

UPTAKE OF CARDIAC GLYCOSIDES BY HELA CELLS

David Owler

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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UPTAKE OF CARDIAC GLYCOSIDES BY HeLa CELLS

A thesis submitted to the University of St. Andrews for the
degree of Doctor of Philosophy.

by

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University of St. Andrews,
February 1986.



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ABSTRACT

Methods for the microcarrier culture of HeLa cells were developed and optimum culture conditions established. Methods were also established for the isolation of HeLa plasma membranes on beads, based on the principle of cell lysis followed by the shearing away of the internal cell components, leaving the plasma membrane attached to the beads. The HeLa plasma membranes isolated on beads showed 5-8 fold enrichment of plasma membrane markers, while intracellular markers were depleted to the point of non-detection (except for some slight lysosomal contamination). The preparation time for isolation of HeLa plasma membranes on beads was about 1.5 hrs.

The uptake of cardiac glycosides by HeLa cells was investigated. HeLa cells were labelled with [3 H]-glycoside, the non-specific binding washed off and the cells returned to normal growth medium. At various time intervals the amount of [3 H]-glycoside bound to HeLa plasma membranes and HeLa cells was determined. The rate of loss of ouabain and digoxin from HeLa plasma membranes was found to be about $10\%hr^{-1}$. The excretion rates of ouabain and digoxin from HeLa cells were $4\%hr^{-1}$ and $10\%hr^{-1}$ respectively. The nature of the uptake and excretion processes were investigated using specific inhibitors of receptor-mediated endocytic processes. No inhibitor affected the rate of loss of cardiac glycoside from HeLa plasma membranes but monensin and chloroquine slowed the excretion rate of digoxin from HeLa cells.

Modulation of sodium pump numbers by various chronic stress conditions - high serum concentration, low K medium and high K medium - were investigated. High concentrations of serum had no effect on the rate of loss ouabain from HeLa plasma membrane, but low K medium reduced the rate of loss of ouabain from membranes from $10\%hr^{-1}$ to $4\%hr^{-1}$. The low K medium also caused a x2 increase in sodium pump numbers in the plasma membrane. Increasing the serum concentration abolished the effects due to low K medium. High K medium also caused a reduction in the rate of loss of ouabain from plasma membranes from $10\%hr^{-1}$ to $4\%hr^{-1}$.

The results obtained for low K medium were consistent with a model whereby sodium pumps in the plasma membrane are regulated by alteration of the sodium pump turnover rate (or internalisation rate).

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TO MUM AND DAD

SUMMARY

Chapter 1.

1. Procedures have been developed for the microcarrier culture of HeLa cells.

2. Culture conditions were established for cells based on the findings of others. Optimal initial culture conditions were found to be: a cell/ microcarrier ratio of 5, a continuous stirring speed of 30 rpm, 200 ml initial culture volume and an inoculation density of 15 million cells.

3. Maximum cell yield was achieved after 5-7 days. This maximum yield was found to be 150 million cells, well short of the theoretical maximum of about 400 million cells. The shortfall was accounted for by sub-maximal binding of cells to beads and the existence of bare patches on beads on which cells would not proliferate. The reasons for the existence of such bare patches were not clear.

4. Methods were then established for the isolation of HeLa plasma membranes on beads, based on the principle of cell lysis followed by the shearing away of internal components, leaving the plasma membrane attached to the beads. The use of hypotonic buffers of pH 8 during the procedure prevented internal components sticking to the beads and thus

contaminating the membrane preparation.

5. Such a membrane isolation procedure enriched the plasma membrane markers : ouabain binding, 5' nucleotidase and adenylate cyclase by 7.9, 4.8 and 5.3 respectively, relative to whole cell homogenates.

6. These enrichment or purification factors compared favourably with other HeLa plasma membranes isolated on sucrose gradients.

7. Some lysosomal contamination (purification factor just less than 1) was measured in membranes on beads, but no other intracellular marker activity could be detected.

8. It was concluded that this rapid method (time 1.5 hours) for the isolation of HeLa plasma membranes, compares favourably with other more traditional methods and should prove useful in the study of plasma membrane function.

