and ovoid with small surface indentations.	Usually smaller and of variable shape with indentations of the surface. Some nuclei ring-shaped in section.			

Endoplasmic reticulum (ER)	Represented by short lengths of rough ER, often in close association with mitochondria.	More extensive rough ER with expanded cisternae. Closer association with mitochondria.	Rough ER has some slight expansions. Extensive smooth ER present.	Smooth ER less extensive.	Rough ER often branched and expanded Smooth ER present.	Very rarely any expansions of rough ER cisternae.	Same as CEF.
Golgi complex	Not extensive	Slightly more extensive.	Extensive and multicentric	Less extensive.	Not extensive, poorly differentiated.	Better differentiated	Same as CEF
Mitochondria	Sparse.	More numerous	Plentiful	More numerous Matrix less dense. More longer ones.	Numerous with many elongated.	Less numerous with very few elongated.	Same as CEF
Lysosomes	Common. No residual bodies.	More numerous with many residual bodies.	Abundant with great numbers of residual bodies.	Less numerous owing to scarcity of residual bodies which were, however, larger when they did occur.	Numerous.	Far fewer autolysosomes and residual bodies.	Fewer lysosomes than CEF but more than ts infected CEF at 35°C.
Other features.				Occasional very large vacuole present.	Virus particles present. Formation of masses of vacuoles in cytoplasm which are expelled from the cell, and appear like bubbling at the surface.	Virus particles present. Vacuole formation and "bubbling" persists.	

It is clear from Table 1. that differences between normal and transformed cells are not the same in the three systems investigated, the lysosomes providing a good example. This makes it very difficult to arrive at general statements about the changes which occur when normal cells are transformed and poses problems when attempting to relate the results to carcinogenesis *in vivo*. The changes in ultra-structural organisation observed when baby hamster kidney cells undergo transformation may be quite unrelated to the changes which occur when human breast cells, for instance, become cancerous.

Chapter 3

DISTRIBUTION AND ACTIVITY OF HYDROLYTIC ENZYMES IN CULTURED CELLS

In order to examine the distribution of a number of hydrolytic enzymes in the cultured cells under investigation a light microscopic enzyme cytochemical survey was carried out. This study would not only indicate which enzymes were present in each cell line but would also provide useful information in planning the electron microscopic investigation. In addition it would establish standards with which to compare the results of the ultrastructural enzyme cytochemistry. Such a survey would also allow the visual assessment of the levels of enzyme activity in each of the cell lines, hence providing data about the role of these enzymes in the transformation process. In an attempt to evaluate the validity of such a visual assessment one pair of cell lines, the normal and transformed 3T3 cells, was also analysed quantitatively with respect to enzyme activity so that a direct comparison with the cytochemical results would be available. Two distinct methods of quantitative enzyme determination were used for this study. In the first the dye deposited in the cells during the cytochemical study was measured by microdensitometry and in the second a direct biochemical assay of the enzymes in extracts obtained from cell homogenates was performed. The direct biochemical approach enabled the results obtained in this study to be compared with the results of other workers and also allowed a comparison with the results of the microdensitometry, thus providing a double check on the cytochemical assessment. In addition, it allowed the effect of fixation on enzyme activity to be determined and indicated whether enzymes which could not be demonstrated cytochemically could be detected by biochemical means.

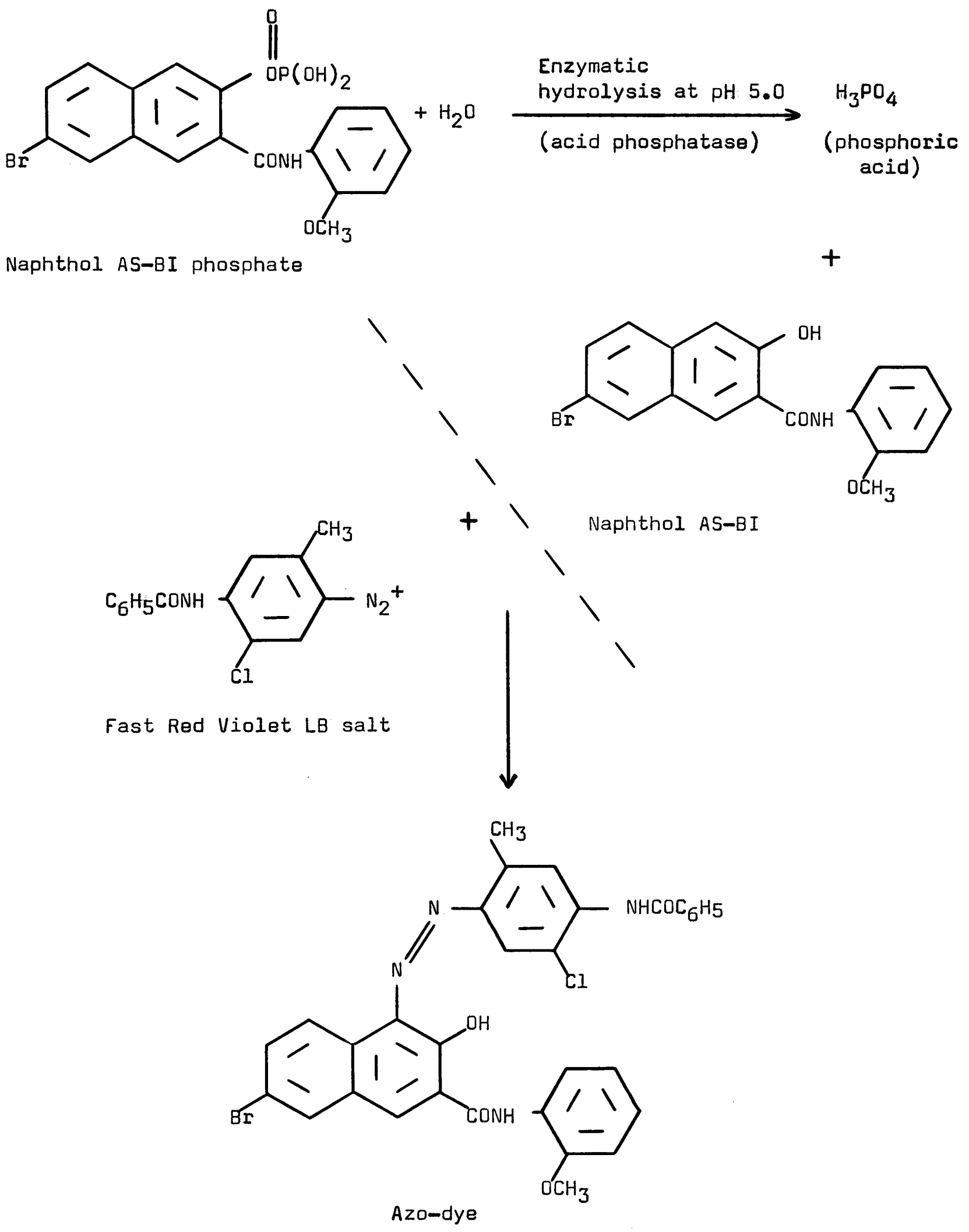
3.1 Light microscopic enzyme cytochemistry

The commercial availability of a variety of suitable substrates made it possible to test cells for the presence of a wide range of hydrolytic enzymes. In this investigation all the techniques used, with two exceptions, made use of azo-dye methods which were modified from those described by Burstone (1962) and Pearse (1968, 1972). Whenever possible, the substrates used were Naphthol AS compounds and in every case Fast Red Violet LB salt was the diazonium coupling reagent.

Azo-dye techniques depend upon the coupling of a diazonium salt with a naphthol released as a result of enzymatic hydrolysis of a naphthol ester substrate, the diazonium salt reacting only with the free naphthol and not with the ester. This coupling produces an insoluble, substantive, highly coloured azo-dye at the site of the enzyme activity. For example, in the demonstration of acid phosphatase the substrate, Naphthol AS-BI phosphate, is hydrolysed by the enzyme at pH 5.0 and the released Naphthol AS-BI is coupled to the Fast Red Violet LB to yield a bright red azo-dye. These reactions are illustrated in Fig. 3.1.1. The coupling salt can be present in the incubation medium so that as soon as the naphthol is released the coupling reaction can take place, or the tissue can be introduced to the coupling salt on completion of incubation. The former is known as simultaneous coupling and the latter post-coupling. Simultaneous coupling is a more convenient procedure and ensures that the naphthol has no opportunity to diffuse away from the site of its production before coupling. There is, however, the possibility that the presence of a diazonium salt may inhibit enzyme activity to some extent. If post-coupling is employed then this possibility is obviated and the coupling can be performed under optimum conditions (pH 7 at $0^{o}C$) rather than under the conditions dictated by

Fig. 3.1.1.

Enzymatic hydrolysis of Naphthol AS-BI phosphate and subsequent coupling of free Naphthol AS-BI with Fast Red Violet LB salt.



the incubation. It has been shown, though, that many enzymes can be successfully demonstrated using simultaneous coupling procedures and it was decided to utilise such procedures for the light microscopical survey.

Cells were cultured on glass coverslips and when they had reached a state of confluence they were removed from the culture medium and rinsed briefly in cold (4°C) cacodylate buffer (pH 7.4, 0.1M) containing 4.5% sucrose. They were then transferred to a fixative solution consisting of 2.5% glutaraldehyde in cacodylate buffer (pH 7.4, 0.1M). Glutaraldehyde fixation was utilised, rather than that by formolcalcium (Baker, 1946) or any other fixative, as this would allow direct comparison with electron microscopic studies in which glutaraldehyde was, of necessity, used. Fixation was for 30 minutes at 4°C after which the cells were washed overnight (16-18 hours) in several changes of cacodylate buffer containing 4.5% sucrose, also at 4°C. The cells were then incubated according to one of the procedures described below.

For the majority of the enzymes to be investigated the same basic procedure was utilised. A 20 mg-% solution of the substrate was prepared in the appropriate buffer after first dissolving the substrate in a small volume of dimethyl formamide (DMF). To this solution was added 50 mg-% Fast Red Violet LB salt. The mixture was shaken and filtered. Cells were then incubated in this simultaneous coupling medium. The details for each enzyme are given in Table 1.

Table 1

Details of incubation media for the demonstration of hydrolytic enzymes. Substrates obtained from Sigma Chemical Company Ltd. (S) or Koch-Light Laboratories Ltd. (K.L.).

Enzyme	Substrate	DMF (ml)	Buffer	pH	Incubation Time (hrs)
Acid Phosphatase EC 3.1.3.2	Naphthol AS-BI Phosphate (S)	0.25	0.2M Acetate	5.0	1
Alkaline Phosphatase EC 3.1.3.1	Naphthol AS-BI Phosphate (S)	0.25	0.2M Tris HCl	9.2	1, 3
Non-specific Esterase EC 3.1.1.-	Naphthol AS-D Acetate (S)	1.00	0.2M Tris HCl	7.1	1
β-D-Glucuronidase EC 3.2.1.31	Naphthol AS-BI-β-D-Glucuronide (S)	0.25	0.2M Acetate	4.5	1
N-acetyl-β-D-Glucosaminidase EC 3.2.2.30	Naphthol AS-BI-2-Acetamido-2-Deoxy-β-D-Glucopyranoside (K.L.)	0.25	0.2M Acetate	5.0	1
β-D-Glucosidase EC 3.2.1.21	1-Naphthyl-β-D-Glucopyranoside (K.L.)	0.25	0.2M Acetate	5.4	1, 3
β-D-Glucosidase	2-Naphthyl-β-D-Glucopyranoside (K.L.)	0.25	0.2M Acetate	5.4	1, 3
β-D-Glucosidase	Naphthol AS-BI-β-D-Glucopyranoside (K.L.)	0.25	0.2M Acetate	5.4	1, 3
α-D-Glucosidase EC 3.2.1.20	2-Naphthyl-α-D-Glucopyranoside (K.L.)	0.25	0.2M Acetate	5.4	1, 3
β-D-Galacto-sidase EC 3.2.1.23	Naphthol AS-BI-β-D-Galactopyranoside (K.L.)	0.25	0.2M Acetate	5.4	1, 3

β-D-Galactosidase	2-Naphthyl-β-D-Galactopyranoside (K.L.)	0.25	0.2M Acetate	5.4	1, 3
β-L-Fucosidase	Naphthol AS-BI-β-L-Fucopyranoside (K.L.)	1.00	0.2M Acetate	5.6	1, 3
α-L-Fucosidase EC 3.2.1. -	2-Naphthyl-α-L-Fucopyranoside (K.L.)	0.25	0.2M Acetate	5.6	1, 3
α-D-Mannosidase EC 3.2.1. 24	1-Naphthyl-α-D-Mannopyranoside (K.L.)	0.25	0.2M Acetate	5.0	1, 3

Some cells were incubated in control media containing no substrate.

Azo-dye techniques were also utilised in attempts to demonstrate certain proteolytic enzymes. Methods for chymotrypsin (EC 3.4.4.5)-like enzymes were described by Lagunoff and Benditt (1964) and involved the use of three benzosalicylanilide ester substrates: Naphthol AS Phenylacetate (S), Naphthol AS-BI Chloropropionate (S) and Naphthol AS Benzoate (S). 1mM substrate solutions were prepared in 0.2M Tris HCl-absolute methanol (70:30, v/v) buffer at pH 8.0 after first dissolving the substrates in small volumes (< 1ml) of absolute methanol. To these solutions were added 50 mg-% Fast Red Violet LB salt. The media were then filtered and cells incubated in them for up to 3 hours at 37°C or similarly in control media containing no substrate.

For the demonstration of a trypsin (EC 3.4.4.4) -like proteolytic enzyme an incubation medium was prepared by dissolving 30 mg of substrate, N-α-Benzoyl-DL-Arginine-β-Naphthylamide Hydrochloride (BANA, K.L.) in 20 ml of 0.1M pH 7.0 Tris HCl buffer and adding 10 mg of Fast Red Violet LB salt. Cells were incubated in this medium, or in a control medium containing no substrate, for up to 3 hours at 37°C. After incubation cells were rinsed in distilled water and then transferred for 2 minutes

to 0.1M copper sulphate solution. They were then, as were all the above preparations, rinsed in distilled water at $4^{o}C$ and mounted in Farrant's Medium on glass slides.

Two enzymes, acid phosphatase and alkaline phosphatase, were also investigated using alternative, non-azo-dye methods. This was undertaken primarily to determine whether different methods for a particular enzyme would demonstrate the same distribution. The methods in question were the metal-salt techniques of Gomori, both of which utilise sodium-β-glycerophosphate (S) as substrate. The enzyme hydrolyses the glycerophosphate, liberating phosphate ions which are captured by metal ions present in the incubation medium to yield an insoluble precipitate at the site of enzyme action. This precipitate is colourless and must be transformed into an easily observable, coloured compound. To achieve this the preparation is treated with a soluble sulphide, resulting in the formation of a blackish, exceedingly insoluble metal sulphide, the final end product of the enzyme cytochemical procedure. For acid phosphatase, lead ions are usually used to capture the phosphate whilst for alkaline phosphatase, calcium ions are utilised.

The incubation medium for acid phosphatase demonstration was prepared in the following manner:

Solution A: 25 ml 0.2M acetate buffer at pH 5.0

75 ml distilled water

0.12 gm lead nitrate

Solution B: 10 ml distilled water

0.3 gm sodium β-glycerophosphate

Solution A was added, with stirring, to solution B and the resulting solution left overnight at $37^{o}C$. It was filtered immediately before use. A control medium was prepared without substrate. Cells were

incubated for 30 or 60 minutes in these media, rinsed in distilled water and then immersed for 1 minute in a saturated solution of hydrogen sulphide to bring about conversion of deposits of lead phosphate to lead sulphide.

The alkaline phosphatase incubation medium had the following composition:

10 ml 3% sodium β-glycerophosphate

10 ml 2% sodium diethyl barbiturate (barbitone sodium)

9 ml distilled water

20 ml 2% calcium chloride

1 ml 5% magnesium sulphate

A no-substrate control medium contained no sodium β-glycerophosphate. Cells were incubated in these media for up to 3 hours at 37°C, rinsed in running water and then treated with 2% cobalt nitrate for 5 minutes to convert calcium phosphate into cobalt phosphate. After a further rinse in distilled water the cobalt phosphate was converted into the final end product, cobalt sulphide, by immersing the cells for 1 minute in a saturated solution of hydrogen sulphide.

On completion of the two procedures outlined above the cells were rinsed in distilled water at 4°C and mounted in Farrant's Medium on glass slides. These, and all the azo-dye slides were examined and photographed with a Carl Zeiss Standard RA microscope fitted with photomicrography attachments.

Of the azo-dye techniques, only those for the demonstration of the proteolytic enzymes were not applied to all the cell lines under study. These were applied solely to the normal and transformed 3T3 cells whilst the two Gomori methods were only applied to the normal and transformed 3T3 and BHK21 cells.

On examination of the preparations it was found that the test incubations resulted in the demonstration of just four enzymes, acid phosphatase (by both the azo-dye and Gomori methods), non-specific esterase, β-D-glucuronidase and N-acetyl-β-D-glucosaminidase. All the no-substrate controls were negative. Apart from non-specific esterase which was absent from the chick embryo cells, the four enzymes were present in all the cell lines. Azo-dye end product was deposited in the form of bright red particles whilst the end product of the Gomori acid phosphatase technique appeared as very dark brown or blackish particles. All four established cell lines (normal and transformed BHK21 and 3T3) exhibited similar azo-dye deposition, both in terms of localisation and amount present for any one enzyme. The enzymes were localised in a particulate fashion throughout the cytoplasm and especially in the perinuclear region. When these preparations were compared with the phase-contrast morphological preparations the distribution of end product in the former appeared to coincide with the distribution of cytoplasmic particles in the latter. Acid phosphatase activity was similarly localised by both azo-dye and Gomori techniques (compare Figs. 58 and 60). Transformed cell cultures contained a number of cells which had rounded up for division and it was noticeable that these often displayed more intense activity than the non-mitotic cells. Due to the differences in manner of growth, cell thickness, shape, refractility and the nature of the end product deposits it proved extremely difficult to assess the levels of enzyme activity in the normal as compared to the transformed cells. For example, there were more particles of azo-dye in 3T3 cells than in SV40 3T3 cells but in the latter the particles were often larger and more intensely coloured. The situation in the normal and ts virus-infected chick embryo fibroblasts was

basically similar to that in the established cell lines although non-specific esterase was not demonstrated. In the uninfected cultures all the cells exhibited some degree of end product deposition, but certain of them which were rounded, possibly for division though this was far from certain, displayed much larger, intensely coloured particles of azo-dye. In the ts virus-infected cultures kept at 35°C (transformed cultures) many of the cells exhibited very little or no end product deposition. However, the cells which did display activity contained large, intensely coloured azo-dye deposits. When infected cultures were kept at 41°C all the cells once again exhibited enzyme activity and the overall level of this activity appeared to remain similar to that of the transformed cells at 35°C rather than reverting to the level in the uninfected cells.

Table 2 attempts to indicate the comparative levels of enzyme activity in the various cell lines, based on azo-dye end product deposition after incubation for 1 hour in the relevant test media. This is a visual assessment and ascribes each enzyme activity to one of the five positions on a scale increasing from + to ++++. This scale represents only gradual increases in levels of activity so that ++++, for instance, does not signify twice as much activity as ++.

Table 2

Visual assessment of hydrolytic enzyme activities.

Cell line	Enzymes			
	Acid phosphatase	Non-specific esterase	β-D-glucuro-nidase	N-acetyl-β-D-glucosaminidase
3T3	+++	++++	++	+
SV40 3T3	+++	++++	++	+
BHK21 C13	++++	++++	+	+
BHK21 J1	++++	+++++	+	+
Chick embryo fibroblast (CEF) kept at 35°C	++	0	++	+
ts 29 infected CEF at 35°C	++++	0	+++	++++
CEF at 41°C	+++	0	++	+
ts 29 infected CEF at 41°C	++++	0	+++	+++

Figs. 58-64 illustrate the major features of the results outlined above.

Of the thirteen enzymes studied nine could not be demonstrated by the cytochemical procedures employed. This implies that the enzymes were not present in the cells or at least, if present not active, or that the methods employed were incapable of detecting them. It is unlikely that all these enzymes would be absent from the cells and Bosmann (1969) has demonstrated by biochemical methods that some of them are present. It seems probable, therefore, that the cytochemical methods used were not adequate or that the optimal conditions for the activity of the enzymes were not being met. In order to determine whether this was the case, all the enzymes that had not been demonstrated were reexamined using different fixation procedures and changes in the

conditions of incubation. As an alternative to glutaraldehyde fixation, cells were fixed overnight (16 hrs.) in formol-calcium (Baker, 1946) at 4°C. After washing in distilled water, the cells were incubated in media prepared as previously described (p. 52). This change of fixative did not, however, lead to any positive results. Another factor which could be altered was the pH of incubation media. It is possible that the pH optima for enzyme activities in the cultured cells were different from the optima derived histochemically or biochemically for similar enzymes in other systems. pH was, therefore, altered by up to 3 pH units either side of the value used in the original tests. In certain cases this necessitated changing the buffer. The proteolytic enzyme media were prepared in phosphate buffer rather than Tris HCl when pH values of 7 and 6 were required and acetate buffer for pH 5. Only in the case of chymotrypsin-like proteolytic enzyme incubations was any end product deposition achieved as a result of pH changes. Cells incubated in media at pH 6.0, utilising Naphthol AS-phenylacetate or Naphthol AS-BI chloropropionate as substrate, exhibited a small amount of azo-dye deposition whilst cells incubated in similar media at pH 5.0 exhibited larger deposits. The deposition was greatest when Naphthol AS-BI chloropropionate was the substrate. At pH 7.0 and 4.0 tests were negative. The particles of azo-dye were distributed throughout the cytoplasm but most concentrated in the perinuclear region. In fact the distribution of end product was very similar to that of the four enzymes previously demonstrated (p. 56) although it was present in less abundance. There was no difference in level of end product deposition between the normal and transformed 3T3 cells.

As this enzyme activity was demonstrated at pH 5.0 it is unlikely that the enzyme in question was chymotrypsin-like since this pH is so

far removed from the accepted optimum for chymotrypsin activity (pH 8.0). It is possible, therefore, that the enzyme was an acid esterase or perhaps an esterase-like proteolytic enzyme such as Cathepsin C (EC 3.4.4.9), a lysosomal exopeptidase which is known to act upon synthetic amino-acid esters at acid pH levels, optimal activity occurring between pH 5.0 and 6.0.

Bosmann (1969, 1972), in biochemical studies, showed that the specific activity of N-acetyl-β-D-glucosaminidase was similar to that of acid phosphatase in 3 T3 cells and much higher in transformed 3T3 cells, clearly a very different situation from that demonstrated by the cytochemistry where glucosaminidase was the least active of the enzymes detected. Since acetate has been shown to be a competitive inhibitor of N-acetyl-β-D-glucosaminidase (Pugh, 1973) it was likely that the use of acetate buffer in the incubation medium could be responsible for the low activity of this enzyme. Therefore, the procedure for N-acetyl-β-D-glucosaminidase demonstration was repeated as previously described (p. 52) except that 0.1M citrate buffer at pH 5.2 replaced the acetate buffer. Incubation was for 1 and 2 hours. This change resulted in an increase in the amount of azo-dye deposition, indicating greater enzyme activity. However, this increased level of activity was still lower than that of acid phosphatase, a fact that was particularly noticeable when preparations incubated for 2 hours were compared. Also, there was still no difference in levels of glucosaminidase activity between the normal and transformed cells.

3.2. Microdensitometry

The light microscopic enzyme cytochemistry resulted in the demonstration of five hydrolytic enzymes, all of which could be further studied at the electron microscope level. It was possible to compare

the localisation and distribution of enzyme activities in corresponding normal and transformed cells but not to make satisfactory comparative assessments of the levels of this activity. A means of analysing the azo-dye deposits was required. Altmann (1969) found that deposits of formazan, the insoluble end-product of dehydrogenase demonstration using tetrazolium salts as hydrogen acceptors, could be quantitatively eluted from liver sections and measured in a spectrophotometer. However, since azo-dyes are much more substantive than formazan it is doubtful whether such an elution technique would have been applicable. The highly coloured nature of the azo-dye end product deposits meant that they were suitable for analysis by microdensitometry, a method which involves measuring the optical density of coloured deposits, <u>in situ</u>, at an appropriate wavelength. A Vickers M86 scanning microdensitometer was utilised for this purpose. In this instrument a selected region of the material on the slide is masked off from the surrounding field, scanned by light at the desired wavelength and measurements of optical density and area made every 100 μsec. A single scan produces a great number of measurements which are automatically integrated to give the total optical density and area of the coloured product.

Unfortunately for practical reasons it was possible only to compare the levels of activity of two enzymes, acid phosphatase and N-acetyl-β-D-glucosaminidase, in normal and transformed 3T3 cells. Slides were prepared as previously described with incubation times of 1 and 2 hours. Fields containing a small number of cells were selected and measurements made with light of wavelength 553 nm. This process was continued until the total number of cells scanned gave a sample size that was statistically valid.

It was found that only the 2 hour acid phosphatase preparations

had sufficiently high levels of azo-dye deposition to be suitable for accurate analysis. The end product deposits in the other preparations had much lower colour intensity and measurements taken from them were similar to measurements made on cells with no azo-dye present. It appeared, therefore, that, under the conditions used, the instrument could not distinguish between lightly coloured end product and the surrounding cytoplasm.

Table 3

Scanning microdensitometry data obtained from cells incubated for 2 hours in medium for the demonstration of acid phosphatase. Azo-dye measured at 553 nm.

Cell line	No. of scans	Sum of Optical Density Measurements	Sum of Area Measurements	Total No. of cells.	Average Area/Cell	Ratio of Optical Density/Area
3T3	31	1040	850	154	5.52	1.22
SV40 3T3	28	1981	1044	203	5.14	1.90

$$\text{The ratio of } \frac{\text{Optical Density/Unit Area, SV40 3T3}}{\text{Optical Density/Unit Area, 3T3}} = \frac{1.90}{1.22} = 1.56$$

$$\text{and the ratio of } \frac{\text{Average area per 3T3 cell}}{\text{Average area per SV40 3T3 cell}} = \frac{5.52}{5.14} = 1.07$$

These results show that the average area taken up by azo-dye deposits is slightly greater in the 3T3 cells than it is in the SV40 3T3 cells but that in the transformed cells the intensity of colouration of the deposits is greater. Thus the level of activity of acid phosphatase is 1.56 times higher in SV40 3T3 cells than it is in 3T3 cells but this higher activity in the transformed cells is confined to fewer sites. This latter fact fits the observed appearance of the azo-dye deposits in the two cell lines.

3.3. Biochemical assays of enzyme activity

Although scanning microdensitometry provides a useful means of comparing the levels of azo-dye deposition, andhence enzyme activity, in normal and transformed cultured cell preparations, the end product must be of a sufficiently high colour intensity for accurate measurement which could render the technique inapplicable to enzymes of very low activity. Such limitations do not apply to biochemical assays of enzyme activity which, in addition, require no prior fixation of cells so that more activity is likely to be retained. As biochemical assays are applied to extracts obtained from homogenised cell preparations the enzyme activity which is recorded cannot be directly related to ultra-structure or to the organisational changes which occur on transformation. Without an accompanying cytochemical study, therefore, the biochemical approach is, on its own, inadequate but within the present investigation it can supply valuable additional information and also provides a means of assessing the effect on enzyme activity of the fixation procedure.

Assays for three enzymes, one of which had high activity as demonstrated cytochemically, one with lower activity and one which was not detected at all, were performed on extracts prepared from fixed and unfixed normal and transformed 3T3 cells. The enzymes were, respectively, acid phosphatase, N-acetyl-β-D-glucosaminidase and β-D-glucosidase. The extraction and assay procedures were modified from those described by Bosmann (1969, 1972) and Bosmann and Pike (1970). Cells were grown in 90 mm plastic Petri-dishes and those destined for fixation were put up 24 hours earlier than those which were to remain unfixed. This ensured that the first batch could be fixed, washed overnight ($23\frac{1}{2}$ hours) and harvested at the same time as the unfixed cells. Five dishes of 3T3 and five of SV40 3T3 comprised each of the

two batches. The cells to be fixed were grown until they achieved confluence (about 4 days), after which they were rinsed in 0.1M, pH 7.4 cacodylate buffer at 4°C, containing 4.5% sucrose. They were then fixed for 30 minutes at 4°C in 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.4 and washed in several changes of buffer with sucrose for 2$3\frac{1}{2}$ hours at 4°C. At the end of this period the washing solution was poured off and replaced by ice-cold PBS. At the same time the culture medium was removed from the second batch of cells and also replaced by PBS. The cells were scraped from the dishes with a silicon rubber policeman and the resulting suspensions pooled in four large centrifuge tubes. Centrifugation at low g yielded cell pellets. The supernatants were discarded and to each of the four pellets were added 30 volumes (about 5 ml) of 0.1% Triton X-100 at 4°C. The pellets were then homogenised at the temperature of melting ice by 30 strokes of a Potter-Elvehjem homogeniser. Homogenates were transferred to small flasks and left to extract overnight (16 hours), with stirring, at 4°C after which the extracted mixtures were centrifuged at 30,000 g for 1 hour. The supernatants were decanted off, frozen and stored at -20°C until required for the assays.

Three 0.5 ml samples of each extract were utilised for total protein determination by the method of Lowry et al.(1951) using crystalline bovine serum albumin as a standard. Enzyme activities were determined using p-nitrophenyl-phosphate, p-nitrophenyl-N-acetyl-β-D-glucosaminide and p-nitrophenyl-β-D-glucoside as substrates, each determination being performed in triplicate. To 0.25 ml of extract were added 1.75 ml of 500 μM substrate solution in 0.05 M citrate buffer at pH 4.3. This mixture was incubated for 2 hours at 37°C. The reaction was terminated by the addition of 2 ml of 0.4M glycine:NaOH

buffer at pH 10.5. The mixture was agitated and then left for 30 minutes after which time the released p-nitrophenol was measured at 420 nm in a Cecil CE 343 spectrophotometer. A parallel enzyme blank was also determined. From these data and a standard p-nitrophenol curve the enzyme specific activities, expressed in terms of n moles of p-nitrophenol released/hour/mg protein, were calculated.

This series of determinations was performed twice more on extracts from further cultures of cells so that three sets of results were obtained. These results are set out in Tables 4 - 8 and have deliberately not been combined since to do so would hide a number of significant features which emerge from them.

Table 4

Enzyme specific activities, expressed as n moles of p-nitrophenol released/hour/mg protein, from 1st series of determinations.

Enzyme	Cell Sample			
	3T3	SV40 3T3	Fixed 3T3	Fixed SV40 3T3
Acid phosphatase	260	447	17	2.0
N-acetyl-β-D-glucosaminidase	593	322	<1	<1
β-D- glucosidase	0	0	0	0

Table 5

Percentage enzyme activities lost during fixation (1st series)

Enzyme	Cell line	
	3T3	SV40 3T3
Acid phosphatase	93.5	95.5
N-acetyl-β-D-glucosaminidase	~100	~100

Table 6

Enzyme specific activities, expressed as n moles of p-nitrophenol released/hour/mg protein, from 2nd series of determinations

Enzyme	Cell sample			
	3T3	SV40 3T3	Fixed 3T3	Fixed SV40 3T3
Acid phosphatase	235	394	<1	<1
N-acetyl-β-D-glucosaminidase	791	391	<1	<1
β-D-glucosidase	0	0	0	0

The fixation process led to the loss of ~100% of the enzyme activities.

Table 7

Enzyme specific activities, expressed as n moles of p-nitrophenol released/hour/mg protein, from 3rd series of determinations

Enzyme	Cell sample			
	3T3	SV40 3T3	Fixed 3T3	Fixed SV40 3T3
Acid phosphatase	546	433	23	35
N-acetyl-β-D-glucosaminidase	541	289	0	5
β-D- glucosidase	0	0	0	0

Table 8

Percentage enzyme activities lost during fixation (3rd series)

Enzyme	Cell line	
	3T3	SV40 3T3
Acid phosphatase	95.8	91.9
N-acetyl-β-D-glucosaminidase	100	98.3

Apart from the absence of β-D-glucosidase activity, which seemed

to endorse the cytochemical findings, the most noticeable feature of the results was the drastic effect of glutaraldehyde fixation upon the activities of acid phosphatase and N-acetyl-β-D-glucosaminidase, the latter enzyme appearing particularly sensitive to the action of the fixative. The cytochemistry indicated that N-acetyl-β-D-glucosaminidase had a lower level of activity than acid phosphatase whereas the biochemistry demonstrated that this was not so and showed that the almost complete loss of the glucosaminidase activity during fixation was responsible for creating this situation. Although the cytochemistry may, therefore, have given results which were in some respects misleading, it was evident that the cytochemical techniques were able to detect enzyme activity in fixed material more successfully than the biochemical techniques.

Of the specific activities demonstrated in the extracts obtained from unfixed cells, that of 3T3 acid phosphatase of the 3rd series appeared to be out of step with the rest of the results. This value was more than twice as high as those in the first two sets of results and was also higher than the values for SV40 3T3 cells. It was undoubtedly significant that the 3T3 cultures used for the 3rd series of determinations contained far more cells than the previous 3T3 cultures. This was obvious from the total protein estimations carried out on the extracts. The cells had overgrown the monolayer stage and the greatly increased acid phosphatase activity may have been associated with the increased cell density or the fact that the cells were still growing and dividing. The other two batches of 3T3 cultures had ceased growth and division at the monolayer stage. The possibility that acid phosphatase activity was directly related to cell density or stage of growth was likely since the highest specific activity was in the culture

with most cells and the lowest in the culture with least cells. The correlation also held good for the values between these extremes. The ratio of $\frac{\text{SV40 3T3 specific activity}}{\text{3T3 specific activity}}$, disregarding the 3rd set of results for the reasons discussed, was 1.70. Under the conditions employed, the transformed cells, therefore, had 1.7 times more acid phosphatase activity than the normal cells. This figure compared well with that derived by microdensitometry (1.56).

N-acetyl-β-D-glucosaminidase activity remained consistent even despite the abnormal growth of 3T3 cells in the final cultures. This enzyme, therefore, appeared to react in a different manner to acid phosphatase when changes in cell density and growth occurred. In the 3T3 cells specific activity was greatest in the culture with least cells and was at its lowest in the most crowded culture. The same situation applied to the SV40 3T3 cultures. The ratio of $\frac{\text{3T3 specific activity}}{\text{SV40 3T3 specific activity}}$ was 1.91. Therefore, whether comparing a 3T3 monolayer with a transformed culture or an overgrown 3T3 culture with a less dense transformed culture, there was consistently almost twice as much N-acetyl-β-D-glucosaminidase activity in 3T3 cells as there was in SV40 transformed 3T3 cells.

Bosmann (1969) studied glycosidases in normal and SV40 transformed 3T3 cells and found that β-D-glucosidase was present with a very low specific activity (14 units, expressed as n moles p-nitrophenol released/hour/mg protein) in the normal cells. On transformation this activity was increased threefold to 42. N-acetyl-β-D-glucosaminidase had a high specific activity (520) in 3T3 cells which more than doubled to 1350 when the cells were transformed. Acid phosphatase was not included in this study but in a subsequent one (Bosmann, 1972) in which enzyme activities in normal and RSV transformed 3T3 cells were compared, this

enzyme was included and its specific activity increased only very slightly, from 482 to 509, on transformation. These results are clearly very different from those of the present study, particularly in respect of N-acetyl-β-D-glucosaminidase. The reason for the difference is probably that Bosmann harvested all his cells in the logarithmic phase of growth whereas in the present investigation the 3T3 cultures, with the exception of the final batch, were confluent and therefore consisted of non-growing or stationary cells. Horvat and Acs (1974) compared the specific activities of three lysosomal enzymes (N-acetyl-β-D-glucosaminidase, aryl sulphatase and DNase II) in 3T3 cells during the logarithmic phase of growth and in stationary cultures. They showed that after 3T3 cells had achieved confluence there was a very marked increase, of the order of tenfold, in the specific activities of these lysosomal enzymes. Therefore, although Bosmann (1969) has shown that N-acetyl-β-D-glucosaminidase has a higher specific activity in logarithmically growing SV40 3T3 cells than it does in logarithmically growing 3T3 cells, it is to be expected that when 3T3 cells become confluent the glucosaminidase specific activity increases so much that it rises above the level in the proliferating transformed cells. This would substantiate the present biochemical results. As it is known that proliferating normal cells resemble transformed cells in many respects (Stoker, 1972), it is very important to ensure that when carrying out comparative studies of normal and transformed cells, the comparison is always between confluent, non-proliferating normal cells and transformed cells.

Acid phosphatase must be subject to different control mechanisms from N-acetyl-β-D-glucosaminidase since its specific activity is greater in proliferating cells than in stationary normal cells.

CHAPTER 4

ELECTRON MICROSCOPIC ENZYME CYTOCHEMISTRY

The light microscope-level enzyme cytochemical survey demonstrated the presence of five hydrolytic enzymes in the cell lines investigated. These were acid phosphatase, non-specific esterase, β-glucuronidase, N-acetyl-β-D-glucosaminidase and an esterase-like proteolytic enzyme. Since methods were available for the ultrastructural demonstration of all five enzymes each could be studied further with a view to elucidating more clearly its localisation at the fine structural level. All five were suitable for demonstration by appropriate azo-dye procedures and as it has been reported that one type of cytochemical method often results in the demonstration of only a limited number of isoenzymes (Maggi et al., 1966; Bowen, 1968; Beadle and Gahan, 1969) it is also desirable to apply alternative methods when these are available in order to study a wider range of isoenzymes. Such alternative methods include the Gomori lead salt method for acid phosphatase and the thiolacetic acid method for esterase (Barrnett and Palade, 1959).

The Gomori lead salt method (see p. 54) is directly applicable at the ultrastructural level since the primary end product, lead phosphate, is electron dense. Similarly, the thiolacetic acid procedure for the demonstration of esterase can be directly applied to electron microscopy since the enzyme hydrolyses thiolacetic acid (CH_3COSH) with the liberation of hydrogen sulphide which, in the presence of lead ions, is converted to electron dense lead sulphide. The azo-dye methods are used routinely in light microscopy (see p. 49) and can be adapted for electron microscopic studies provided that the end-product is electron dense. The necessary density can be imparted by the diazonium coupling salt which may contain a heavy metal component or have a molecular configuration

which renders it electron opaque. In the former category of diazonium salts are the diazonium chloride of triphenyl-p-aminophenethyl lead (LPED), diazotised lead phthalocyanin and p-(acetoxymercuric) aniline diazotate whilst in the latter are hexazonium pararosaniline which produces an amorphous, electron opaque azo-dye when coupled with a substituted naphthol and p-nitrobenzene diazonium tetrafluoroborate which gives rise to an electron dense, crystalline azo-dye. In the present study the LPED procedure (Livingston et al., 1970) was used since this gives a fine, particulate end-product that is very electron dense but does not obscure ultrastructural detail and appears to be free of many of the problems encountered with other metal-based methods (Bowen, 1973).

4.1. The Gomori lead-salt method for acid phosphatase.

As the study of cell ultrastructure had shown that the centrifugation of unfixed cells into a pellet was likely to lead to the production of artifacts the Gomori method was applied principally to cells which were cultured on Melinex squares and subsequently embedded in situ. However, this method of preparing cells did not readily facilitate examination of the cell surface where it was in contact with the substratum and as this surface is of importance some cells were prepared for examination in other ways. The first of these was to centrifuge the cells into a pellet prior to fixation, followed by the application of the Gomori method to the pellet. In an attempt to identify any artifacts that this method might have caused, other cells were fixed and incubated prior to scraping from the culture dish and centrifuging into a pellet.

Two versions of the Gomori method were used: the standard procedure described by Gomori (1952) and the modified scheme of Brunk

and Ericsson (1972) which was developed particularly for use with cultured cells. The standard incubation medium was prepared as previously described (p. 54) and the Brunk and Ericsson medium was prepared in the same way but was then made 0.22 M with respect to sucrose and to this solution was added 10% v/v dimethyl sulphoxide (DMSO). This modification was specifically designed to eliminate non-enzymically produced lead deposits. The sucrose increases the osmolality of the medium, a measure intended to reduce diffusion of the enzyme and reaction products within the cell. The role of the DMSO is to increase the permeability of cellular membranes, thus reducing the incubation time by permitting more rapid entry of substrate to the sites of enzyme activity. As a control, cells were incubated in media containing no sodium -β-glycerophosphate.

4.1.1. Cells grown on Melinex squares

All the cell lines under study were used in this investigation. Cells were grown to confluence on Melinex squares, rinsed in cacodylate buffer (0.1M, pH 7.4, 4°C) containing 4.5% sucrose and then fixed for 30 minutes in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4, 4°C). After fixation the cells were washed overnight in several changes of buffer containing 4.5% sucrose and then incubated for 30 or 60 minutes in the standard Gomori medium, the modified medium of Brunk and Ericsson or a no-substrate control medium. On completion of incubation cells were rinsed in cold acetate buffer, post-fixed for 1 hour in 1% cacodylate-buffered osmium tetroxide at 4°C, dehydrated through a graded ethanol series (stained in 1% uranyl acetate for 20 minutes while in 70% ethanol), infiltrated with Araldite and then surface embedded in Araldite. Silver-thin sections were cut from the trimmed, polymerised blocks and examined, with or without further staining by lead citrate, in

the electron microscope.

BHK21C13 and BHK21J1

When these cells were examined most of them exhibited dense deposits, presumed to be lead phosphate, the end product of the cytochemical procedure. In the cells which were incubated for 60 minutes the deposits were often present in such quantities that underlying ultrastructural details were hidden. Cells incubated for 30 minutes, however, did not, in general, display such excessive end product deposition. Fine structural details were rarely masked and there was less evidence of deposits, identified as non-specific lead, which were not produced as a result of enzyme activity. The results are, therefore, based on the appearance of cells incubated for 30 minutes.

In both normal and transformed BHK21 cells lead deposits were found in five distinct sites. Two types of secondary lysosome were the main sites of this deposition. The first type was small, up to 1 µm in diameter, with a homogeneous, dense matrix except in those which also had denser regions within the matrix. These denser regions were not associated with the end product which was present throughout the rest of the lysosome (Fig. 65). The organelle was bounded by a single membrane and the amount of end product deposited was variable, some lysosomes exhibiting only a trace or none at all whilst others were almost completely filled (Fig. 65). The second type was the autolysosome. Particulate and membranous material and matter that was identifiable as of endogenous cytoplasmic origin was found within autolysosomes which were, in general, larger than the first type of secondary lysosome, being up to about 2 µm in diameter. The amount of end product deposition was variable. Most autolysosomes were bounded by a single membrane but certain of them (Fig. 70) were surrounded by

sections of double membrane in which end product occurred between the two membranes. Golgi complexes were also sites of end product deposition in both cell types although some complexes were totally devoid of activity. The end product was contained within Golgi saccules and vesicles (Figs. 65, 71). No-substrate control sections exhibited no deposition in lysosomes or Golgi complexes, showing that these organelles were true sites of acid phosphatase activity.

Lead deposits were also seen in the nucleus and cytoplasm of some cells (Figs. 67-69). Study of control sections showed that the nuclear lead was often present when the substrate was omitted so that its presence was not due to enzyme activity. The occurrence of cytoplasmic lead was more difficult to interpret. It occurred even when the Brunk and Ericsson modified incubation medium, which was designed to eliminate non-enzymically produced lead, was used and could be present in one cell but not its immediate neighbour or would be absent for one experiment but manifest itself in an identical repeat. This was typical of the capricious nature of the Gomori method, such behaviour having been encountered by Gomori (1952) and many other workers. However, by careful examination of controls it was possible to eliminate most of the cytoplasmic deposits as non-enzymic, bound lead. In a few significant cases, though, (Fig. 68) apparently genuine end product resulting from acid phosphatase activity occurred around, but outside, autolysosomes.

3T3 and SV40 3T3

An incubatiom time of 2 to 3 hours was required in order to produce deposition of end product comparable to that in BHK21 cells (i.e. 4 to 6 times longer). The distribution of lead deposits was essentially similar to that seen in the BHK21 cells, secondary lysosomes being the major sites of lead phosphate deposition. In addition residual bodies contained

end product. These organelles were most frequent in the 3T3 cells although those that were present in the SV40 3T3 cells tended to be much larger. Golgi complexes, which were more extensive in the 3T3 cells with greater development of components, also gave a positive reaction for the presence of acid phosphatase (Fig. 74). Nuclear and cytoplasmic lead occurred but similar deposits in the no-substrate control sections showed that all the nuclear lead and virtually all the cytoplasmic lead could not have been deposited as a result of enzyme action. A small number of SV40 3T3 cells had a distinct surface coat, up to 0.1 μm thick, which contained end product of acid phosphatase activity (Fig. 76). In places this coat had become detached from the cell surface. Its nature was unclear.