Chapter 2

1. The rate of loss of cardiac glycosides from HeLa plasma membranes were measured and found to be about $10\%.\text{hr}^{-1}$ for both ouabain and digoxin.
2. The excretion rates of ouabain and digoxin from HeLa cells were found to be about $4\%.\text{hr}^{-1}$ and $10\%.\text{hr}^{-1}$ respectively.
3. The kinetic data for glycoside uptake by HeLa cells and the direct measure of the sodium pump turnover rate, support the idea that loss of cardiac glycosides from HeLa plasma membranes occurs by internalisation and not dissociation.
4. The nature of the receptor-mediated internalisation and excretion of cardiac glycosides was studied in HeLa cells by use of specific inhibitors reported to inhibit other such processes.
5. Chloroquine did not affect the rate of loss of glycoside from the plasma membrane or the rate of loss of ouabain from HeLa cells, but slowed the rate of loss of digoxin from HeLa cells.
6. Cycloheximide had no effect on the rate of loss of glycoside from HeLa cells, but slightly slowed the rate of loss of digoxin from HeLa cells.

7. The other reagents/conditions studied: chlorpromazine, monodansylcadaverine, Ca, cytochalasin B and nocodazole, all had no effect on the rate of loss of glycoside from HeLa plasma membranes or HeLa cells.

8. Modulation of sodium numbers by various stress conditions were investigated by measuring the rate of loss of ouabain from HeLa plasma membranes (or internalisation rate of ouabain).

9. High serum concentration in growth medium had no effect on rate of loss of ouabain from HeLa plasma membranes.

10. Incubation of HeLa cells in low K medium caused a reduction in the rate of loss of ouabain from the plasma membrane, from $10\%.\text{hr}^{-1}$ to $4\%.\text{hr}^{-1}$. The low K medium also caused a 50% increase in the specific activity of ouabain binding.

11. When HeLa cells in low K medium were returned to normal medium (5 K), the rate of loss of ouabain from the plasma membrane immediately returned to the control rate.

12. Increasing concentrations of serum abolished the reduction in the rate of loss of ouabain from HeLa plasma membranes caused by the low K stress

conditions.

13. High K medium (135 mM K, 12 mM Na) similar to low K medium, caused a reduction in the rate of loss of ouabain from HeLa plasma membranes, from $10\%.\text{hr}^{-1}$ (controls) to $5\%.\text{hr}^{-1}$.

14. Chronic glycoside stress did not cause any significant increase in sodium pump site numbers.

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CHAPTER 1

MICROCARRIER CELL CULTURE

ISOLATION OF HeLa PLASMA MEMBRANES ON MICROCARRIER BEADS

INTRODUCTIONTISSUE CULTURE

Tissue culture developed from techniques of embryology used last century. The first recorded instance of a successful explanation was in 1885 when Wilhelm Roux maintained the medullary plate of a chick embryo in warm saline for several days. In 1898 the possibility that animal tissues might be kept alive for longer periods was demonstrated, when Ljunggren showed that human skin could survive in vitro for many days when stored in ascitic fluid.

Many of the early experiments proved difficult to repeat though, because of the unsatisfactory media used, and doubts were cast as to whether genuine cell survival was occurring or merely delayed cell death. However in 1907 Harrison explanted small pieces of frog medullary tube into clots of frog lymph and observed that in aseptic conditions the fragments survived for several weeks and axones grew out from the cells. This experiment showed unequivocally continuation of normal function in vitro. The reproducible techniques used by Harrison have generally been regarded as the true beginning of tissue culture.

Tissue culture techniques were quickly established: Carrel pioneered aseptic techniques, the important constituents of growth media identified, virus culture and plant tissue culture established. For review of early tissue culture work see Paul (1970).

Nearly all of the methods developed by 1940, depended on the growth of tissue in either plasma, serum or embryo extract. (These were thought to be important for physical support and nutritional requirements of cells in culture). Evans and Earle (1947)

demonstrated that cells could be grown on cellophane and glass. In 1952, Moscona and Moscona (1952) developed methods for dispersion of tissues to give single cell suspensions and demonstrated the viability of such preparations, with embryonic cells prepared by tryptic digestion in Ca/Mg-free medium. The work of Evans and Earle (1947) and Moscona and Moscona (1952) were major advancements and provided the impetus for the subsequent establishment of permanent cell strains of uniform populations.