Normal and ts virus-infected chick embryo fibroblasts

Four categories of cells were used: uninfected (set up at 35°C, shifted to 41°C and then back to 35°C), ts 29 infected (maintained at 35°C), ts 29 infected (35-41-35°C) and ts 29 infected (35-41°C). The first and last categories were untransformed whilst the second and third were transformed. An incubation time of 30 minutes resulted in a level of end product deposition similar to that achieved in the BHK21 cells. Apart from the absence of surface deposits, the localisation of end product was similar to that seen in the hamster and mouse cells. Secondary lysosomes were the main sites of enzyme activity. In the transformed cells which had been maintained at 35°C there appeared to be little enzyme activity owing to the scarcity of secondary lysosomes but in the other three categories of cells end product deposits occurred in auto-lysosomes and small, dense, homogeneous secondary lysosomes. In a number of cases apparently intact, end product-containing secondary lysosomes were present amongst extracellular material (Figs. 82, 86) and

may have arrived there as a result of exocytosis (Fig. 83). Residual bodies exhibited only sparse deposits. There was no strong evidence to indicate that acid phosphatase activity was associated with virus particles or the vacuoles which contained them although in at least two cases there was a trace of end product in virus-containing organelles (Figs. 81, 84). Golgi saccules and vesicles also displayed lead phosphate deposition. Comparison of test and no-substrate control material showed that whereas lysosomes and Golgi were true sites of acid phosphatase activity, the nucleus and cytoplasm were not since lead deposits occurred at these sites after incubation in media containing no substrate.

4.1.2. Cells centrifuged to a pellet

BHK21 cells were utilised for this part of the study. When the cells had grown to confluence in Petri-dishes the culture medium was removed and replaced with PBS. The cells were then scraped off with a silicon rubber policeman and centrifuged at low g to a pellet which was fixed for 3 hours in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4, 4^{o}C). After fixation the pellets were washed overnight in several changes of buffer containing 4.5% sucrose and then incubated for 30 or 60 minutes in the Gomori medium, or in the Gomori medium minus substrate, followed by processing as previously described (p. 17) and embedding in Taab capsules. Ultrathin sections were cut and examined unstained in the electron microscope.

In addition to lead deposits in lysosomes, Golgi complex, nucleus and cytoplasm, similar sites to those seen in the flat-embedded material, many cells also had distinct deposits on the cell surface (Figs. 87, 88). These surface deposits were of irregular thickness and, unlike those encountered on the flat-embedded SV40 3T3 cells, were not obviously

associated with a surface coat. When no-substrate control sections were examined lead deposits were found in the nucleus and cytoplasm, indicating that these were not sites of enzyme activity in the test material. Cell surface lead was absent from the control cells in some experiments but in other, identical repeats surface deposits were found which were similar to those of the tests. In certain experiments the surface deposits appeared in the controls but not in the tests. Therefore, although some experiments suggested that the process of centrifugation had caused the appearance of acid phosphatase activity on the cell surface, others seemed to suggest that the surface had been changed in such a way that it sometimes simply bound lead. This situation highlights the necessity for constant and careful use of controls and also provides further evidence of the capricious nature of the Gomori technique. To obviate ambiguous results it is essential, when utilising the Gomori procedure, to ensure that experiments are repeated a number of times.

4.1.3. In situ incubation followed by centrifugation of cells to a pellet

Normal and SV40-transformed 3T3 cells were utilised in this section of the investigation. They were grown in 90 mm Nunc plastic Petri-dishes and when they had reached confluence the culture medium was removed and the cells rinsed in cacodylate buffer (0.1M, pH 7.4, 4°C) containing 4.5% sucrose prior to fixation for 30 minutes in 2.5% cacodylate buffered glutaraldehyde. The fixed cells were washed overnight in several changes of buffer with sucrose and then incubated for 90 minutes in either the complete Gomori medium or in the no-substrate control medium. At the end of the incubation period cells were rinsed in acetate buffer (0.2M, pH 5.0, 4°C), scraped from the plastic with a silicon rubber policeman and centrifuged to a pellet. Pellets were post-fixed for 2 hours in 1% osmium tetroxide at 4°C, ethanol dehydrated,

infiltrated and embedded in Araldite in Taab capsules. Ultrathin sections were cut from the trimmed, polymerised blocks and examined in the electron microscope.

The majority of cells appeared to have been sectioned in a vertical, longitudinal plane. They were narrow and elongated, the 3T3 cells being generally narrower than the SV40 3T3 cells. Of the two free surfaces, one was usually much flatter than the other and it was likely that this flatter surface was the one which had been in contact with the culture dish. As was the case in the surface embedded preparations of these cells the lead phosphate end product indicating acid phosphatase activity was found in secondary lysosomes (both the dense homogeneous type and autolysosomes), residual bodies and Golgi complexes (Figs. 89-92). The lysosomes and residual bodies were more frequent in the 3T3 cells but in the SV40 3T3 cells residual bodies tended to be larger. Nuclear and cytoplasmic lead was sparse and occurred in both test and no-substrate controls. It was, therefore, not produced as a result of enzyme activity. No surface coat containing end product deposits was found in the SV40 3T3 cells but a number of 3T3 cells had a distinct layer of end product upon their surface (Fig. 91). This surface end product may have been associated with a cell coat and occurred largely on the flatter of the cells' surfaces, the one which had probably contacted the culture dish. The cell coat/end product layer was approximately 0.08 μm thick. No-substrate control material did not exhibit the surface lead deposits.

4.2. Acid phosphatase demonstration using the LPED azo-dye procedure

This procedure was developed by Livingston et al. (1970) and applied initially to rat and mouse kidney tissue for the demonstration of acid phosphatase activity. The technique was subsequently utilised by Beadle et al. (1971) for the demonstration of acid phosphatase, non-specific esterase and β-glucuronidase activities in the midgut

epithelium of the mealworm (Tenebrio molitor), the desert locust (Schistocerca gregaria), and the stick insect (Carausius morosus). The principle of the method is exactly the same as that described on pp. 49-51 but in order that the azo-dye end product is detectable in the electron microscope the Fast Red Violet LB is replaced as coupling agent by LPED, the diazonium chloride of triphenyl-p-aminophenethyl lead. When LPED couples with an enzymically released naphthol a bright red azo-dye is formed which, since it contains lead, is also electron dense and, therefore, visible in the electron microscope. Details of the synthesis and chemical properties of LPED are given by Livingston et al. (1970).

BHK21 C13 and BHK21 J1 cells were grown on 20mm^2 Melinex slips. On the attainment of cell confluence the slips were removed from the culture medium, briefly rinsed in cacodylate buffer (0.1M, pH 7.4, 4^{o}C) containing 4.5% sucrose and then fixed for 30 minutes at 4^{o}C in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4). After fixation the cells were washed in several changes of buffer containing sucrose for 18 hours at 4^{o}C prior to incubation in a simultaneous coupling medium containing 20 mg-% naphthol AS-BI phosphate and 2.5 mg-% LPED in acetate buffer (0.2M, pH 5.0). Both substrate and LPED were dissolved in 0.25 ml DMF prior to addition of buffer. Incubation was for 30 minutes at 37^{o}C. A number of cell preparations were incubated in a medium containing no substrate. At the end of this period the cells were washed for 30 minutes in acetate buffer (0.2M, pH 5.0, 4^{o}C) and then post-fixed for 2 hours in 1% osmium tetroxide in cacodylate buffer (0.1M, pH 7.4, 4^{o}C) containing 4.5% sucrose, after which they were dehydrated in Durcupan and infiltrated with Araldite as previously described (p. 38). Durcupan dehydration was necessary because the azo-dye was sparingly

soluble in ethanol (Livingston et al., 1970). The cells were surface embedded in Araldite and silver-thin sections were cut from the trimmed, polymerised blocks. The sections were examined unstained in the electron microscope operating at 50 KV and with an objective aperture of 25 µm.

When cells were examined with the light microscope at the end of the incubation period those which had been incubated in the test medium exhibited red, particulate end-product deposition, similar to that produced in the studies using Fast Red Violet LB salt as the coupler (Figs. 60-64). Cells incubated in the no-substrate control medium exhibited no end-product. It was clear, therefore, that the enzyme cytochemical reactions had taken place successfully. When sections of test material were examined with the electron microscope very little end product was found to be present although a small amount was detected in certain secondary lysosomes. It was in the form of fine, dense particles. When the no-substrate control sections were examined apparent end product was found in identical situations to that in the test sections (Fig. 93). There was no evidence in either tests or controls of end product, or end product-like material, associated with organelles other than secondary lysosomes. The presence of these deposits in the lysosomes of the controls indicated, perhaps, that the LPED had an avidity for some component of the lysosomes. It was essential at this stage that these two problems should be investigated in some detail. Experiments were carried out, therefore, to study the loss of end-product during processing for electron microscopy and the occurrence of end product-like deposits in control material.

4.2.1. Loss of end product during processing for electron microscopy.

Observations with the light microscope of cells at each stage of

the processing after incubation in the test medium demonstrated clearly that it was during Durcupan dehydration that the loss was sustained. The particulate end product was prominent after osmication but became progressively fainter until by the time the pure Durcupan stage was reached, very little or no end product was visible. As this azo-dye procedure necessitated the use of a water-soluble dehydrating agent (Livingston et al., 1970) it was not possible to employ traditional agents such as alcohol or acetone. The only feasible approach was, therefore, to reduce the dehydration time to the minimum necessary to still retain ultrastructural integrity. All attempts to produce a dehydration schedule that would not lead to end product loss were, however, unsuccessful.

Livingston et al. (1970) pointed out that if excessively large deposits of azo-dye were allowed to form or if post-fixation was inadequate then loss of dye might take place during Durcupan dehydration. This did not prove a problem in their experimental tissues, however. In order to counter this possibility incubation times were reduced to produce smaller dye deposits and post-fixation times were varied. These measures failed to prevent the loss of the dye. In case this problem was peculiar to BHK21 cells or to this particular coupling salt, other cell lines and alternative coupling salts, such as diazotised lead phthalocyanin and p-nitrobenzene-diazonium-tetrafluoroborate, were utilised. Details of these experiments are given later (pp. 87,95) but in each case the azo-dye produced, as seen with the light microscope, was lost during dehydration. It seemed that solubilisation of azo-dye was facilitated to a much greater extent in the conditions presented by a layer of cultured cells than it was in blocks of tissue. Clearly, if these procedures were to be used for the study of enzymes in cultured cells a more favourable

processing scheme would be required. Recently a method using GACH, a water-miscible, lipid-retaining embedding polymer, has been described by Heckman and Barrnett (1973). As this obviates the use of ethanol, Durcupan and Araldite it could prove valuable for use with azo-dye procedures.

Although end product of the azo-dye methods could not be retained in monolayers of cells it has been shown by Beadle et al. (1974) that azo-dye could be retained in cells that had been centrifuged into a pellet prior to glutaraldehyde fixation. However, since this procedure results in both ultrastructural and cytochemical artifacts, as shown previously in the present study and also by Lucky et al. (1975), it was not utilised. Instead, cells were fixed and incubated in situ and then scraped from the culture dish and centrifuged at low g into a pellet which was post-fixed, dehydrated in Durcupan and embedded in Araldite. On examination in the electron microscope, cells prepared in this manner were found to contain little or no end product of the cytochemical test. This indicated that the loose pellet formed when fixed cells are centrifuged also allows solubilisation and extraction of azo-dye.

4.2.2. Occurrence of end product-like deposits in controls

Despite the lack of success in developing a method that would retain the azo-dye in cultured cells, the investigation of the occurrence of end product-like deposits in control material was still considered to be important in view of the possible wider implications of this phenomenon. Since cultured cells have their own unique problems it was decided to utilise mouse kidney and mealworm midgut tissues for this investigation. The use of these tissues, which were employed in the earlier LPED studies, would also indicate whether this phenomenon was peculiar to the cultured cells or was more general.

Kidneys were removed from freshly killed mice and cut into 1mm thick transverse slices from which small blocks of cortex tissue were obtained, of maximum size 1 mm^3. These operations were conducted in cacodylate buffer (0.1M, pH 7.4, 4^oC) containing 4.5% sucrose. The small blocks were fixed for 3 hours at 4^oC in 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4) and then washed for 18 hours in several changes of the cold buffer containing sucrose. After washing, the blocks of kidney were incubated for 30 or 60 minutes in the test and no-substrate control media, prepared as previously described (p. 79). Two additional control systems, sodium fluoride inhibition and heat inactivation of the enzyme, were also utilised. To achieve the former, sodium fluoride to a concentration of 10^{-3}M was added to the test medium whilst for the latter, glutaraldehyde-fixed tissue blocks were heated in water at 100^oC for one minute prior to incubation in the test medium. Post-fixation, dehydration and Araldite infiltration were also carried out as previously described (p. 38). Embedding in Araldite took place in Taab capsules. Silver ultrathin sections were cut from the trimmed, polymerised blocks and examined, unstained, in the electron microscope.

Mealworms (Tenebrio molitor) were decapitated, their alimentary tracts removed and placed in cacodylate buffer (0.1M, pH 7.4, 4^oC) containing 4.5% sucrose, where the midgut regions were separated. The midguts were cut into lengths of about 2mm and these were treated in a similar manner to the blocks of mouse kidney cortex. Transverse ultrathin sections of part of the midgut wall were cut and examined, unstained, in the electron microscope.

Examination of the kidney and midgut sections revealed an abundance of end product or end product-like material compared with that seen in the cultured cells. This suggested that loss of azo-dye or diazonium

salt by solubilisation in Durcupan was not significant in blocks of tissue whereas it was of serious proportions in the thin layers of cultured cells. It was noticeable that the test tissues reddened during incubation whilst the controls did not, indicating that end product was produced in the former but not the latter. In the test sections of mouse kidney cortex the end product was deposited in the form of fine, dense particles and occurred solely in secondary lysosomes. The deposits were of a homogeneous nature (Fig. 94), more irregularly distributed (Fig. 95) or associated with the membranous structures of myelin figures (Fig. 96). When the no-substrate, sodium fluoride inhibited and heat denatured control sections were examined they were found to have secondary lysosomes containing dense, end product-like deposits which were indistinguishable from those found in the test material (Fig. 97). Similar results were obtained when test and control sections of mealworm midgut were examined, the deposits being mainly associated with myelin figures (Figs. 98, 99). In both kidney and midgut material there were a great many secondary lysosomes which did not contain dense deposits.

The presence of lead deposits in the lysosomes of material incubated in media containing LPED alone suggested that LPED, or at least a lead-containing part of the molecule, had an affinity for secondary lysosomes and bound itself to them. The fact that these deposits appeared identical to those in the test material made interpretation of the tests impossible. Therefore attempts were made to prevent the occurrence of non-enzymically derived lead deposition.

The first of these attempts involved using a post-coupling rather than a simultaneous-coupling system. When the latter is utilised the tissue is in the presence of LPED for 30 or 60 minutes and this might not only cause some inhibition of the enzyme but might also facilitate the

binding of the diazonium salt to secondary lysosomes. When post-coupling is used tissues are in contact with the diazonium salt for a much shorter period regardless of the incubation time. Mouse kidney and mealworm midgut tissues were prepared as previously described (p. 83) and incubated in the test and control media without LPED. After incubation they were transferred to a solution containing 2.5 mg-% LPED in acetate buffer (0.2M, pH 5.0) where they were treated for 10 minutes at $4^{o}C$ and then rinsed in acetate buffer for 30 minutes followed by processing as described previously (p. 38).

Secondly, extended post-incubation washing was employed in the hope that this procedure would remove uncoupled LPED whilst having no effect on the azo-dye produced as a result of the cytochemical reactions. After incubation in test and control media, mouse kidney and mealworm midgut tissues were washed for periods varying from 1 to 48 hours in acetate buffer (0.2M, pH 5.0, $4^{o}C$) prior to processing for electron microscopy.

If the apparent affinity of LPED for lysosomes was due to the fact that it was a diazonium salt, as opposed to its being a lead or heavy metal containing diazonium salt, then it could be assumed that a non-heavy metal containing diazonium salt might also have such an affinity. If this was the case, pre-incubation of tissues with a non-heavy metal containing diazonium salt might saturate all the available sites for diazonium attachment so that LPED would be unable to bind and hence the controls would be negative and in the tests only LPED coupled to the substantive naphthol AS-BI would be detected at the sites of enzyme activity. Prior to incubation in test and control media, therefore, the mouse kidney and mealworm midgut tissues were pre-incubated for 30 minutes at $37^{o}C$ in a solution containing 30 mg-% Fast Red Violet LB salt.

Incubation and processing were carried out as previously described (pp. 79, 38).

Examination of the test and control sections produced in these three experiments showed that in every case secondary lysosomes could be found which contained the characteristic deposits of dense end product-like material. The nature and quantity of this deposition appeared to be similar to that seen in the previous experiments (pp. 83,84). These attempts to prevent the occurrence of non-enzymically derived lead deposition were, therefore, unsuccessful.

A further measure which was considered was the use of formol-calcium fixation in place of glutaraldehyde. Smith and Fishman (1969) found that the use of glutaraldehyde often led to uneven fixation and caused greater enzyme inhibition than formol calcium, especially when used in conjunction with cacodylate buffer. In addition they found that in no-substrate controls, glutaraldehyde-fixed tissues developed a stronger avidity for a mercury-containing diazonium coupling salt, p-(acetoxymercuric) aniline diazotate, than did formalin fixed tissues and hence exhibited greater background metal deposition. Complete absence of background was never achieved, in spite of a variety of precautions, and deposition occurred in lysosomes and along membranes. A trial of formol-calcium fixation of the material under investigation was carried out. Mouse kidney, mealworm midgut and BHK21 C13 cells were fixed in formol-calcium at pH 7.4 for 18-20 hours at 4^{o}C. They were then incubated in test and control media and processed for electron microscopy as previously described (p. 38). On examination of the tissues it was found that the preservation of ultrastructure was poor, resulting in amorphous, disorganised material in which no definate end product could be positively identified (Fig. 100), although the reaction

had been successful as the tissues in the test medium had reddened during incubation. The use of formol-calcium fixation, therefore, was not pursued.

The experiments conducted in the investigation of the occurrence of end product-like deposits in controls showed that, in spite of a number of precautions, when tissues were introduced to a solution of LPED these deposits were consistently encountered when the tissues were subsequently examined in the electron microscope. The deposits were, characteristically, confined exclusively to secondary lysosomes and this highly specific deposition contrasted with the more general cytoplasmic and nuclear lead deposits which often accompanied the Gomori method. It was not clear whether the apparent avidity for secondary lysosomes was peculiar to LPED or was a property common to related compounds such as other lead-containing diazonium salts, heavy metal-containing diazonium salts or even diazonium salts in general. It was also possible that a particular constituent of the LPED molecule, such as the phenyl, triphenyl or phenethyl group might have been responsible for the avidity. Since Smith and Fishman (1969) have shown that a mercury containing diazonium salt, p-(acetoxymercuric) aniline diazotate, exhibited a similar avidity, this suggested that the phenomenon was not confined to compounds containing lead. For the further investigation of this problem several experiments were carried out.

4.2.3. Avidity of various compounds for secondary lysosomes.

In order to ascertain whether other lead-containing diazonium salts exhibited an avidity for secondary lysosomes a procedure for acid phosphatase demonstration was carried out utilising naphthol AS-BI phosphate as substrate and diazotised lead phthalocyanin as coupling agent. This procedure, described by Tice and Barrnett (1965) and

subsequently modified by Livingston et al. (1969), was performed upon glutaraldehyde fixed mouse kidney cortex blocks, prepared as previously described (p. 83). The tissue was incubated for 90 minutes at 37°C in a medium containing 20 mg-% naphthol AS-BI phosphate in acetate buffer (0.2M, pH 5.0) or in buffer alone (no-substrate control) and then post-coupled for 10 minutes at 4°C in diazotised lead phthalocyanin prepared as follows: 100 mg of lead phthalocyanin were suspended in 3 ml of 20% hydrochloric acid at 0-5°C with stirring. To this were added 2 ml of 1% sodium nitrite (0.5 ml at a time over 5 minutes). The diazotisation was complete in 10 minutes and the solution filtered. The pH was adjusted to 5.0 with cold 1M sodium hydroxide and the solution diluted to 10 ml with 0.2M acetate buffer at the same pH. On completion of the coupling step the tissue blocks were rinsed in acetate buffer, post-fixed in 1% cacodylate-buffered osmium tetroxide for 2 hours, dehydrated through Durcupan and embedded in Araldite as previously described (p. 38). Ultrathin sections were cut and examined, unstained, in the electron microscope.

The appearance of the sections was very similar to that of sections of LPED-treated tissue. In both tests and controls secondary lysosomes were found which contained deposits of fine, dense, particulate material (Figs. 101-104). It appeared, therefore, that diazotised lead phthalocyanin also had an avidity for secondary lysosomes and, like LPED, occurred at no other sites.

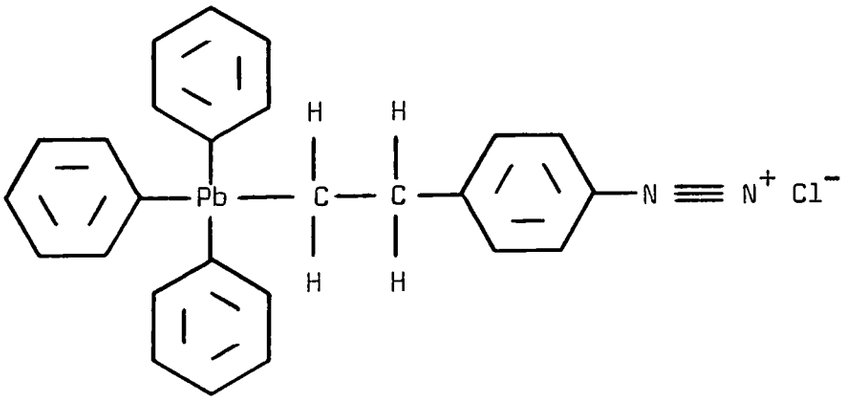
By incubating blocks of mouse kidney cortex in the presence of stable precursors of LPED it was possible to determine whether related non-diazonium compounds exhibited the avidity for secondary lysosomes and also to gain some indication of which constituent part or parts of the LPED molecule might be responsible for the avidity. The two stable

precursors in question were hexaphenyl lead and triphenyl-p-amino phenethyl lead. Media were prepared containing 2.5 mg-% of these compounds in acetate buffer (0.2M, pH 5.0). Blocks of glutaraldehyde-fixed mouse kidney cortex were incubated for 30 minutes at 37°C in these media and subsequently processed for electron microscopy as previously described (p. 38). On examination of ultrathin sections of material incubated in hexaphenyl lead and material incubated in triphenyl-p-amino phenethyl lead it was found that some secondary lysosomes contained the characteristic end product-like deposits which, although less abundant, were similar to those observed in tissues treated with LPED and diazotised lead phthalocyanin (Figs. 105, 106).

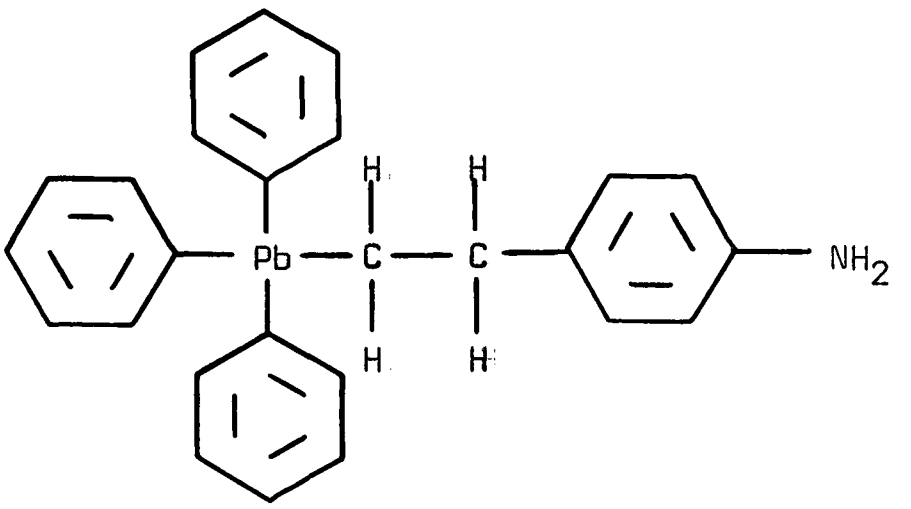
The experiments carried out so far, therefore, have shown that four compounds, whose structures are illustrated in Fig. 4.2.3.1., appeared to have an avidity for secondary lysosomes since when tissues were treated with these compounds fine, dense, particulate deposits were found exclusively in secondary lysosomes. The deposits withstood washing and processing for electron microscopy, suggesting that there was a reasonably strong bond between the compounds and the lysosomes.

Fig. 4.2.3.1. Structures of compounds having an avidity for secondary lysosomes.

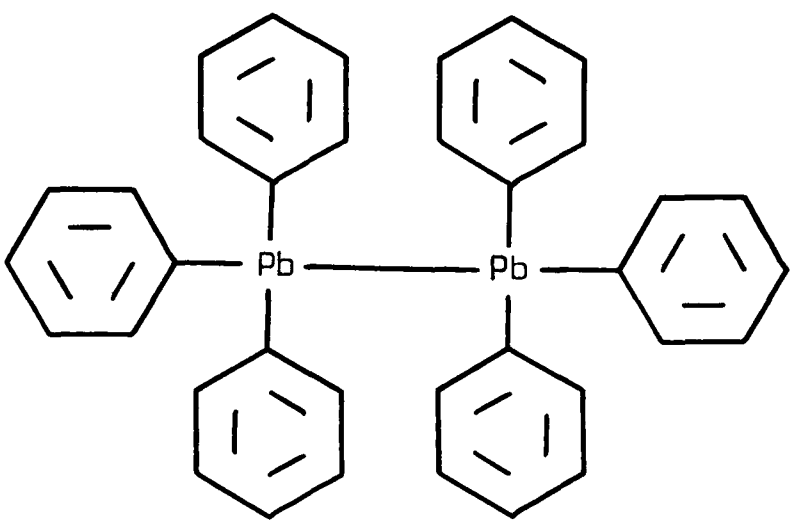
LPED (the diazonium chloride of triphenyl-p-amino phenethyl lead)



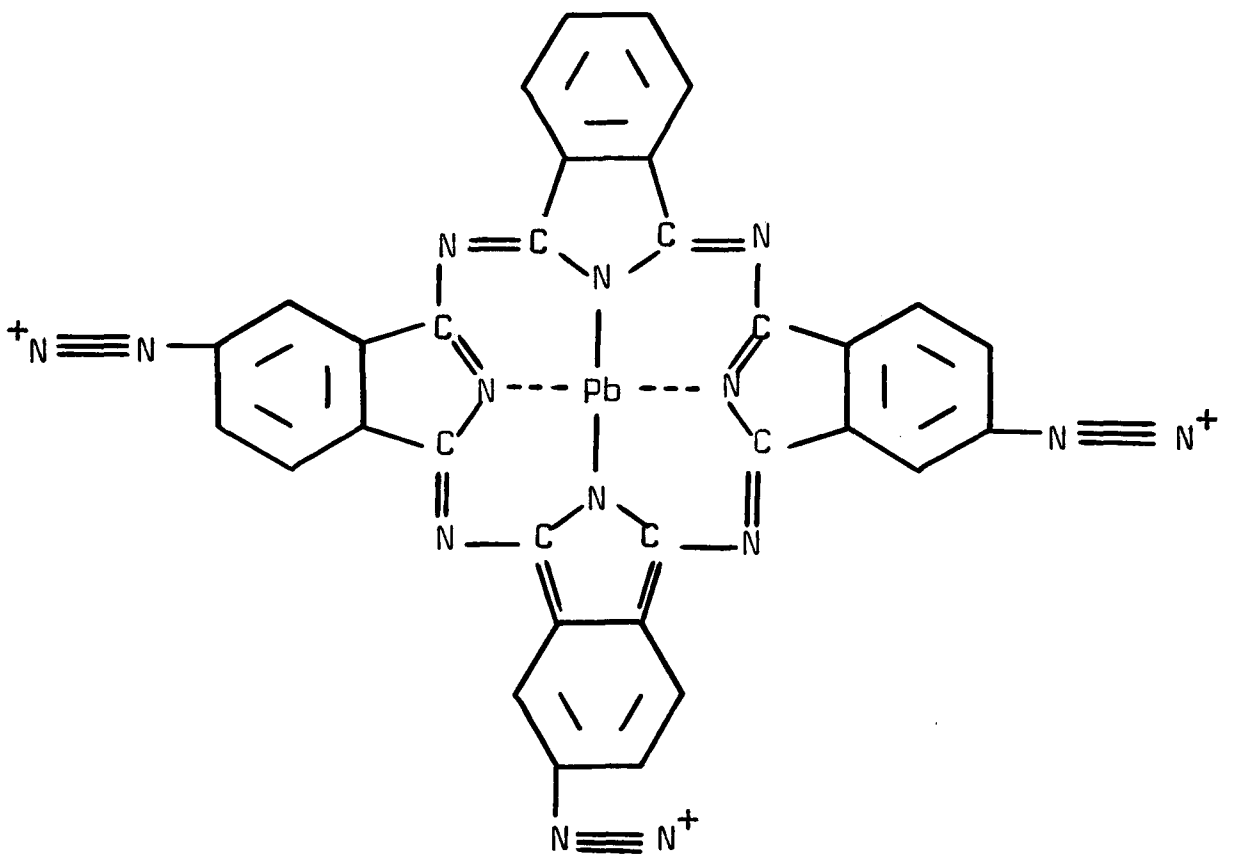
Triphenyl p-amino phenethyl lead



Hexaphenyl lead

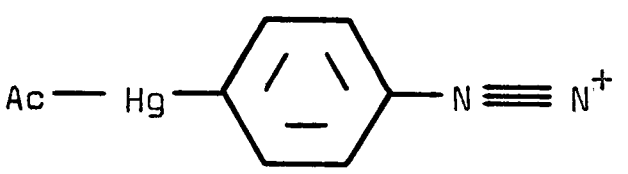


Diazotised tri (4-amino) lead phthalocyanin



It can be seen that each of these compounds possesses not only at least one lead atom in the molecule but also a number of aromatic benzene ring structures. The coupling salt employed by Smith and Fishman (1969), p-(acetoxymercuric) aniline diazotate, is a much smaller molecule but is similar in that it has a heavy metal atom and a benzene ring structure (Fig. 4.2.3.2.).

Fig. 4.2.3.2. Structure of p-(acetoxymercuric) aniline diazotate.



All these compounds have features which might be responsible for an attraction to cellular components. Burstone (1962) has mentioned several ways in which dyes may be held in tissue sections, including chemical combination with tissue proteins, Van der Waal's forces, hydrogen bonding and the formation of insoluble pigment particles which are mechanically held in the tissue section, and it is possible that one or more of these factors could be operating in the present situation. Van der Waal's attraction is likely between aromatic nuclei and hydrophobic protein chain molecules whilst hydrogen bonding could take place between peptide groupings of protein and amino or azo groups. It has been reported (Hugo, 1967) that triphenyl methane dyes are lipid soluble and can penetrate lipid membranes. This might explain the presence of the compounds possessing the triphenyl arrangement (viz LPED, hexaphenyl lead and triphenyl-p-amino phenethyl lead) at lipid-rich sites such as myelin figures. There is also the possibility of attraction of heavy metals by sulphydryl groups in membranous structures. The fact

that LPED has aromatic nuclei, an azo group, the triphenyl arrangement and a lead atom means that all the above mentioned potential sources of attraction are present and one or a combination of them could, therefore, be acting. This does not, however, explain why LPED and the other compounds used in the investigation were localised exclusively in secondary lysosomes. A localisation at other sites, particularly membranes, would be expected if these attractive forces were operating. Smith and Fishman (1969) obtained lysosomal and general membrane localisation in material incubated in the presence of p-(acetoxymercuric) aniline diazotate whilst Beadle et al. (1974) reported that when lead phthalocyanin was used as coupling salt in ripe fruits and in insect midgut it was found in the Golgi apparatus and a variety of vacuoles in tests and controls of the former and in lysosomes, Golgi, endoplasmic reticulum and nuclear membrane in both tests and controls of the latter. It is possible that, in the present situation, LPED was, in fact, localised at a number of sites in the controls but during processing was removed from all of them except the lysosomes where it was more tightly bound, perhaps because of a high lipid content or some other feature of the lysosomal environment. The presence in the lysosomal membrane of high levels of cholesterol and sphingomyelin, lipids which are virtually absent from all other cytomembranes except the plasma membrane (Thines-Sempoux, 1973), could also be significant.

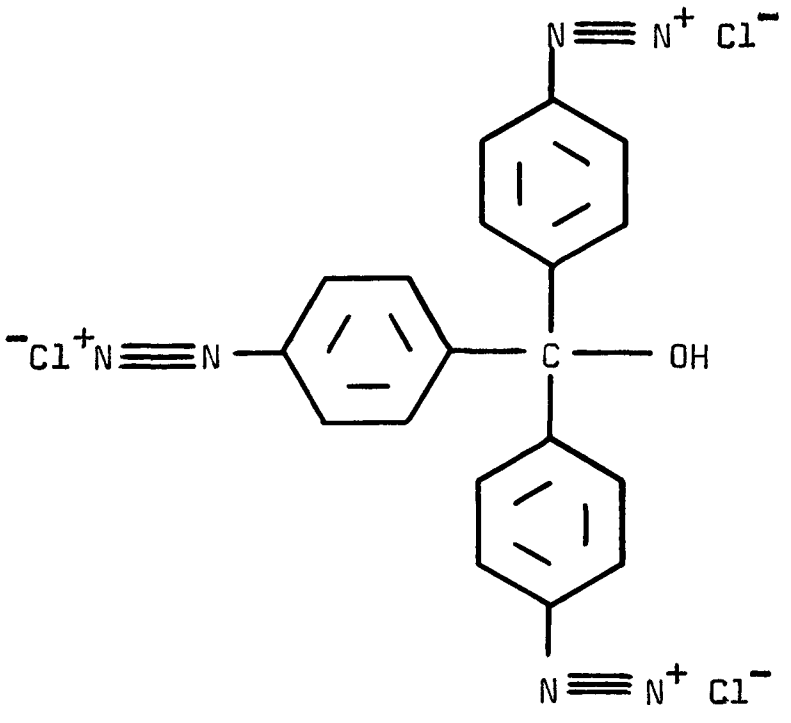
A further problem to be considered is why the phenomenon of the appearance of the end product-like deposits in control material was not encountered in the earlier LPED studies of Livingston et al. (1970) and Beadle et al. (1971). The coupling salt was synthesised from the stock amine preparation of Livingston et al. (1970) and the described procedure for its use was closely followed, although slight procedural differences

may have gone unnoticed. If the LPED had started to break down for some reason then it is possible that the lead became exposed and free to bind with tissue components. In the intact molecule all the valencies of lead are taken up so that it should not be reactive.

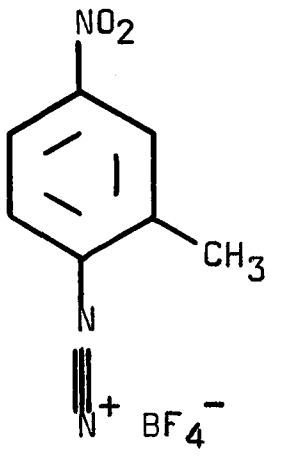
Not until the problems inherent in the use of heavy metal-containing diazonium salts are solved can the azo-dye techniques which utilise these compounds be applied with confidence. Bowen (1973) maintains that the incorporation of lead into a diazonium salt means that nonspecific staining similar to that obtained with the Gomori lead-salt technique can occur and that particular attention must be given to no-substrate controls. He further adds that metal-based azo-dye methods are on the whole as open to similar criticisms as the Gomori-based methods are. The procedures advocated by Bowen (1968, 1971, 1973) utilise hexazonium pararosaniline (HPR) or p-nitrobenzene diazonium tetrafluoroborate (NDFB) as coupling salts. The structures of these compounds are illustrated in Fig. 4.2.3.3.

Fig. 4.2.3.3. Structures of HPR and NDFB.

Hexazonium pararosaniline (HPR)



p-nitrobenzene diazonium tetrafluoroborate (NDFB)



When coupled with an enzymically released Naphthol AS the HPR gives an amorphous end product whilst the NDFB end product is crystalline when viewed in the electron microscope. Bowen (1973) expresses reservations about the use of the HPR technique. The end product is amorphous and electron opaque and leads to a slight increase in the density of the organelles with which it is associated. Interpretation of the results depends upon a laborious comparison of the relative density of organelles and necessitates very careful parallel recording of test and control sections. Since certain organelles and lysosomes in particular often exhibit a wide range of densities (see Figs. 52-55 for example) there could be difficulties in establishing whether end product was present. The published photographs of NDFB end product, however, show it to be much more distinctive, having a very fine particulate appearance reminiscent of LPED. It was decided to apply these two techniques to cultured cells and mouse kidney in order to assess the feasibility of using them for further experiments and also to determine whether HPR and NDFB exhibited any avidity for secondary lysosomes or other sites. Neither compound possesses a heavy metal but both have at least one azo group and a benzene ring structure which could conceivably be attracted to cellular

components. The enzyme to be examined was acid phosphatase.

Glutaraldehyde-fixed preparations of normal and transformed BHK21 and 3T3 cells and of mouse kidney cortex were prepared as previously described (pp. 72, 83). They were incubated in media prepared in the following manner:

Naphthol AS-BI phosphate/hexazonium pararosaniline method (after Bowen, 1968)

A solution containing 20 mg-% Naphthol AS-BI phosphate was prepared by dissolving 10 mg of substrate in 0.25 ml DMF and 50 ml acetate buffer (0.2M, pH 5.0). To this was added 2 mM hexazonium pararosaniline, made up immediately before use from two solutions:

Solution A: 1 gm of pararosaniline (Taab Laboratories Ltd.) was dissolved in 30 ml hot 2M HCl, cooled and filtered.

Solution B: 1 gm of sodium nitrite was dissolved in 30 ml distilled water.

Equal volumes of A and B were mixed. Diazotisation was completed in about a minute at room temperature and resulted in a clear straw-coloured solution.

Cells and kidney blocks were incubated in the complete medium for 30 or 60 minutes at 37°C or in a control medium containing no substrate.

Naphthol AS-BI phosphate/p-nitrobenzene-diazonium tetrafluoroborate method (after Bowen, 1971).

To 50 ml of a solution containing 20 mg-% Naphthol AS-BI phosphate (as above) were added 30 mg of p-nitrobenzene diazonium tetrafluoroborate (Eastman Kodak Co.). Cells and kidney blocks were incubated in this medium or in a no-substrate control medium for 30-150 minutes at 37°C.

On completion of incubation the material was rinsed, post-fixed in 1% buffered osmium tetroxide, dehydrated through a graded ethanol series,

infiltrated with Araldite and surface embedded in Araldite, all as previously described (p. 25). Ultrathin sections were cut and examined unstained in the electron microscope.

It had been observed that at the end of the incubation period the blocks of kidney from the test medium had reddened whilst those from the no-substrate control medium had not. This suggested that the enzyme cytochemical reaction had been successful. Likewise, when the cultured cells were examined in the light microscope after incubation, those from the test medium exhibited particulate deposition of red azo-dye whilst those from the no-substrate control medium did not. These deposits in the cells from the test medium were still distinct after osmication but during the subsequent alcohol dehydration and Araldite infiltration they became progressively fainter until they were no longer visible. When the ultrathin sections of the cells were examined in the electron microscope no definite evidence of end product was detected in any of the preparations. It appeared that, as in the case of the earlier LPED experiments, diazonium salts and azo-dyes were readily solubilised and extracted from cell monolayer preparations. When sections of the mouse kidney cortex were examined, however, there was evidence of deposition of end product or end product-like material in some of the secondary lysosomes although it was difficult to identify with certainty, particularly in the case of the HPR material. The deposits in the tests and no-substrate controls of the NDFB material were fine and particulate, associated mainly with myelin figures and closely resembled LPED deposits (Figs. 107, 108) whilst the secondary lysosomes of the HPR tissues exhibited, in both tests and controls, components, largely membranous, of varying density and also a certain amount of fine particulate deposition, not normally characteristic of HPR

(Figs. 109-113). It is possible that the appearance of these lysosomes was not due to the presence of HPR and that they were examples of a lysosomal population which displayed great variation in density of its components. An examination of sections of kidney which had not been subjected to cytochemical tests endorsed this possibility since lysosomes of varying density, some containing dark particulate matter, were observed (Fig. 114).

These experiments showed that the azo dye procedures utilising HPR and NDFB as coupling salts led to the formation of end product which was lost from monolayer cell preparations during dehydration. When the procedures were applied to blocks of mouse kidney cortex end product was difficult to identify and there was evidence that end product-like deposits occurred in no-substrate controls. Both procedures were, therefore, subject to the same problems as was the LPED technique. It was clear that the forces which bound the various diazonium salts to secondary lysosomes were not strong enough to resist the extraction which occurred during processing of cell monolayers for electron microscopy. When Pitt (1975) utilised the NDFB procedure with plant material he found it to be of only limited value since even using a wide range of substituted naphthol substrates the azo-dye chromogens were soluble in both Araldite and Durcupan embedding media. The lead phosphate end product of the Gomori method, however, was completely unaffected by processing for electron microscopy and this technique, despite its often capricious nature, could be relied upon to demonstrate reasonably unambiguously the localisation of acid phosphatase in <u>in situ</u> cultured cell preparations. As the present investigation has shown that the azo-dye techniques currently available did not appear to be suitable for the demonstration of the hydrolytic

enzymes of cultured cells at the ultrastructural level, then alternative procedures such as the metal-salt techniques of which the Gomori method is an example, had to be utilised where possible. Enzymes such as β-glucuronidase, which can only be demonstrated by azo-dye methods, could not be further examined. One of the alternative procedures, the thiolacetic acid method for esterase, has already been referred to (p. 70). Another metal-salt technique which was applicable to electron microscopical studies was the barium-nitrocatechol sulphate method for arylsulphatase (Hopsu-Havu et al., 1967). Although this enzyme had not previously featured in the investigation it was decided to include it at this stage since it was a lysosomal hydrolase which could be demonstrated by a metal-salt technique, a type of procedure which has been shown to be successful when applied to cultured cells. As the number of enzymes which could be demonstrated was now severely limited, the inclusion of a demonstration of arylsulphatase activity would broaden the scope of the investigation.

4.3. The thiolacetic acid method for esterase.

This procedure was applied to normal and transformed BHK21 cells.

The principle of the procedure is that hydrolysis of thiolacetic acid by the esterase liberates hydrogen sulphide which, in the presence of lead ions, is converted to lead sulphide. Four variations of the technique were utilised. The first was that of Wachstein et al. (1961) which was based on that of Crevier and Belanger (1955), also used. Both these methods were derived for light microscopical work but are applicable at the electron microscope level. The other two techniques, those of Barrnett (1962) and Barrnett and Palade (1959), were designed for electron microscopical use, though still based on the original Crevier and Belanger (1955) method. Cells were cultured on Melinex

squares and prepared for incubation as previously described (p. 72).

The four incubation schemes were as follows:

Wachstein et al. (1961) procedure

The incubation medium was prepared from two solutions:

Solution A: 0.15 ml of thiolacetic acid was pipetted into 5 ml of water and brought to pH 5.5 with 0.1M sodium hydroxide. 0.2M acetate buffer at pH 5.5 was then added to bring the total volume to 100 ml.

Solution B: 500 mg of lead nitrate was dissolved in 100ml of distilled water.

1 ml of solution B was added drop by drop to 20 ml of solution A. After short standing the solution was centrifuged, filtered and the clear supernatant used. No substrate control medium contained no thiolacetic acid. Cells were incubated for 15-120 minutes at room temperature. The test incubation medium was replaced every 15 minutes by fresh medium to minimise the formation of spontaneous precipitates which were a hazard with this procedure.

Crevier and Belanger (1955) procedure

The test medium was prepared by dissolving thiolacetic acid (0.12 M) and lead nitrate (0.001M) in 83 ml of 0.1M disodium hydrogen ortho-phosphate and adding 17 ml of McIlvaine phosphate-acetate buffer at pH 6.2. The control medium contained no thiolacetic acid. Cells were incubated for 30-60 minutes at room temperature.

Barrnett (1962) procedure

Test medium was prepared in the following manner:

0.25 ml of thiolacetic acid was titrated to pH 7.2-7.4 with 1M sodium hydroxide and enough 0.05M cacodylate buffer at the same pH was added to bring the volume to 20 ml. 48 mg of lead nitrate (0.006M) in 5 ml of

cacodylate buffer were added slowly, with constant stirring, to the previous ingredients. The complete medium was then filtered. No substrate control medium was prepared without thiolacetic acid. All the liquids used were pre-cooled to 1°C. Incubation was carried out in the cold (1°C) until the first sign of a staining reaction was seen with a dissecting microscope. This was normally after 30 minutes.

Barrnett and Palade (1969) procedure

The incubation medium was basically as above except that a pH of 6.0 was used and lead carbonate was substituted for lead nitrate. Incubation was performed at 37°C for 10 minutes.

At the end of the incubation periods cells were prepared for electron microscopy as previously described (p. 72). Ultrathin sections were cut and examined unstained in the electron microscope.

During the course of all incubations some spontaneous precipitate formation occurred. This was in the form of fine black particles and was most prevalent at room temperature or 37°C. When sections were examined it became clear that the frequent changes of incubation medium had not prevented the formation of the precipitate within the cells. It appeared as randomly dispersed, comparatively large lumps of very dense material (Fig. 115). Despite the presence of varying amounts of precipitate, true end product of thiolacetic acid esterase activity was detected in all but the Crevier and Belanger preparations which, significantly, were subject to greatest precipitation. The clearest results were obtained with the Wachstein et al. procedure (Fig. 115). The end product, which was absent from the no-substrate control sections, was in the form of very fine dense particles and was quite distinctive in the unstained preparations. It was localised within cisternae of the endoplasmic reticulum, the mitochondrial matrix,

secondary lysosomes (Fig. 115) and also within Golgi saccules and vesicles. This distribution appeared to be common to both normal and transformed cells.