From primary cell cultures, primary cell lines and eventually established cell lines were developed. Established cell lines, which have the ability to be subcultured indefinitely, develop from primary cell lines by cell alteration or transformation (not to be confused with the transfer of genetic material in bacteria). Established cell lines often differ from primary cell lines in that they often have different properties from original cells, have shorter doubling times, are aneuploid, grow to much higher densities, grow in suspension and show a great reduction of contact inhibition. Transformation has been shown to be caused by chemical carcinogens, DNA viruses and RNA viruses such as the Rous sarcoma virus (spontaneous transformation can also occur). Primary cell lines derived from tumours, very often behave as though they have already been transformed, in that they grow rapidly, are already aneuploid and do not show contact inhibition. Paradoxically, the established cell lines obtained from tumours often show greater retention of properties than established cell lines obtained from normal (non-tumour) tissue.

Microcarrier cell culture

The idea of culturing anchorage-dependent animal cells on small spheres (microcarriers) kept in suspension was first conceived by van Wezel (1967). The advantages he envisaged, would be the bringing together of both suspension and monolayer culture while retaining all advantages of both culture systems. Microcarrier culture would also offer an elegant cultivation technique for large scale production of animal cells, especially those which did not multiply in free suspension, such as most primary cell lines.

The first bead used was beaded ion-exchange gel DEAE-Sephadex A-50, and proved useful in initial experiments since it provided a charged surface, large surface area/volume ratio, good optical properties and a suitable density- glass beads proved unsuitable because their high density required high stirring speeds for suspension, which were not compatible with cell growth, (van Wezel, 1967).

These initial experiments by van Wezel illustrated the potential of microcarrier cell culture.

An early problem uncovered by van Wezel was that if the DEAE-Sephadex A-50 concentration was increased from 1 mg/ml to 2 mg/ml, poor cell growth and limited cell yields were obtained. It is now known that this toxicity of 2 mg/ml DEAE-Sephadex A-50 is due to the degree of substitution of DEAE, which was not optimal for cell growth (Horng and McLimans, 1975; Levine, Wang and Thilly, 1979; Pharmacia Fine Chemicals, Technical Booklet Series, 1978). It is likely that the toxicity is due to excessive ion-exchange capacity in the micro-environment of the cell, rather than too large a total ion-exchange capacity in the culture.

In early experiments, microcarrier culture conditions were not controlled, however many studies started to appear, establishing optimal conditions for various parameters of microcarrier culture.

Microcarriers: Inooka (1969) and Horng and McLimans (1975) suggested that alteration of the ion-exchange capacity of DEAE-Sephadex A-50 could lead to improved cell attachment in growth. The work of these authors formed the basis for development of microcarriers with a much lower degree of substitution than DEAE-Sephadex A-50, which proved optimal for cell growth and attachment (van Wezel, 1977; Levine et al, 1979; van Wezel and van der Velden-de Groot, 1978; Levine, Wong and Wang, 1977). Eventually, commercially made microcarriers composed of a spherical matrix of cross-linked dextran with charged DEAE groups, became available. These microcarriers allowed optimal cell attachment and growth, as well as satisfying other requirements for an optimum microcarrier: optimum density of 1.030 - 1.045 g/ml facilitating easy separation of cells on beads and medium, a size of 100 - 230 μ m diameter for optimal growth and large surface area to volume ratio, transparent microcarriers for easy routine observation, non-toxic for high cell yield, non-rigid for good cell growth when the culture is stirred (a compressible matrix reduces the possibility of damage to microcarriers and cells, van Wezel, 1977). An additional development has been the use of collagen-coated microcarriers to promote the growth of primary cell lines and cells normally difficult to grow in culture (Reid and Rojkind, 1979).

Cell attachment: The adhesion of cells to culture surfaces is fundamental to both traditional monolayer culture techniques and microcarrier culture. The proliferation of anchorage-dependent cells can only occur after adhesion to a suitable culture surface (Grinnell, 1978). Cell attachment is a multistep process involving: (a) absorption of attachment factors to culture surfaces, (b) contact between cells and the surface, (c) attachment of cells to coated surface and (d) spreading of attached cells. It has been shown that the culture surface must be hydrophilic and correctly charged for successful cell attachment to occur Grinnell (1978). It has been shown by Maroudas (1975) and Hirtenstein, Clark and Lindgren (1980) that it is not the polarity of charge, rather the density of charge that is important in optimising cell attachment. Maroudas (1977) using chemically defined culture surfaces, has reported that a minimum of 5 charged groups per 100 \AA^2 are required for a culture surface to be effective for cell attachment and proliferation. It is not only charge that is important for cell attachment, two factors in culture medium are also essential for cell adhesion - divalent cations and attachment protein. Grinnell, Hays and Minter (1977) have shown that in the absence of protein and divalent cations, cells attach to a cell surface only by non-specific absorption. A protein molecule essential for attachment has been isolated from serum and identified as cold-insoluble globulin (CIG), a glycoprotein (Grinnell and Feld, 1979; Laterra, Ansbacher and Culp, 1980; Yamada and Olden, 1978). An attachment glycoprotein has also been shown to be secreted from certain cells as fibronectin (Hughes, Pena and Clark, 1979).