4.4. The barium-nitrocatechol sulphate method for arylsulphatase (Hopsu-Havu et al., 1967)

This technique was applied to normal and ts virus infected chick embryo fibroblasts and also to 3T3 and SV40 3T3 cells. The chick embryo fibroblasts were of three types: normal uninfected at 35°C, ts 29 infected at 41°C and ts 29 infected at 35°C, only the latter being transformed. They were grown to confluence on Melinex squares and prepared for incubation as previously described (p. 72). After incubation in the manner detailed below the cells were processed for electron microscopy, also as previously described (p. 25). 3T3 and SV40 3T3 cells were subjected to in situ incubation followed by centrifugation to a pellet. With the exception of the incubation this procedure was carried out exactly as described for the Gomori method (p.77). The preparations were incubated for 60 minutes at 37°C in medium made up as follows: 160 mg of 2-hydroxy-5-nitrophenyl sulphate (p-nitrocatechol sulphate) were dissolved in 4 ml of distilled water. To this were added 12 ml of 0.1 M acetate buffer at pH 5.5 and 5 ml of 5% barium chloride. The pH was adjusted to 5.5 with 0.2 M acetic acid. Some preparations were incubated in control medium containing no substrate.

The hydrolysis of p-nitrocatechol sulphate by arylsulphatase leads to the release of sulphate which is "captured" by barium ions in the incubation medium, thus leading to the formation of insoluble barium sulphate, the end product of the procedure. When preparations were examined in the electron microscope this end product was found in the

test sections as dense black deposits. No-substrate controls were devoid of such deposits.

In the chick embryo fibroblasts arylsulphatase activity, as indicated by the end product deposition, was localised at several sites, chiefly the various types of secondary lysosomes which were most abundant in the perinuclear region (Figs. 116-118). Homogeneous dense bodies, multivesicular bodies, signet ring lysosomes and autolysosomes all exhibited some degree of deposition although by no means the whole population of these secondary lysosomes gave a positive reaction. The non-transformed cells, particularly the uninfected ones, had much larger populations of these organelles and consequently appeared to have higher overall levels of enzyme activity. It was noticeable that in most cases the end product was concentrated into discrete, rounded zones within the lysosomes and could occupy up to a third of the area, though usually less. In the signet ring lysosomes (Fig. 119) end product was found between the two membranes. Some residual bodies contained end product as also did a number of the organelles, presumably secondary lysosomes, which held virus particles (Fig. 121). Myelin figures which occurred in residual bodies and some autolysosomes often appeared very dense but whether or not this density was imparted by end product deposition was not certain. The only other site of enzyme activity was the inner surface of the large vacuole-like bodies which occurred in certain cells (Figs. 118, 119, 122). There was no evidence of activity within Golgi complexes.

In the normal and SV40 transformed 3T3 cells end product of arylsulphatase activity appeared to be present at three other sites in addition to those in which it was observed in the chick cells. These were the Golgi complex, lipid droplets and cell surface. In the Golgi

complex the cisternae of a small number of saccules contained very dense material which may have been end product. The dense material in lipid droplets was almost certainly end product and was localised on the inner surface of the limiting membrane where it formed a discontinuous layer, sometimes with also a larger area of more intense activity. At the cell surface there were a number of small, discrete aggregations of very dense material, possibly end product, which were usually found where two cells were separated by a narrow intercellular space (Fig. 127).

4.5. Discussion

In this section of the investigation a number of cytochemical techniques for the demonstration at the ultrastructural level of certain acid hydrolases were applied to the cultured cells. These techniques were of two types, the azo-dye procedures and the metal-salt methods. Each of the azo-dye procedures was in theory applicable to a whole range of enzymes, provided suitable substrates were available, but as it proved impracticable to utilise these techniques, for the reasons previously discussed (p. 97), only enzymes which could be demonstrated by metal-salt methods were studied. These enzymes were acid phosphatase, thiolacetic acid esterase and arylsulphatase.

As so few enzyme cytochemical studies have been applied to cultured cells by other workers there are few results with which to compare the findings of the present investigation. No other work at all has been traced which compares, by means of cytochemical methods, enzymes of normal and virus-transformed cells. All three enzymes have, however, been studied in a variety of animal tissues. Only acid phosphatase has been cytochemically demonstrated in cultured cells. Brunk and Ericsson (1972) showed that in cultured human diploid glia

cells and embryonic rat fibroblasts this enzyme was associated exclusively with lysosomes, Golgi elements and related structures whilst Wilson (1973) reported that, in addition to these sites, acid phosphatase was present on the surface of spontaneously transformed mouse fibroblasts. Maciera-Coelho et al. (1971) demonstrated the presence of the enzyme in the cytoplasm around vacuoles that appeared in cultured human embryonic diploid cells that had been maintained in the resting phase for several days. Acid phosphatase localisation at all these sites was demonstrated in the present study. Non-specific esterase, which is demonstrated by the thiolacetic acid method, has not been studied in cultured cells but several workers have shown it to be present in various animal tissues at similar sites to those in which it was localised in the BHK21 cells. Deimling and Madreiter (1972) in particular have demonstrated the presence of this enzyme in a wide range of situations in liver and kidney. All the studies indicate that non-specific esterase is a ubiquitous enzyme. In the chick embryo fibroblasts arylsulphatase activity was localised only in lysosomes and related structures, a distribution which agrees with the findings of Hopsu-Havu et al. (1967) who studied this enzyme in cells of the proximal convoluted tubule of the rat. In the 3T3 cells it appeared that arylsulphatase activity might also be associated with lipid droplets, Golgi and cell surface. Rappay et al. (1973), studying the adrenal cortical cells of the rat, demonstrated this enzyme in lysosomes, Golgi elements and in the endoplasmic reticulum where this organelle was close to the cell membrane or the nuclear membrane. They also found barium precipitate around lipid droplets but suggested that this deposition was non-specific since it was also observed in no-substrate control material.

Acid phosphatase was the only enzyme studied in all three cell systems and is, therefore, the only one whose levels of activity can be compared between the three cell types. In the BHK21 cells and the chick embryo fibroblasts a similar level of end product deposition was achieved after 30 minutes incubation but in the normal and transformed 3T3 cells this level of deposition was only achieved after incubation for 4 to 6 times longer, suggesting that acid phosphatase has a lower level of activity in the latter cells.

For any one enzyme, no differences in localisation were found when a normal cell line was compared with its transformed counterpart, unless the acid phosphatase activity associated with the surface coat in SV40 3T3 cells is regarded as distinct from the activity observed on the surface of 3T3 cells. Enzyme differences were in terms of level of activity rather than of localisation. As the chief sites of acid phosphatase and arylsulphatase activity were the lysosomes it appeared that the cells with highest activity were those with the most extensive lysosomal system. This was, however, partially misleading since although 3T3 cells had far more lysosomes per cell than SV40 3T3 cells and appeared to have a higher level of acid phosphatase activity, the light microscopic, microdensitometric and biochemical results showed that the SV40 3T3 cells had a higher level of enzyme activity even though there were fewer lysosomes. The work of Horvat and Acs (1974) appears to confirm that, for arylsulphatase at least, the level of enzyme activity is proportional to the extent of the lysosomal system. They demonstrated that the activities of three lysosomal acid hydrolases, including arylsulphatase, were ten times greater in stationary 3T3 cells (i.e. cells in a confluent monolayer) than in non-stationary proliferating cells. Normal chick embryo fibroblasts, like 3T3 cells,

have far more lysosomes than their transformed counterparts and this larger population appeared to endow the cells with greater arylsulphatase activity, in agreement with the results of Horvat and Acs (1974).

This study has shown that electron microscopic enzyme cytochemistry in its present state of development cannot be reliably used on its own for comparative studies of hydrolytic enzymes in normal and transformed cells since it can only be confidently applied to a limited range of enzymes and even then may prove misleading. When used in conjunction with light microscopic enzyme cytochemistry, microdensitometry and biochemistry, however, an unambiguous picture of the status of these enzymes can be obtained.

DISCUSSION

The aim of this investigation was to relate changes in ultrastructural organisation occurring when normal cells are transformed by tumour virus to any changes in enzyme activity. It was intended to achieve this mainly by combining a morphological survey of several cell lines with a cytochemical examination of a number of hydrolytic enzymes. The results of such a study might then help to determine the role of enzyme and ultrastructural changes which occur when a normal cell *in vivo* becomes a cancer cell.

When glutaraldehyde-fixed cell cultures were examined, unstained, by phase-contrast light microscopy it was found that all the cell lines exhibited the characteristics described by the originators of the lines and by other workers who have studied them. The nature of the cells did not appear to have changed throughout their histories in culture. Significant features which were emphasised were the sensitivity of 3T3 cells to density-dependent inhibition of growth and division, the similarity of proliferating normal cells to transformed cells and the temperature dependence of transformation in ts virus-infected chick embryo fibroblasts. Light microscope-level descriptions of cultured cell growth and morphology have appeared frequently in the literature but this is not true of ultrastructural studies. Although a number of workers have examined various aspects of cultured cell ultrastructural morphology there is a lack of comprehensive comparative investigations of normal and transformed cell fine structure. The present study has, therefore, incorporated such an investigation of three cell systems.

A number of procedures have been described for the preparation of cultured cells for electron microscopy. A method similar to that of Firket (1966) was utilised. This was an *in situ* embedding technique

which involved growing the cells on Melinex polyester film, allowing the preservation of spatial relationships and ultrastructural integrity. Such a technique was essential since it had been shown that if cells were detached from the substratum and centrifuged to a pellet prior to fixation and processing, they exhibited artifacts and distortion. Similar findings were subsequently published by Lucky et al. (1975). Morphological artifacts in a pellet of cells were also found to be accompanied by enzyme cytochemical artifacts since when the Gomori method for acid phosphatase was applied to a pellet of BHK21 cells, enzyme activity sometimes appeared to be induced at the cell surface. The Melinex procedure was successfully applied to all the cell lines studied and only that part of the cell surface which had been in contact with the substratum could not be readily examined by this method. To achieve this end requires the use of a method which enables cells to be sectioned in a vertical longitudinal or vertical transverse plane. The sandwich-embedding techniques (p. 22) or a method similar to that of Goldman (1972) (p.21) can be used to obtain such sections and when, during the enzyme cytochemical investigation, a similar procedure to the latter was employed, vertical longitudinal sections were obtained. In these sections the lower cell surface could be distinguished.

In each of the three cell systems studied it was found that there were distinct differences in ultrastructural morphology between the normal and transformed cells. However, when the three systems were compared it was apparent that the ultrastructural differences between normal and transformed cells were not consistent. This meant that it was difficult to formulate general statements about the ultrastructural changes which occur on transformation and, therefore, equally difficult to relate the results to carcinogenesis in vivo. If the components of

the GERL system i.e. Golgi complex, endoplasmic reticulum and lysosomes, are considered this situation can be examined more closely. In BHK21 cells the endoplasmic reticulum (ER) was more extensive, with many expanded cisternae, in the transformed (J1) cells than it was in the normal (C13) whereas in the 3T3 and chick embryo fibroblast (CEF) cells the opposite was true. Here, the more extensive and expanded ER was found in the normal cells. Similarly, the Golgi complex was more extensive and better differentiated in transformed BHK21 cells than it was in the normal whilst in 3T3 cells the reverse situation applied. Finally, the lysosome systems followed the same pattern. Lysosomes and residual bodies were more numerous in transformed BHK21 cells than in normal cells and in normal 3T3 and CEF cells than in their transformed derivatives. It is clear that a well-developed system of ER is usually accompanied by an extensive Golgi complex and lysosomal system. A well-developed GERL system indicates higher levels of both synthetic and catabolic activity and why such activity should decrease when 3T3 and CEF cells are transformed but increase when BHK21 cells are transformed is unclear. Such a situation implies that there must be basic differences in the metabolism of BHK21 cells compared with that of 3T3 and CEF cells, though what these differences might be is not known.

Because of the great sensitivity of 3T3 cells to density-dependent inhibition of growth and division they have been widely utilised for transformation studies since it is the loss of this property which is responsible for tumour-forming ability <u>in vivo</u>. The results of a number of such studies provide an explanation for the decreased GERL activity of transformed 3T3 cells. Weber and Rubin (1971) have shown that in non-proliferating, confluent, density-inhibited 3T3 cells the rate of transport of certain small molecules, such as uridine and

inorganic phosphate, into the cell is significantly reduced as compared to transformed cells and proliferating, sub-confluent normal cells. In addition, Horvat and Acs (1974) have demonstrated that in similar non-proliferating 3T3 cells the activity of three lysosomal hydrolases is increased tenfold as compared to proliferating normal and transformed cells. These findings suggest that, in order to compensate for the reduced supply of metabolites, the non-proliferating 3T3 cells must increase autodigestion which results in the appearance of greater numbers of autophagosomes, lysosomes and residual bodies. The consequent increased demand for lysosomal hydrolytic enzymes is met by the well-developed ER and Golgi systems. When the cells are transformed, surface and membrane changes occur which, amongst other effects, lead to increased transport of metabolites. A high level of autodigestion is obviated so that the GERL system can be reduced. The cell's population of residual bodies and lysosomes is diluted by cell division whilst residual bodies may additionally fuse and perhaps be exocytosed from the cell, a process which could be facilitated by the surface and membrane changes. Brunk (1973) has shown that non-proliferative, contact-inhibited human glia cells are unable to dispose of material in this manner. Further evidence which appears to confirm such a scheme is provided by Maciera-Coelho et al. (1971), who demonstrate an increase in the number of lysosomes when human fibroblasts enter the stationary phase, and Shanfield and Pinsky (1972) who have shown higher levels of lysosomal enzyme activity in non-proliferating monolayers of human skin fibroblasts than in proliferative cells. Lipetz (1973), studying human diploid fibroblasts, has demonstrated that as cells with a limited capacity to divide approach the end of their division potential, they accumulate autophagic vacuoles and show an increase in Golgi-lysosomal activity whereas cells

just beginning to divide or capable of unlimited division have low Golgi-lysosomal activity.

As normal BHK21 cells are less sensitive to density-dependent inhibition of growth and division than 3T3 cells and tend to continue growth after the attainment of a monolayer, they might be expected to have a less well-developed GERL system than normal 3T3 cells but why this should then become more, rather than less, active on transformation is not understood. In fact, normal BHK21 cells could be expected to behave in similar fashion to normal CEF cells which they resemble in many ways.

In view of these results it would be desirable, in future studies, to include sub-confluent, proliferating normal cells in comparative morphological investigations of normal and transformed cells. They would be expected, if the above explanation is correct, to exhibit certain similar ultrastructural features, particularly in the GERL system, to transformed cells. This does not seem unlikely since Stoker (1972) points out that features such as cell shape, movement, membrane glycolipids and transport characteristics of isolated growing normal cells resemble those of transformed cells.

The GERL systems of the normal and transformed cells are especially interesting since they are intimately involved with hydrolytic enzymes. The relationship between ultrastructure and enzyme activity will be discussed later. Other aspects of the ultrastructure did not provide such pronounced differences between normal and transformed cells. The latter tended to have smaller, more irregular nuclei although in the CEF cells there was little nuclear variation. Transformed BHK21 and 3T3 cells had more numerous mitochondria than normal cells. This was probably, in part at least, a consequence of their greater mobility.

This trend was not followed by transformed CEF cells which were of quite a different form and not as mobile as the other transformed cells. Cell surface differences were not extensive enough to permit interpretation of possible organisational differences. Since studies such as that of Kimoto et al. (1970), however, seem to indicate that an increase in the extent of pinocytosis and micropinocytosis could accompany increased autophagy and lysosomal activity as a means of compensating for the reduced entry of metabolites through the cell membrane when normal cells cease proliferating and become confluent, a detailed examination of these phenomena ought to detect a reduction in their extent on transformation.

Most of the available information on the hydrolytic enzymes of cultured cells has been provided by biochemical studies. In the present investigation a number of these enzymes were examined by light and electron microscopical cytochemistry. In addition, a quantitative approach was made by means of microdensitometry and biochemical assays. The results help to clarify some of the apparently conflicting findings of other workers concerning the behaviour of hydrolytic enzymes on transformation.

In the light microscopic enzyme cytochemical investigation, techniques for thirteen hydrolytic enzymes were applied to glutaraldehyde-fixed normal and transformed cells. However, only five of these enzymes could be demonstrated. These were acid phosphatase, non-specific esterase, β-D-glucuronidase, N-acetyl-β-D-glucosaminidase and an esterase-like proteolytic enzyme. It is doubtful whether all the other enzymes would be absent from the cells and more likely that the azo-dye techniques employed were inadequate or unable to detect low levels of activity. Bosmann (1969, 1972) and Bosmann and Pike (1970) have, in fact,

demonstrated biochemically that some of these enzymes are present, albeit with very low activity levels. The possibility that enzyme activity was lost due to fixation was later examined during the biochemical studies. All five demonstrated enzymes had a similar, particulate localisation, the distribution of particles corresponding to the granularity observed earlier by light microscopy. That this was largely a lysosomal localisation was subsequently confirmed by the electron microscopical investigation. Comparative visual assessment of levels of enzyme activity proved both difficult and unsatisfactory but a successful quantitative analysis by scanning microdensitometry of azo-dye end product of acid phosphatase activity was carried out on normal and transformed 3T3 cells. This showed that SV40 3T3 cells had about one and a half times more acid phosphatase activity than 3T3 cells but that this higher activity was confined to fewer sites. It is clear, therefore, that a larger lysosome population does not automatically mean a higher level of lysosomal enzyme activity. It was unfortunate that it was not possible to carry out a similar microdensitometric analysis for N-acetyl-β-D-glucosaminidase since this enzyme was later shown to behave quite differently from acid phosphatase.

Biochemical assays of the activities of three enzymes were performed on extracts obtained from homogenates of fixed and unfixed normal and transformed 3T3 cells. One of these enzymes, β-D-glucosidase, was not detected, thus endorsing the cytochemistry, but the other two, acid phosphatase and N-acetyl-β-D-glucosaminidase, exhibited high specific activities in the unfixed material. In the glutaraldehyde fixed material, however, enzyme activity was drastically reduced or, in the case of N-acetyl-β-D-glucosaminidase, virtually eliminated. The extreme

sensitivity of the latter enzyme to fixation probably explains why it appeared to have a lower level of activity than acid phosphatase in the cytochemical study whereas the biochemistry showed it, in fact, to have a higher level. Brunk and Ericsson (1972) also demonstrated a great loss of acid phosphatase activity, of the order of 90%, due to glutaraldehyde fixation of cultured human diploid glia cells. The extent of such losses of enzyme activity seems to warrant a change to a less inhibitive fixative or perhaps to a method of physical fixation such as freeze-drying or freeze-substitution. However, James (1974), working with plant tissue, showed that in cytochemical studies enzyme activity was only slightly inhibited in glutaraldehyde-fixed material compared to fresh material prepared by frozen sectioning, whereas in biochemical studies nearly 90% of the activity was lost in fixed material. Clearly, cytochemical techniques are better able to detect enzyme activity in fixed material than are biochemical techniques. Such results appear to support the use of glutaraldehyde fixation for enzyme cytochemical investigations.

The enzyme specific activities derived from the biochemistry were approximately of the same order as those shown by Bosmann (1969, 1972). The activity of acid phosphatase was found to be related to cell density. The denser the culture, the higher was the activity. Transformed cells exhibited 1.7 times more activity than non-proliferating, confluent normal cells, a result which compared well with that obtained by microdensitometry (1.56 times more activity in transformed cells). In a normal cell culture which had escaped from growth control and was, therefore, not subject to density-dependent inhibition of growth and division, there were more cells than in the corresponding transformed culture and these cells had a higher activity than that of the transformed cells. In the case of N-acetyl-β-D-glucosaminidase there was almost

twice as much activity in normal cells as there was in transformed cells, even in the normal culture which had overgrown. Clearly this enzyme was subject to different control mechanisms from acid phosphatase.

Bosmann (1969) studied glycosidase and proteolytic activity in normal and SV40 transformed 3T3 cells and subsequently (Bosmann, 1972) glycosidase, acid phosphatase and proteolytic activity in normal and Rous Sarcoma virus-transformed 3T3 cells. In both these studies he reported that in the transformed cells there were elevated levels of glycosidase and proteolytic activity and in the latter study a slight increase in the level of acid phosphatase activity. N-acetyl-β-D-glucosaminidase was the most active enzyme and in the transformed cells the level of its activity was about two and a half times higher than that of the normal cells. Bosmann suggests that these elevated levels of hydrolytic enzyme activity in transformed cells could be responsible for bringing about the modifications in the surface architecture which are, in turn, responsible for the all-important changes in the social behaviour of cells on transformation. Such a process has been termed sublethal autolysis (Poste, 1971; Bosmann, 1974). Bosmann's findings, especially for N-acetyl-β-D-glucosaminidase, are at great variance from those of the present investigation and the probable reason for this discrepancy becomes clear when his experimental procedure is examined. He harvested all his cells in the logarithmic phase of growth whereas in the present study the 3T3 cultures, with the exception of the final batch, were confluent monolayers of stationary, non-proliferating cells. Horvat and Acs (1974) compared the specific activities of three lysosomal enzymes (N-acetyl-β-D-glucosaminidase, aryl sulphatase and DNase II) in 3T3 cells during the logarithmic phase of growth and in stationary cultures. They showed that after 3T3 cells had reached

confluence there was a very marked increase, of the order of tenfold, in the specific activities of these lysosomal enzymes. Therefore, although Bosmann (1969) has demonstrated a higher N-acetyl-β-D-glucosaminidase specific activity in logarithmically growing SV40 3T3 cells than in logarithmically growing 3T3 cells, it is to be expected that when 3T3 cells become confluent the N-acetyl-β-D-glucosaminidase specific activity increases to such an extent that it rises well above the level of the proliferating transformed cells. This would substantiate the present biochemical results. As there are known to be a number of similarities between proliferating normal cells and transformed cells (Stoker, 1972) it is essential to ensure that, when making comparative studies of normal and transformed cells, the comparison is between confluent, non-proliferating normal cells and transformed cells. The study made by Elligsen et al. (1975) of the release of lysosomal enzymes from normal and transformed BHK cells into the culture medium demonstrated that the transformed cells released more acid phosphatase and glycosidases than did the normal cells. They advanced these results as further evidence for sublethal autolysis but they, like Bosmann, did not compare non-proliferating normal cells with transformed cells. Nevertheless, the fact that they did demonstrate the release by cultured cells of lysosomal enzymes into the extracellular environment is significant since such enzymes would presumably be able to act upon suitable substrates on the cell surface.

If Bosmann's hypothesis, that elevated levels of hydrolytic enzymes in transformed cells are responsible for surface modification by sublethal autolysis, is correct then the present biochemical results for acid phosphatase could certainly support this concept, although the role of the enzyme in such a process is unclear, whilst those for N-acetyl-β-D-

glucosaminidase appear not to. However, increased total levels of enzyme activity do not necessarily reflect increased levels at the cell surface and it is conceivable that despite a general overall drop in the activity of N-acetyl-β-D-glucosaminidase in transformed cells there could still be an increased release of enzyme at the cell surface or even the first appearance of activity at the surface. In such a situation the biochemical approach is found lacking and cytochemistry can contribute. This becomes clear when the electron microscopic cytochemical results for acid phosphatase are considered since they indicate that enzyme activity is present on the cell surface of both normal and transformed cells and, therefore, that sublethal autolysis might occur in both cell types.

No surface activity of any enzyme was detected in any cell line at the light microscope level. The electron microscope study, however, did furnish a certain amount of significant information. End product of acid phosphatase activity, as demonstrated by the Gomori method, was found on the cell surface of a number of normal and transformed 3T3 cells. In the SV40 3T3 cells this activity was definitely associated with a surface coat whereas in the 3T3 cells it was not clear whether such a coat was present. The role of acid phosphatase at the cell surface is unclear. Its activity is unlikely to be related to that of two other phosphatases which have been demonstrated on the surface of cultured cells, ATPase and alkaline phosphatase. Both these enzymes are involved in plasma membrane active transport mechanisms. Epstein and Holt (1963) localised ATPase activity on the cell surface of HeLa cells, the end product being associated only with the areas of the surface which were thrown into folds, processes and microvilli. Alkaline phosphatase was one of a number of enzymes examined in a series of

studies by Wilson (1973; 1974a; 1974b). She demonstrated surface alkaline phosphatase on *in vivo* mouse embryo cells, *in vitro* tissue culture cells derived from various organs of young, old and embryo mice, some mouse tumours of *in vivo* origin and cultured cells derived from these tumours. This enzyme activity was greatly decreased or completely lost in cultured cells which became spontaneously transformed and in tumours derived from these transformed cells. These changes in alkaline phosphatase activity were accompanied by changes in the activity of ATPase and 5' nucleotidase, another surface-active enzyme, and it seems that on the acquisition of tumour-producing capacity there is a change in the surface active transport mechanism of the cells (Wilson, 1974b). Several workers have reported acid phosphatase activity at the cell surface, the most relevant studies being those of Wilson (1973; 1974a; 1974b) since these are concerned with cultured cells. She does not, however, speculate on the enzyme's role at this site. She has demonstrated cell surface acid phosphatase in normal and spontaneously transformed cultured cells derived from various organs of young, old and embryo mice and in tumours derived from the spontaneously transformed cells. There was no such surface activity in normal or tumour cells *in vivo*, cultured cells derived from *in vivo* tumours or in the tumours formed on reimplantation of these cultured cells. These results indicate that *in vitro* culture conditions induce surface acid phosphatase activity in normal mouse cells and that this activity persists after spontaneous neoplastic transformation and in the cells of tumours derived from the spontaneously transformed cells. Miyayama *et al.* (1975) detected acid phosphatase on the brush border and basal membranes of tubule epithelial cells of normal mouse kidney using p-nitrophenyl phosphate, rather than β-glycerophosphate, as

substrate. They believed that it localised an extralysosomal isoenzyme of acid phosphatase. Braten (1975) demonstrated acid phosphatase activity over the entire surface membrane of gametes and zoospores of the green alga Ulva mutabilis. The enzyme has also been reported on the surface membrane of spermatozoa from sea urchins (Anderson, 1968) and mammals (Gordon, 1973) where it may bring about some of the changes which occur prior to fertilisation. Braten (1975) speculates that in the gametes of the green alga, acid phosphatase could be involved in intercellular recognition by means of enzyme-substrate reactions on the cell surface. Such a role is not inconceivable for the surface acid phosphatase of cultured cells since it is unlikely to play a major part in sublethal autolysis. It is possible, of course, that acid phosphatase has no function at the cell surface and that its presence is a result of being released along with other lysosomal enzymes which do have a function. A positive role for aryl sulphatase, which also appeared to be active on the cell surface of 3T3 cells, is more likely since on transformation there are known to be changes in the complex sulphates which are constituents of the cell surface glycosaminoglycans (Underhill and Keller, 1975).

There was a possibility that azo-dye techniques such as the LPED procedure, although they had been ruled out for general use due to the apparent avidity of the diazonium salts for secondary lysosomes, could be used to detect enzyme activity at non-lysosomal sites such as the cell surface. However, this was not achieved and glycosidases such as β-D-glucuronidase and N-acetyl-β-D-glucosaminidase could not be demonstrated at the cell surface. The cytochemical and biochemical results in the present investigation, therefore, especially those for acid phosphatase, provide some evidence for sublethal autolysis but

since the latter enzyme was found to be active on the surface of both normal and transformed 3T3 cells there is no evidence to support the concept that sublethal autolysis is instrumental in maintaining the transformed cell state.

If acid phosphatase and aryl sulphatase are found to be active on the cell surface, there seems to be no reason why other lysosomal enzymes should not be similarly active at this site. It is clear that lysosomes contain a mixed complement of hydrolases since, even if initially they contained only one or a small number of enzymes, the mixing of the lysosomal population which occurs due to fission and fusion (Biberfeld, 1971; Brunk, 1973) would ensure that many lysosomes had a heterogeneous enzyme content. Therefore if, as appears likely, acid phosphatase arrives at the cell surface by the release of lysosomal contents (Fig. 83) such a process would also involve the release of other lysosomal hydrolases. Of course, if the vehicles of enzyme transport to the cell surface were the Golgi vesicles or primary lysosomes then it is possible that specific enzymes could be synthesised, packaged and delivered to the surface without coming into contact with other enzymes. How far acid phosphatase can be considered to be a typical lysosomal enzyme is unclear but it appears from the present biochemical results that acid phosphatase and N-acetyl-β-D-glucosaminidase behave in distinctly different fashions in response to transformation and changes in cell density.

A possibility which does not seem to have been considered is that the cell surface of transformed cells could be modified by a reduction or loss of some hydrolytic enzyme activity rather than an increase. Such a course would be supported by the present results for N-acetyl-β-D-glucosaminidase and the findings of Horvat and Acs. Until the exact

nature of the changes at the cell surface are elucidated this possibility cannot be discounted. However, the evidence that transformed cells produce proteases (Unkeless et al., 1973; Ossowski et al., 1973) and that mild treatment of normal cells with proteolytic enzymes (Sefton and Rubin, 1970) or neuraminidase (Vaheri et al., 1972) induces changes characteristic of transformed cells suggests that it is unlikely and that surface modification is probably brought about by new or increased degradative enzyme activity. The other class of enzymes which have been associated with cell surface changes are the glycosyl transferases and these have been shown to have decreased activity on transformation (Cumar et al., 1970). However, their role is quite distinct from that of the glycosidases although they could presumably act in conjunction with them. There is a further facet of the sublethal autolysis question which does not appear to have been much discussed by those who believe that lysosomal degradative enzymes are involved. Given that these enzymes are released onto the cell surface it is not clear how much activity they would exhibit outside the lysosomal environment. All the biochemical and cytochemical demonstrations of enzyme activity have been carried out at or very close to the pH optima for activity but it is unlikely that the enzymes would encounter such optimum conditions on the cell surface. Therefore, although activity of an enzyme like acid phosphatase can be demonstrated at the cell surface this does not necessarily mean that this activity would be exhibited under normal conditions. Whether the presence of abundant sialic acid residues and acid mucopolysaccharides on the cell surface could reduce the pH to create a favourable local environment for acid hydrolase activity is not certain but such a situation could provide a means of controlling enzyme activity since sialic acid is much reduced in transformed cells

(Vaheri *et al.*, 1972). This could, of course, imply that surface conditions are less amenable to acid hydrolase activity in transformed cells as compared to normal cells. Poole (1973) has assessed the evidence for the activity, in the extracellular environment, of lysosomal enzymes from tumours. This indicates that in the peripheral zone around the tumour the pH is low enough to permit appreciable acid hydrolase activity and that the favourable environment could be created by the electronegativity of the cell surface and/or the presence of acidic compounds such as carbonic and lactic acid. He also presents evidence that lysosomal enzymes can degrade intercellular material at or near neutral pH.

In addition to the demonstration of acid phosphatase and aryl sulphatase activity at the cell surface the electron microscopic enzyme cytochemical investigation provided some significant information on cytochemical techniques and evidence to support the views of Novikoff *et al.* (1964) and de Duve and Wattiaux (1966) as to the fate and role of hydrolytic enzymes within the cell's vacuolar or GERL system.

As was the case with the morphological work, most of this investigation utilised *in situ* techniques. Just as ultrastructural artifacts were discovered in cells which were prepared by centrifugation to a pellet, so also were cytochemical artifacts found to be present in cells prepared in this manner. This emphasises the desirability of employing *in situ* procedures for ultrastructural and cytochemical investigations of cultured cells whenever possible. Even fewer enzymes were demonstrated at the electron microscope level than at the light level since the azo-dye techniques, on which this investigation was to be largely based, could not be utilised owing to the apparent avidity of the diazonium salts for secondary lysosomes. LPED, for instance,

possessed a number of features, viz. aromatic nuclei, an azo-group, the triphenyl arrangement and a lead atom, which it was considered might be responsible for this avidity. In the event, therefore, three enzymes were demonstrated by well established metal-salt procedures. These enzymes were acid phosphatase, aryl sulphatase and non-specific esterase. It was found that, provided adequate control experiments were conducted, the metal-salt procedures would give a relatively unambiguous picture of enzyme distribution and localisation. Non-specific esterase was studied solely in BHK21 cells and was localised within cisternae of the ER, Golgi saccules and vesicles, secondary lysosomes and the mitochondrial matrix. Its presence at the latter site indicates that non-specific esterase capable of hydrolysing thiolacetic acid is not purely a lysosomal enzyme. These results were common to normal and transformed cells. Acid phosphatase was the only enzyme to be studied in all the cell lines under investigation. Aryl sulphatase was studied principally in the chick embryo fibroblast system and also in normal and transformed 3T3 cells. Both enzymes, apart from their presence on the cell surface, were localised almost exclusively in elements of the GERL system and examination of this localisation together with the associated ultra-structure enables the functioning of the system to be elucidated.

That lysosomal hydrolases are synthesised at the ribosomes of the rough ER and are then passed via this organelle to smooth ER and Golgi complex is generally accepted (Novikoff et al., 1964). In the present study acid phosphatase and aryl sulphatase were not localised in either rough or smooth ER but the former enzyme has been demonstrated at these sites by other workers. Hugon and Borgers (1967) used the Gomori method to demonstrate acid phosphatase in smooth ER of the duodenal mucosa of the mouse whilst Miyayama et al. (1975) localised the enzyme in the ER of tubule epithelial cells of mouse kidney using

p-nitrophenyl phosphate as substrate, a method which is especially suitable for demonstrating non-lysosomal activity. Both acid phosphatase and aryl sulphatase activities have been detected in Golgi saccules and vesicles (Figs. 85, 127) and these vesicles are regarded as synonymous with primary lysosomes, particles whose enzyme has not been engaged in a hydrolytic event (de Duve and Wattiaux, 1966). It also seems likely that primary lysosomes arise from Golgi vacuoles and smooth ER since bodies identical to the Golgi vesicles are produced at these sites (Figs. 42, 43). This is in accord with the views of Novikoff et al. (1964). Four main categories of secondary lysosome were identified, all of which exhibited some degree of acid phosphatase and/or aryl sulphatase activity. These were the dense body type, autolysosomes, multivesicular bodies and hybrids of two or all three types. The dense body type of secondary lysosome is formed by the fusion of endocytic vesicles with primary lysosomes (Straus, 1967) and there are a number of possible examples of this fusion to be seen (Figs. 42, 43). Not all the dense bodies exhibited enzyme activity (Figs. 118, 124) but in those in which arylsulphatase was shown to be active the end product was usually confined to a discrete zone adjacent to the membrane (Figs. 119, 120). According to Hugon and Borgers (1967) this one-sided end product deposition is probably an artifact of post-fixation or dehydration. Unlike the dense body secondary lysosomes, which had a homogeneous matrix, autolysosomes contained membranous fragments, amorphous material, myelin figures and material that was recognisably of cytoplasmic origin. This latter, in varying degrees of breakdown, was predominantly mitochondria and cytoplasmic ground substance with ribosomes. The numbers of these autolysosomes gave a rough measure of the degree of autophagy taking place within the cells and it was clear that the normal 3T3 cells and chick embryo fibroblasts

were most active in this respect. No evidence of heterophagy was encountered. There were a number of features which suggested possible mechanisms of autophagy. The fact that autolysosomes contained endogenous cytoplasmic material in varying degrees of breakdown implies the occurrence of a series of events starting with a vacuole showing little or no autolytic activity and ending with a vacuole where autolysis is complete. According to de Duve and Wattiaux (1966), autophagic vacuoles may arise as autophagosomes containing no hydrolytic enzymes. These they subsequently acquire by fusion with primary lysosomes and thus form autolysosomes. A number of possible autophagosomes (or cytosegresomes) have been observed (Figs. 44, 78), the main criteria for such a classification being absence of hydrolytic enzyme activity and the presence of virtually unchanged cytoplasmic organelles. However, no evidence of the fusion of primary lysosomes with autophagosomes was found and it seems that enzymes might be present during sequestration and thus early on in the autophagic process. If this were so then autophagosomes, as such, would not exist, the bodies in question being autolysosomes from the outset. Evidence for at least three likely mechanisms of sequestration of material for autophagy has been observed. In certain instances (Figs. 13, 55) a number of small vesicles and short lengths of double membrane are arranged around an area of cytoplasm. This could indicate sequestration by the enlargement and fusion of several neighbouring saccules or perhaps the de novo formation of membranes around the area in question. The most frequently observed and most convincing evidence indicates that existing cytomembranes are utilised, particularly those of smooth ER and Golgi saccules (Figs. 42, 55). It is also possible that rough ER could be involved since there are many cases of the intimate surrounding of mitochondria by sections of rough ER (Figs. 27, 33). This could be a first step in sequestration.

Ericsson (1969) has described a similar situation and points out that where the rough ER and mitochondrion are closest, the ER is devoid of ribosomes, a phenomenon also seen in the present study (Figs. 38, 48). The final suggested mechanism could explain the presence of "signet-ring" lysosomes (Figs. 42, 43). These bodies could be formed if an existing lysosome flattened out and then enveloped an area of ground cytoplasm. Again, Ericsson (1969) has described a similar situation.

The acquisition of hydrolytic enzymes by autolysosomes is readily explained if existing cytomembranes and lysosomes are involved in sequestration since the enzymes would already be present within these organelles. There is also a less likely possibility that autophagosomes are formed which then produce their own enzymes at the enclosed, isolated ribosomes. In view of the absence of evidence supporting a fusion of autophagosomes with primary, or indeed secondary, lysosomes, one of these mechanisms, probably the former, seems likely to operate.

Early autolysosomes, or autophagosomes if these exist, are easily distinguished from the later or more mature stages. The early forms usually contain easily recognisable cytoplasmic organelles since lysis at this stage is minimal. In addition the early autolysosome is invariably surrounded by two membranes, derived from the sequestration membranes of ER or Golgi origin. The inner membrane is digested along with the contents so that mature autolysosomes are usually surrounded by a single membrane. Intermediate forms, with the inner membrane only partially digested, are occasionally seen (Figs. 31, 34). In early autolysosomes it is possible to demonstrate enzyme activity solely between the two membranes (Figs. 70, 119) whilst in the later forms and product of enzyme activity is found throughout the organelle (Figs. 66,

67).

The extent of autophagy in cultured cells indicates that it plays an essential part in cellular economy. The hydrolytic enzymes of the autolysosomes are responsible for the controlled intracellular digestion of senescent organelles such as mitochondria and ribosomes which have a comparatively short active life. After undergoing digestion within the autolysosomes, essential compounds from these organelles can be reutilised. Thus autophagy maintains a continuous turnover at the ultrastructural and molecular level. It is also clearly a flexible system since it can be increased to compensate for a reduced supply of metabolites, as when normal 3T3 cells achieve confluence. This situation resembles the increased autophagy demonstrated in the cells of the digestive caeca of starved locusts (Bowen, 1968a).

The third type of secondary lysosome was the multivesicular body. These were less common than the other lysosomes and their lysosomal nature was confirmed by the presence of enzyme activity (Fig. 125). There is no general agreement as to their origin and function and it seems possible that they may be a heterogeneous group, some having an autophagic role whilst others are involved in the digestion of proteins taken up by endocytosis (Daems et al., 1969). Their vesicle content presumably consists of primary lysosomes and small vesicles of endocytic or other origin.

Secondary lysosomes which appeared to be a product of the fusion of two or more smaller lysosomes were commonly observed (Figs. 52, 53) and were often associated with enzyme activity (Figs. 118, 124). A very prominent feature in the normal 3T3 cells and chick embryo fibroblasts, but also found in other cells, were the residual bodies or telolysosomes (Figs. 38, 54). These were derived from senescent secondary lysosomes

which had completed their degradative activities. They contained electron dense debris, broken membranes and myelin figures and often exhibited enzyme activity (Figs. 76, 92). In the chick embryo fibroblasts there appeared to be a great deal of fusion of residual bodies to produce extremely large organelles and this probably also occurred in SV40 3T3 cells since their residual bodies, although far fewer than the 3T3 cells, were much larger. The great numbers of residual bodies in normal 3T3 cells seemed to indicate that these cells were unable to expel them. It has been suggested that the accumulation of such residual bodies in normal cells could contribute to the process of ageing (Brunk et al., 1973). Although chick embryo fibroblasts retain most of their residual bodies they do eject some (Figs. 54, 82), presumably by exocytosis. Whether the scarcity of residual bodies in transformed 3T3 cells and chick embryo fibroblasts is due to simple dilution by cell division or to elimination through exocytosis is not clear.

When the Gomori method was used for the demonstration of acid phosphatase there was often a certain amount of cytoplasmic lead deposition which could be shown, by use of control material, to be unrelated to enzyme activity. However, in a few cases in normal and transformed BHK21 cells acid phosphatase activity was demonstrated in the cytoplasm around autophagic vacuoles (Fig. 68). The vacuoles themselves exhibited little activity and in the rest of the cytoplasm there was very low background lead deposition. The situation appeared to be the result of leakage of enzyme from the vacuoles into the cytoplasm immediately surrounding them. This was a similar finding to that of Maciera-Coelho et al. (1971) who demonstrated acid phosphatase activity around vacuoles which formed in cultured human

embryonic diploid cells after maintenance in the resting phase for several days.

The aim of this investigation was to examine the changes in ultrastructural organisation which occur when normal cells are transformed by tumour virus and to relate these changes to any in hydrolytic enzyme activity. This aim has been achieved with some success although the scope of the study was restricted because the azo-dye techniques could not be used at the electron microscope level which meant that some of the potentially most interesting enzymes could not be demonstrated. Cytochemistry on its own was unable to give reliable information on levels of enzyme activity but when it was applied in conjunction with biochemical studies it was possible to obtain a much fuller picture of the relationship between enzyme activity and ultrastructural organisation.

The currently available biochemical procedures are suitable for the assay of most of the enzymes involved but new developments are needed in enzyme cytochemical techniques for use at the electron microscope level. The p-nitrophenyl phosphate method for acid phosphatase (Ryder and Bowen, 1975; Miyayama et al., 1975) provides significant improvements over the Gomori method and is particularly useful since it means that the same substrate can be used for both cytochemical and biochemical investigations. This is an important feature because it ensures that the same isoenzyme is being studied and not two distinct ones. Ryder and Bowen (1975) have used this technique to study cell death and autolysis in planarian worms and found that it demonstrates the involvement of acid phosphatase whereas the Gomori method failed to do so. In view of these results, the p-nitrophenyl phosphate method appears to be ideal for the study

of sublethal autolysis in cultured cells. However, it is possible that a satisfactory method for one tissue or cell type may not be equally applicable to other tissues or cells. The azo-dye procedures are still potentially the most versatile of the enzyme cytochemical techniques since one basic method can be adapted for a wide range of enzymes provided suitable substrates are available. However, problems such as the avidity for secondary lysosomes must be solved first. Some encouraging demonstrations of lysososomal enzymes have been carried out utilising immunocytochemical techniques (Weston and Poole, 1973) and it seems that if further advances in conventional enzyme cytochemistry are not forthcoming then immunocytochemistry is the logical successor, although whether it can be successfully adapted for use at the electron microscope level remains to be seen. The technique is also open to the criticism that it demonstrates the presence of an enzyme but not necessarily enzyme activity.

The most significant finding of the investigation was that the transformation of normal cells is accompanied by changes in the GERL system, both morphologically and in terms of its enzyme activity. These changes appeared to be primarily in response to the altered entry of metabolites caused by modifications of the cell surface which also accompany transformation. However, the changes did not follow a general pattern and in one pair of cell lines the extent of the GERL system was increased on transformation whilst in two others it was decreased. Similarly, the activity of one acid hydrolase increased on transformation whilst another decreased. The involvement of lysosomal hydrolases with a process of sublethal autolysis at the cell surface was seen to be feasible but the evidence suggested that such a process occurred in both normal and transformed cells, thus not lending support to the

hypothesis that sublethal autolysis has a role in the maintenance of the transformed cell state.

The initial concept of sublethal autolysis, based on results such as those of Bosmann (1972), was that transformed cells have higher levels of lysosomal degradative enzymes which are released from or leak from the cells and bring about modifications on the cell surface, thus leading to altered social behaviour and other characteristics. Current opinion seems to favour the involvement of just one group of enzymes, proteases, in this phenomenon (see review by Hynes, 1974). It has been shown by several workers that transformed cells exhibit proteolytic activities greatly in excess of those of their normal counterparts or even possess proteases which are not found in normal cells. The fact that treatment of normal cells with known proteases brings about changes characteristic of transformation seems to support the idea that transformation can be brought about and maintained by proteolysis. Whereas the involvement of proteases in transformation seems highly likely the evidence of the present investigation and that of Wilson (1974b) suggests that sublethal autolysis by release of a battery of lysosomal hydrolases is not likely to be involved in transformation since one of these enzymes, acid phosphatase, has been demonstrated on the cell surface of both normal and SV40-transformed mouse 3T3 cells and on the surface of normal and spontaneously transformed cells derived from various organs of the mouse (Wilson, 1974b). The localisation of acid phosphatase at this site could indeed provide evidence that sublethal autolysis may occur at the cell surface but it occurs in both normal and transformed cells and is, therefore, unlikely to be involved in bringing about or maintaining the transformed cell state.

The work of Wilson (1973; 1974a; 1974b) includes the study of enzyme levels and localisation not only in normal, spontaneously transformed and tumour cells *in vitro* but also in the *in vivo* tissues from which the cultured cells were derived and in tumours formed from reimplantation of spontaneously transformed cells. This provides the opportunity to compare *in vitro* and *in vivo* results and thus to assess the relevance of studies involving *in vitro* cultured cells. In normal and tumour cells *in vivo* and in cultured cells derived from *in vivo* tumours there was no cell surface acid phosphatase activity but in normal cells cultured *in vitro* acid phosphatase was demonstrated on the cell surface. This indicates that *in vitro* culture conditions induce surface acid phosphatase activity in normal mouse cells. The activity persisted after spontaneous neoplastic transformation and in the cells of tumours derived from the spontaneously transformed cells. Other enzymes were also found to have altered enzyme patterns and activity levels when cells were cultured *in vitro* and it was noted that whatever the origin of the cultured cells their enzyme patterns tended to be similar, even though there were wide variations in pattern between the original tissues (Wilson, 1973). It was also observed (Wilson, 1974b) that the ultrastructural characteristics of cell lines, both tumour-producing and non-tumour-producing, originally derived from different organs of young, old and embryo mice, were strikingly similar after long term culture. The conclusion to be drawn from these results is that the maintenance of cells in culture can induce changes in the localisation and activity of enzymes and that the ultrastructure and enzyme patterns of cells derived from different sites tend to become similar. If this phenomenon is common to all cultured cells then the suitability of such cells as models for the study of some aspects of

cancer and carcinogenesis must be open to question. The differences between normal and virus-transformed fibroblasts demonstrated in the present investigation, for instance, may be quite unrelated to the differences between normal and sarcoma tissue in vivo. Further studies in the manner of those of Wilson may help to clarify this situation but in the meantime normal and transformed cells in culture continue to be a major model system for the investigation of carcinogenesis.

The current interest in the cell surface as the site of growth control and the possibility that surface modifications are engineered by hydrolytic enzymes makes the further investigation of the role of these enzymes in normal and transformed cells desirable. Since it has also been shown (Allison, 1969) that damage to lysosomes can induce transformation and increase malignant potential it is possible that lysosomal hydrolases play a fundamental role in the transformation process. There is a wide literature (see Poole, 1973 for review) concerning the activity of lysosomal enzymes in tumours, many of which exhibit elevated levels of activity compared to normal tissue. Such tumours have also been shown to release hydrolytic enzymes into the extracellular fluid and it is thought that this enzyme activity aids invasive tumour growth.

Lysosomal enzymes clearly play an important role, through their involvement in autophagy and intracellular digestion, in the regulation of the cellular economy of normal and transformed cultured cells. They also seem to be active on the surface of these cells, perhaps being involved in a process of sublethal autolysis postulated originally on the basis of certain biochemical studies. However, the results of the present investigation do not reveal any lysosomal enzyme activity which is exclusive to normal or to transformed cells and, therefore, it is

impossible to conclude, on the basis of these results, that lysosomal enzymes are involved in bringing about or maintaining the transformed cell state.

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THE AVIDITY OF HEAVY METAL DIAZOTATES FOR ANIMAL LYSOSOMES AND PLANT VACUOLES DURING THE ULTRASTRUCTURAL LOCALISATION OF ACID HYDROLASES

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Introduction

Over a period of several years we have been interested in the use of naphthol esters and lead-containing diazonium chlorides for the ultrastructural localisation of acid hydrolases in a number of plant and animal tissues. Acid phosphatase, non-specific esterase and β-glucuronidase have been studied in insect and mouse tissues, baby hamster kidney (BHK/21) and Chinese hamster ovary (CHO) cultured cells, strawberry receptacles and root cells of Vicia faba. Both the diazotates of lead phthalocyanin[1] and triphenyl-p-aminophenethyl lead (LPED)[2, 3] have been used as coupling salts and the appropriate naphthol ester has been used as substrate. Our observations have shown that these heavy metal diazotates have an avidity for lysosomal structures when fixed in glutaraldehyde-cacodylate mixtures, an effect first noticed by Smith and Fishman[4] using a mercury-containing diazotate.

Methods

Small pieces of mouse kidney, midgut from various insects, strawberry receptacles and roots of Vicia faba were fixed at 4°C for 3h. in 2.5% glutaraldehyde buffered with O.1M cacodylate at pH7.2. BHK/21 and CHO cells were spun into pellets and similarly fixed for 2O min. All tissues were then washed in several changes of cold cacodylate buffer before demonstrating enzyme activity. Acid phosphatase was demonstrated in all animal tissues by incubating for 3O min. at 37°C in a medium containing naphthol AS Bl phosphate (2O mg. %) as substrate and LPED (2.5 mg. %) as coupling salt in O.2M acetate buffer at pH 5.O. β-Glucuronidase was similarly demonstrated using naphthol AS- β-D-glucuronide as substrate. Non-specific esterase was demonstrated in plant material using naphthol AS D acetate as substrate in tris-HCl buffer at pH 7.1 and incubating for

8 - 2O min. Acid phosphatase was also demonstrated in insect midgut and non-specific esterase in strawberry receptacles using lead phthalocyanin diazotate (lO mg/ml) as coupling salt. Both simultaneous and post-coupling techniques were used. Control material was reacted in the absence of substrate with an additional sodium fluoride control ($1xlO^{-3}M$) for acid phosphatase.

After incubation all tissues were washed in buffer, post-fixed in 1% osmium tetroxide, dehydrated in Durcapan-water mixtures and embedded in Araldite. All sections were examined unstained.

Results

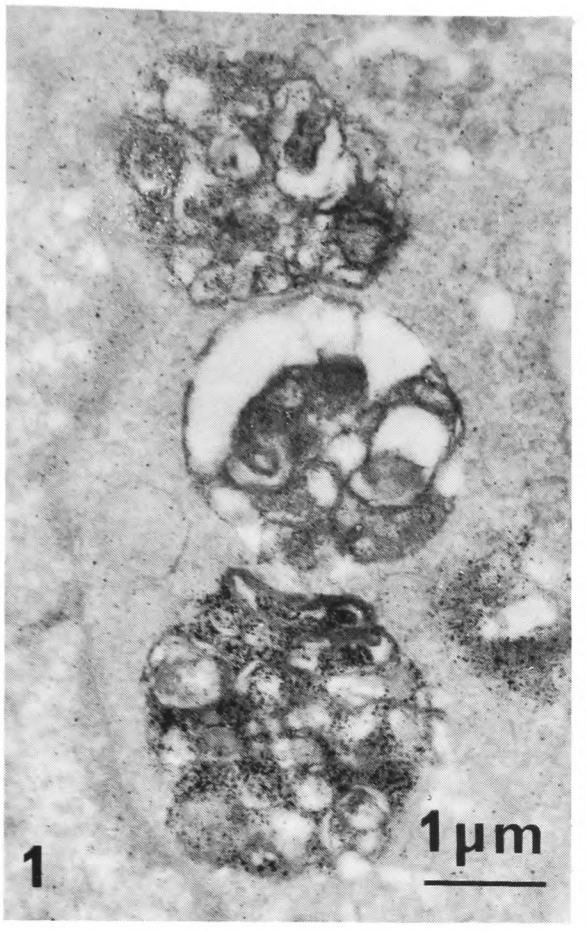
The early experiments using LPED as a coupling salt showed end-product in lysosomes of kidney cells[2] and insect midgut cells[3] in test sections only. No-substrate controls were negative. The end-product was electron opaque and easily identifiable (Fig. 1). However, later work using a wider range of tissues showed LPED deposits in control as well as test material. This proved to be the case with BHK/21 and CHO cells as well as kidney (Fig. 2) and insect tissues. The LPED in control material appeared to be associated only with lysosomes and particularly secondary lysosomes. Any membranous material within the lysosome such as myelin figures showed particularly heavy deposits. No other subcellular organelles or membrane systems showed deposits of LPED. In plant material test sections showed the deposition of LPED in vacuolar structures and endoplasmic reticulum (Fig. 3), although in the latter case it was confined to the membranes aligned with the plane of the newly formed wall. Control sections appeared to be negative.

Fig. 1. Chinese hamster ovary cells incubated in medium to demonstrate β-glucuronidase with LPED. End-product is in secondary lysosomes.

Fig. 2. Mouse kidney incubated in LPED without enzyme substrate. The LPED is bound in a lysosome.

Fig. 3. Epidermal cells of V. faba incubated for non-specific esterase using LPED. End-product is in vacuoles and endoplasmic reticulum.

Fig. 4. Midgut cells of Carausius morosus incubated with lead phthalocyanin and without enzyme substrate showing deposits in the endoplasmic reticulum and nuclear membrane.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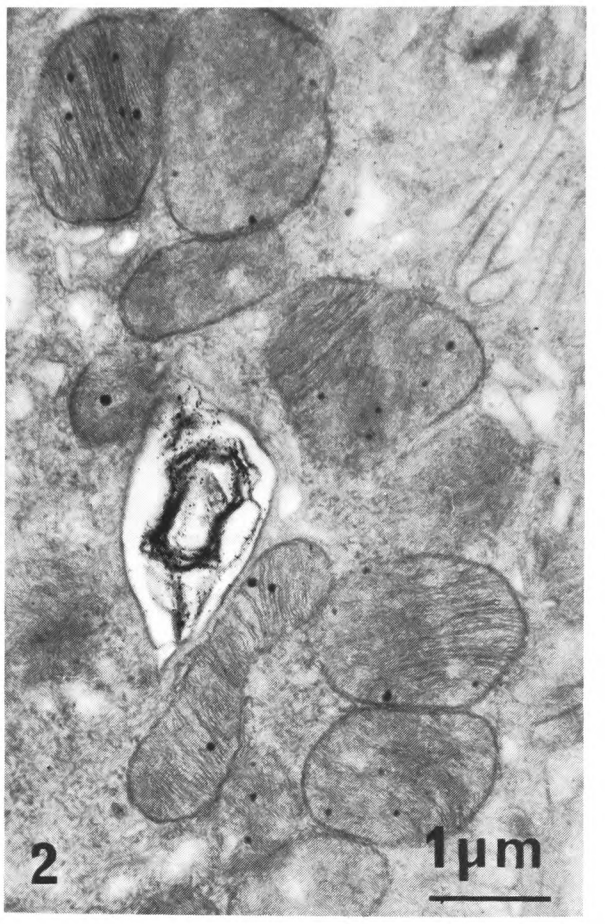
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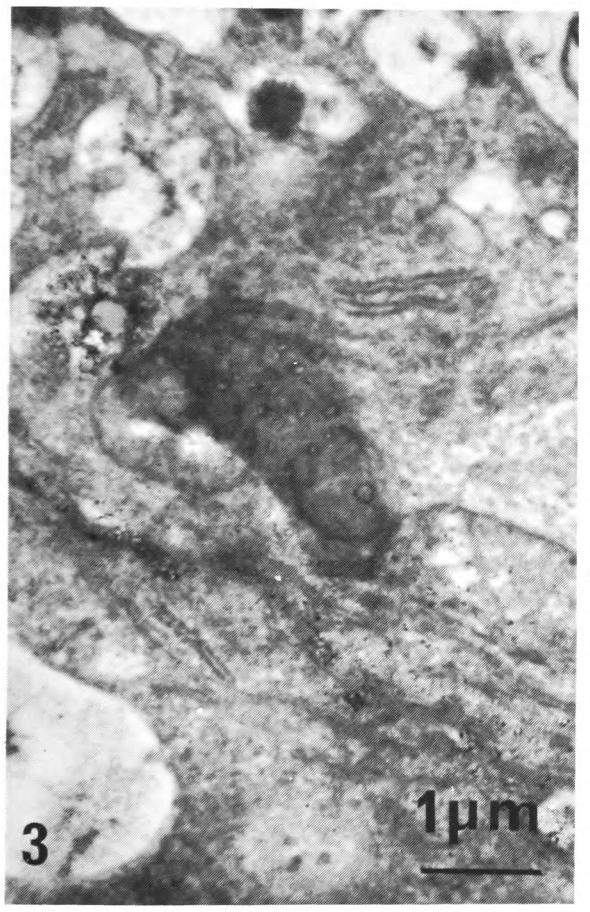
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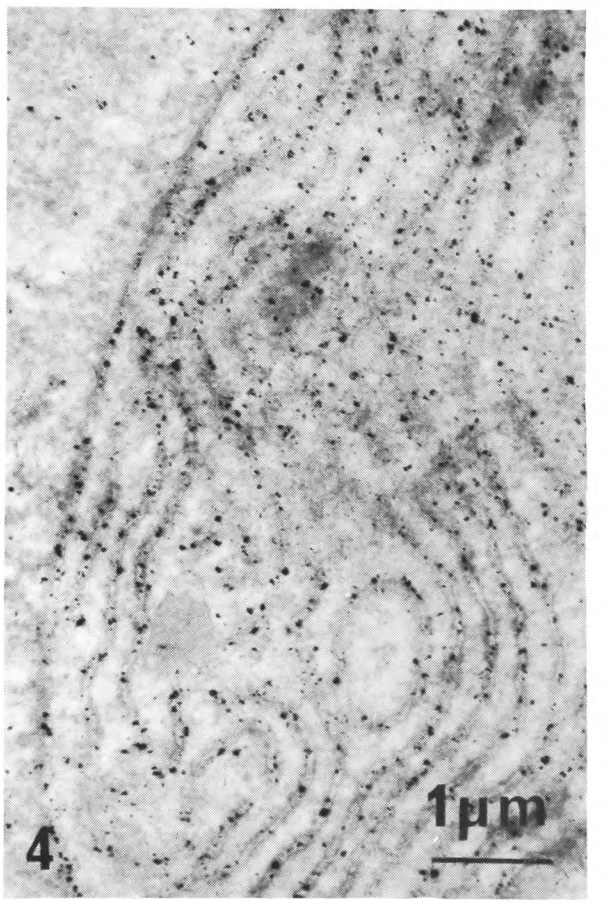
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When lead phthalocyanin was used as a coupling salt in ripe fruits it was found in the Golgi apparatus and a variety of different sized vacuoles in both test and control material. In the insect midgut lead phthalocyanin was found in lysosomes, Golgi apparatus, endoplasmic reticulum and the nuclear membrane in both test and control sections (Fig. 4).

Discussion

These observations endorse the remarks made by Smith and Fishman that the electron density of metal deposits in subcellular organelles, that are unrelated to released naphthols, present a serious limitation to the use of heavy metal diazotates in ultrastructural enzyme cytochemistry. This is particularly true when such compounds are employed in conjunction with glutaraldehyde-cacodylate fixation. With compounds such as lead phthalocyanin that bind to a number of membranous structures it is likely that some component of the membrane, such as sulphydryl groups, might be responsible for the binding but it is more difficult to explain the avidity of LPED for lysosomes. It is possible that when the cause of this binding becomes known some modification of the procedures employed may yet allow these techniques to be used with some confidence.

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A histochemical study of acid phosphatase in normal and virus-transformed cultured fibroblasts

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Synopsis. The distribution of acid phosphatase has been investigated in normal and virus-transformed cultured hamster and mouse fibroblasts. The enzyme was found to be present in lysosomes, autophagic vacuoles and elements of the Golgi apparatus. It was also found to be associated with a surface coat in some virus-transformed mouse cells and in the cytoplasm of both normal and transformed hamster cells.

Introduction

Buck *et al.* (1970), Hakomori & Murakami (1968) and Inbar & Sachs (1969) have shown that a number of phenotypic changes occur in cultured fibroblasts when they are transformed by tumour viruses. Some of the most significant of these are the changes that affect the cell surface since these are almost certainly responsible for the altered social behaviour of transformed cells (for review, see Stoker, 1972). Many of these changes concern the numbers and types of glycoproteins and glycolipids found on the cell surface and it is of interest that such changes are often accompanied by elevated levels of lysosomal glycosidases. Bosmann (1969) has demonstrated increased activity of glycosidases and proteolytic enzymes in fibroblasts transformed by both DNA and RNA tumour viruses and Bosmann & Pike (1970) have found elevated levels of acid phosphatase in polyoma transformed BHK21 cells, although a similar investigation of normal and Rous sarcoma virus transformed 3T3 mouse cells revealed virtually the same levels of acid phosphatase activity in the two cell lines (Bosmann, 1972). These findings have led some workers to suggest that surface changes may be related to the activities of lysosomal enzymes (Bosmann, 1969; Bosmann & Pike, 1970; Sefton & Rubin, 1970).

In view of this work it is surprising that few cytochemical studies appear to have been performed on normal and transformed cultured cells. Allison & Mallucci (1965) used the Gomori acid phosphatase technique to demonstrate the release of lysosomal enzymes into the cytoplasm of virus-infected cells and Wilson (personal communication) has com-

pared the activity and distribution of a number of enzymes in non-neoplastic and spontaneously transformed cultured cells derived from various organs of mice. She, too, used the Gomori acid phosphatase technique and showed that there was a change in the distribution of the enzyme after transformation even though the level of enzyme activity remained relatively constant in both cell lines. The investigation reported here concerns the distribution of acid phosphatase in cultured cells and forms part of a wider study of the role of lysosomal enzymes in the normal and transformed cell state.

Materials and methods

Cell cultures

The cells used in this study were the BHK21 C13 hamster line and its polyoma transformed derivative BHK21 J1 and the 3T3 mouse line and its Simian virus transformed derivative, SV40 3T3. All cells were grown without antibiotics in the Glasgow modification of minimum essential medium supplemented with 10% calf serum on either glass coverslips or plastic Melinex squares in 80 mm Nunc plastic petri dishes at 37°C and in an atmosphere of 90% air and 10% carbon dioxide.

Cytochemistry

For light microscopy, confluent cultures on glass coverslips were used. The medium was removed and the cells rinsed in 0.1 M cacodylate buffer at pH 7.2 containing 4.5% sucrose prior to fixing for 30 min in 2.5% cacodylate buffered glutaraldehyde at 4°C. After washing overnight in buffer, the cells were incubated at 37°C for varying periods in the standard Gomori medium for acid phosphatase (Gomori, 1952) followed by conversion of deposits of lead phosphate to lead sulphide by immersion for 1 min in a saturated solution of hydrogen sulphide. Rinsed coverslips were mounted in Farrant's medium.

For electron microscopy, confluent cultures on plastic Melinex squares were used. After fixation and washing as described above, cells were incubated at 37°C for 30 or 60 min in either the standard Gomori medium or the modified medium of Brunk & Ericsson (1972). After incubation the cells were post-fixed in 1% buffered osmium tetroxide for 1 hr, dehydrated in a graded series of ethanols, infiltrated and surface embedded in Araldite. After polymerization, the Melinex was stripped away leaving the cells at the surface of the Araldite block. Sections were cut on an LKB Ultrotome III and silver sections examined unstained with an AEI EM6B microscope. For both light and electron microscopy, control material was incubated in the appropriate medium either without substrate or with the addition of sodium fluoride to a final concentration of 10^{-3} M.

Results

Light microscopy revealed the presence of acid phosphatase-positive particles in all four cell types after incubation in the Gomori medium (Figs. 1 & 2). Such particles were not seen in the no substrate and fluoride inhibited controls. The 3T3 and SV40 3T3 mouse cells required an incubation period of the order of four to six times longer than the hamster cells to produce a comparable deposition of end-product, suggesting that more acid phosphatase is present in the hamster cells than in the mouse cells, an observation

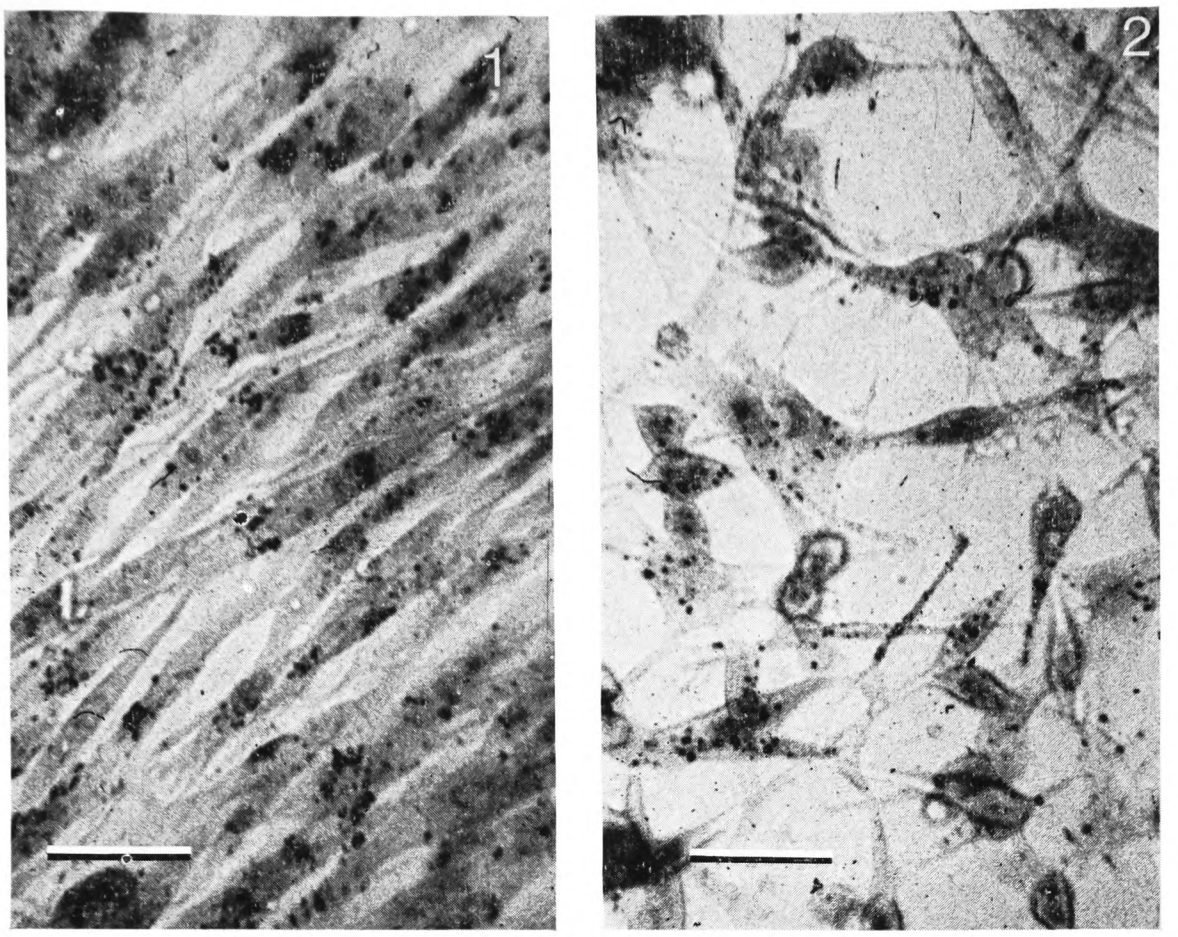
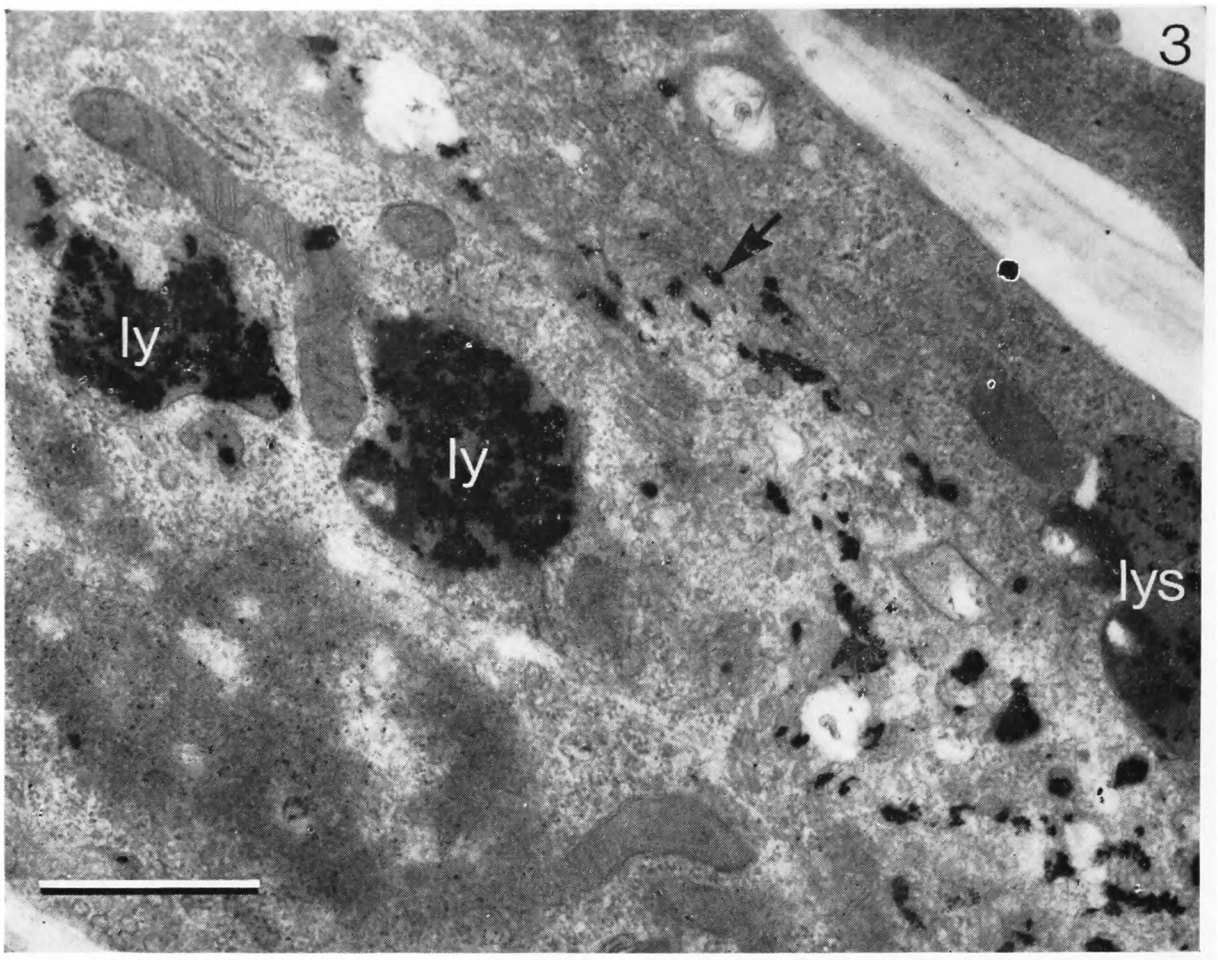


Figure 1. BHK21 C13 cells showing acid phosphatase-positive particles. Bar = 10 μm.
Figure 2. BHK21 J1 cells showing acid phosphatase-positive particles. Bar = 10 μm.

that is supported by biochemical evidence (unpublished observations). The distribution of these particles is similar in all four cell types, the majority occurring in the perinuclear region of the cell with smaller numbers in the peripheral cytoplasm and along the cellular processes that radiate from the main cell body. Apart from the particles no other site of end-product deposition was seen in the cells.

Deposits of lead phosphate were found in five distinct sites when treated BHK21 cells were examined with the electron microscope. In both the normal and transformed cells the main site of acid phosphatase activity was in small membrane-bound lysosomes that had a homogeneous, dense matrix (Fig. 3). These lysosomes were generally spherical, 0.4–0.8 μm in diameter, but occasionally a cylindrical structure was seen, particularly in the elongated cytoplasmic processes, that was 10 μm or more in length and 1 μm in diameter. Also present were lysosomes that contained particulate and membranous material. Such autophagic vacuoles were spherical in shape, 1–2 μm in diameter, and were more numerous in normal than in transformed cells. Fig. 4, for example, shows a typical BHK21 C13 cell with at least eleven autophagic vacuoles present, some of which contain enzyme end-product and some do not. Such areas are rarely, if ever, seen in the BHK21 J1 cells. End-product was also present in many of the Golgi bodies of both cell types although these structures were often without lead. Control sections showed these sites of lead deposition to be true sites of acid phosphatase activity.

Lead deposits were also seen in the nucleus and cytoplasm of some cells. Control



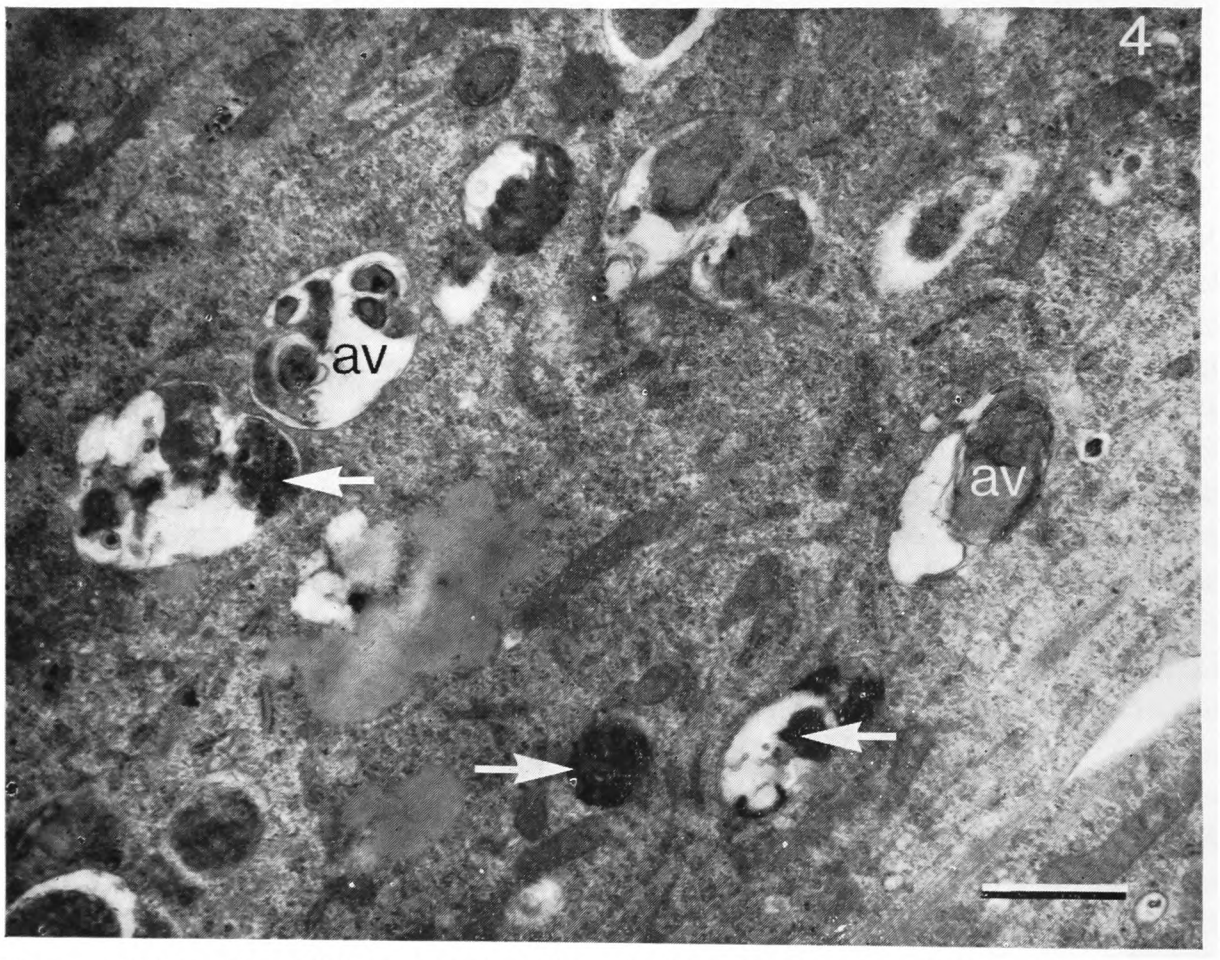


Figure 3. BHK21 C13 cell showing end-product of acid phosphatase activity in lysosomes (ly) and Golgi saccules (arrow). Some cytoplasmic deposits are also present. Bar = 1 μm.
Figure 4. BHK21 C13 cell showing autophagic vacuoles (av) some of which contain lead deposits (arrows) and others do not. Bar = 1 μm.

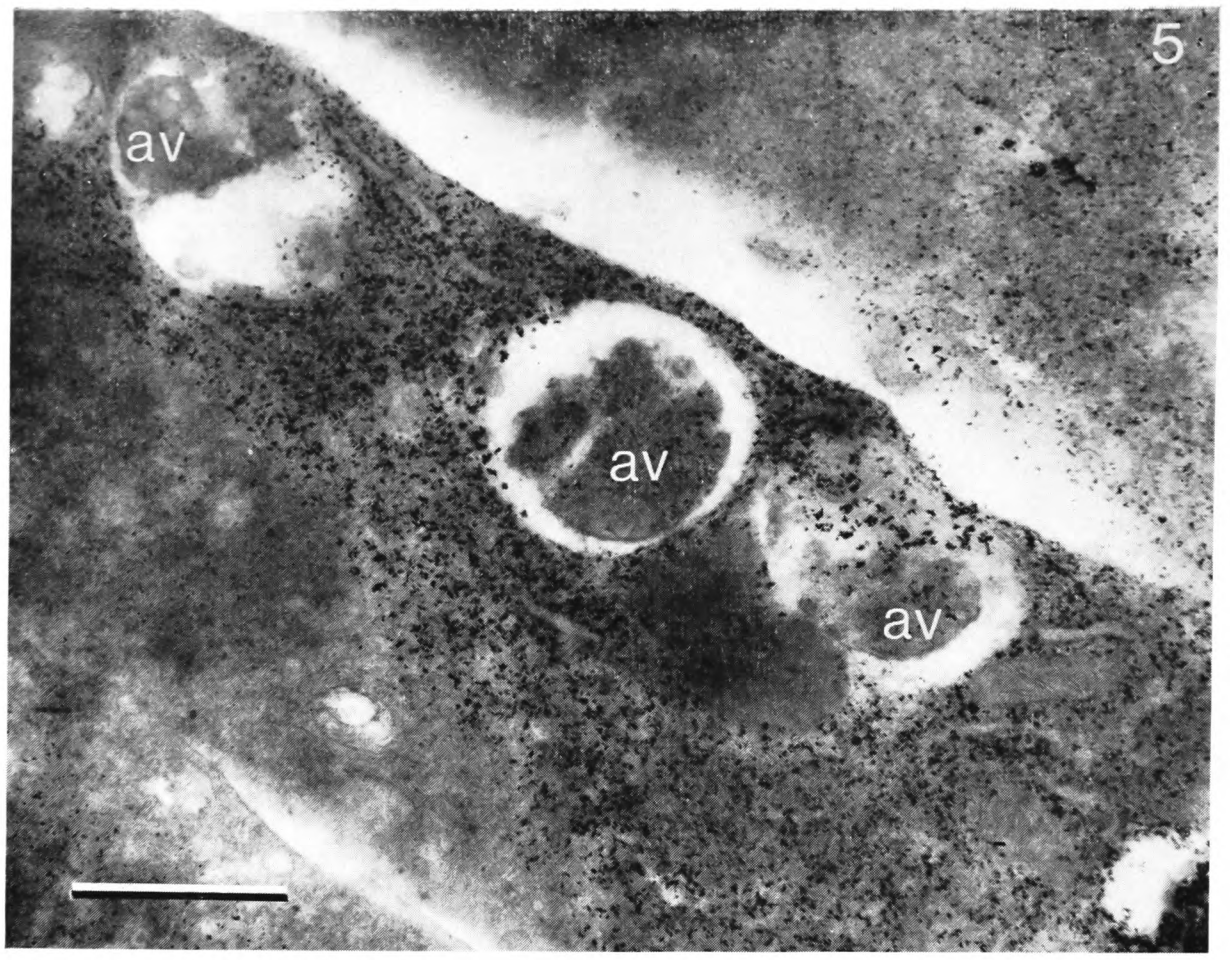
material showed the nuclear lead to be present even when the enzyme was inhibited or the substrate omitted from the incubating medium, indicating that it was not the result of enzyme activity. The presence of cytoplasmic lead was more difficult to interpret. Despite rigid adherence to apparently proven methods cytoplasmic lead would appear in one cell but not its immediate neighbour or it would appear in one experiment but not in an identical repeat. Attempts to vary the methods used so as to prevent or regulate the appearance of lead in the cytoplasm were unsuccessful. However, by careful use of controls it was possible to eliminate most of this as non-enzymic, bound lead. In a significant number of cases, though, cytoplasmic end-product of acid phosphatase activity was seen in both normal and transformed cells around, but outside, autophagic vacuoles (Fig. 5).

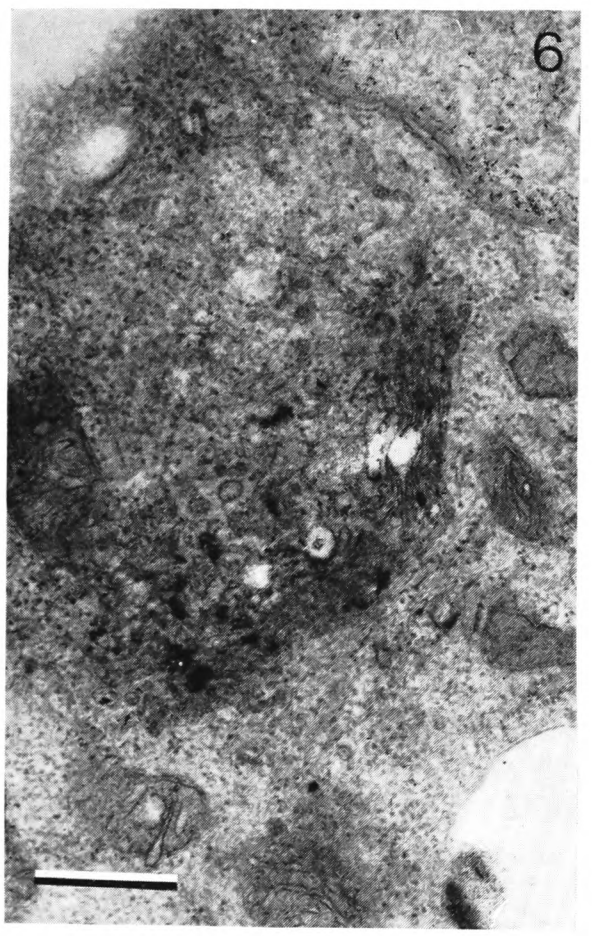
The distribution of lead deposits in treated 3T3 and SV40 3T3 mouse cells when examined with the electron microscope was essentially similar to that seen in BHK21 cells. However, in the 3T3 cells there appeared to be far more lysosomes and autophagic vacuoles than in SV40 transformed cells and also the Golgi apparatus, which was generally a site of heavy lead deposition in these cells, was far more developed in the 3T3 than in the SV40 cells (Fig. 6). On the other hand, the transformed mouse cells often contained autophagic vacuoles that were 4–5 μm in diameter, far larger than those seen in the other three cell types. Also an SV40 3T3 cell was occasionally seen which had a distinct surface coat that contained end-product of acid phosphatase activity (Fig. 7). Such a coat was only seen in a few cells of the hundreds examined and its actual nature is uncertain.

Discussion

Brunk & Ericsson (1972) have shown that in cultured human diploid glia cells and embryonic rat fibroblasts acid phosphatase is an enzyme exclusively associated with lysosomes, Golgi elements and related structures. In general, the results presented here for cultured hamster and mouse fibroblasts are in agreement with their findings. Acid phosphatase was demonstrated in lysosomes, autophagic vacuoles and Golgi elements of all four cell types examined but in addition, enzyme activity was found to be associated with a surface coat in some transformed mouse fibroblasts and free in the cytoplasm of some normal and transformed hamster fibroblasts. The presence of acid phosphatase on the surface of cultured cells has previously been reported by Wilson (1973) in spontaneously transformed mouse fibroblasts and on the surface of mouse tumours. Cytoplasmic acid phosphatase has also been reported previously in cultured human embryonic diploid cells by Maciera-Coelho *et al.* (1971). They showed the enzyme to be present around vacuoles that appeared in the cells after they had been maintained in the resting phase for several days, a location similar to that shown in Fig. 5. An increase in the number of autophagic vacuoles and residual bodies in stationary cultures of diploid normal human glia cells was found by Brunk *et al.* (1973) and in this study normal, contact-inhibited fibroblasts were found to have more autophagic vacuoles than rapidly growing, transformed cells.

Bosmann (1969, 1972) and Bosmann & Pike (1970) have shown that there is a large increase in the activities of lysosomal enzymes in transformed as compared to normal cells. This was particularly the case with lysosomal glycosidases but acid phosphatase does not appear to follow this general pattern. Although Bosmann found that its activity





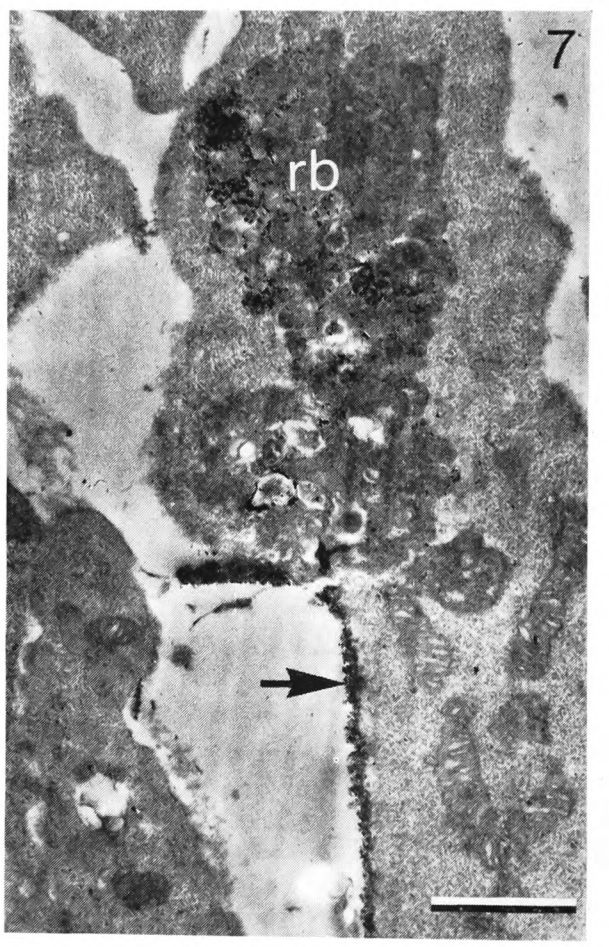


Figure 5. BHK21 C13 cell showing deposits of lead in the cytoplasm outside of autophagic vacuoles (av). Bar = 1 μm.

Figure 6. 3T3 cell showing end-product of acid phosphatase activity in the Golgi apparatus. Bar = 0.5 μm.

Figure 7. SV40 3T3 cell showing lead deposits associated with a surface coat (arrow) and with a residual body (rb). Bar = 0.5 μm.

in polyoma transformed BHK cells was twice that of normal BHK cells, he found no such increase in Rous sarcoma virus transformed 3T3 cells and Wilson (1973) found that phosphatase levels remained the same in normal and spontaneously transformed mouse fibroblasts. Horvat & Acs (1974) have shown a tenfold increase in lysosomal aryl sulphatase, *N*-acetyl-D-glucosaminidase and DNAse during the non-poliferative phase of cultured 3T3 cells as compared to rapidly dividing 3T3 cells. They have also shown that this was not due to ageing but was related to the state of non-proliferation. They suggest that non-proliferative cells maintain their normal metabolism by autodigestion with a resulting increase in autophagic vacuoles and hence lysosomal enzymes. Since Cunningham & Pardee (1969) and Weber & Rubin (1971) have found that the transport of small molecules decreases when cells reach saturation density, this explanation may be substantially correct and it would explain our findings that both BHK21 and 3T3 cells that have grown to confluency contain greater numbers of autophagic vacuoles and lysosomes than their transformed derivatives.

The significance of the reported increases in lysosomal glycosidases in transformed cells is less clear. Bosmann & Pike (1970) have suggested that the increased activity of these enzymes could markedly change the chemical characteristics of the cell surface and lead to changes known to accompany the oncogenic transformation process. The presence of acid phosphatase on the surface of SV40 3T3 cells reported here and in spontaneously transformed mouse fibroblasts and tumours by Wilson suggests that lysosomal enzymes may indeed be involved at the cell surface during or after transformation. However, the surface coat seen on the cells studied here appeared to be present on only a few cells and may not be a normal surface structure, but since Terry & Culp (1974) have reported that 3T3 cells shed surface components into the culture medium and Kapeller *et al.* (1973) have shown that chick embryo cells shed carbohydrate-containing macromolecules from cell surfaces, it is possible that the few cells seen in this study with a surface coat were fixed in the process of shedding it. Why acid phosphatase should be associated with such a process is not known but the extracellular secretion of lysosomal enzymes has been reported for a number of other cell types (see for example Poole *et al.*, 1974) and if these enzymes are secreted during this process in transformed cells it is possible that they could have the effects suggested by Bosmann & Pike. It will be important to investigate the distribution of glycosidases in this respect and work is already underway in our laboratory to determine the distribution of these enzymes in transformed cells.

Acknowledgements

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