Attachment proteins must be absorbed onto the culture surface before they can promote cell attachment and spreading (Carter, Rauvala and Hakomori, 1981). The multistep process of cell adhesion, involves not only specific chemicals but also a rearrangement of cell surface components. Cell adhesion can also be inhibited by specific chemicals (e.g. chemicals which bind to sulfhydryl groups inhibit adhesion). Mutant cells have also been isolated which lack the ability to adhere. The above observations have led to the idea of cells having specific adhesion receptors on their cell surfaces, but conclusive evidence for this has still to be presented (for review see Grinell, 1978). Understanding of the process of cell attachment has led to the development of culture procedures which allow optimal cell attachment. Other culture procedures (stirring speed, cell inoculum, plating efficiency, inoculum condition, concentration of microcarriers) also influence cell attachment as well as subsequent cell growth, and these are reviewed below.

Plating efficiency: In many of the studies cited here, cells in microcarrier culture have been shown to have the same growth kinetics, morphology, population doubling time etc., as they have in other traditional cell culture methods. In short, microcarrier culture is based on traditional culture methods. The basic principles of both, are to ensure maximum initial attachment of cells (to beads), then choose culture conditions which will give rise to the highest possible cell yield. Comparative studies of the conditions required to culture a wide variety of cells in microcarrier culture, has shown that plating efficiency is one of the

most useful parameters to consider when developing a culture procedure (Clark and Hirtenstein, 1981a; Clark, Hirtenstein and Gebb, 1980). Plating efficiency is the proportion of cells which can form colonies when plated at low density. Established cell lines, which have plating efficiencies of greater than 30%, require a low cell:microcarrier ratio (< 5), an initial culture volume 100% of final volume, a continuous stirring speed and no extra medium supplements. Cells with low plating efficiencies (e.g. primary cell lines) require a high cell:microcarrier ratio (> 5), a reduced initial culture volume (30% of final volume), intermittent/static stirring and additional medium supplements. These relationships have been observed for a large number of different cells (Clark and Hirtenstein, 1981a; Clark et al., 1980.)

Initial stirring: It has been shown in many studies that an increase in the efficiency of attachment results in improved final cell yields (Clark and Hirtenstein, 1981a; Clark et al., 1980). These authors have also shown that the rate and proportion of cells attaching to the microcarriers can be increased if the culture remains static with intermittent stirring during the early attachment stage. If at the same time the cell/microcarrier suspension is contained in a reduced volume, then the cells have a greater chance of coming into contact with a microcarrier and the conditioning effects of the medium are greater. These observations are particularly true for primary cell lines with a low plating efficiency (see above), which have an intrinsically slow rate of attachment. Stirring speed (and other initial culture conditions) must allow optimal cell attachment (both rate and proportion) since

the ability of anchorage-dependent cells to attach is decreased, the longer cells are kept in free suspension. For most established cell lines static/intermittent stirring in a reduced volume has not proved necessary for optimum initial attachment. However if the stirring speed is too fast then poor attachment can result. If the stirring speed is too slow, cell/bead aggregation may occur which can reduce final cell yield (Clark and Hirtenstein, 1981a; Clark et al., 1980).

Microcarrier concentration: As stated above the toxic effects of microcarriers at higher concentrations have been solved (Horng and McLimans, 1975; Levine, Wang and Thilly, 1979; Pharmacia Fine Chemicals Technical Booklet Series, 1978) and cultures containing high concentrations of microcarriers (more than 5 mg/ml microcarriers) have been successful in obtaining very high cell yields, although maintaining an adequate nutrient supply, pH and gas control becomes difficult due eventually to the large concentration of cells in a given culture volume. For most established cell lines, a concentration of about 2.5 - 3 mg/ml final volume of microcarriers has proved optimal (Pharmacia Fine Chemicals, Technical Booklet, 1981).

Inoculation density: The initial inoculation density of cells in a microcarrier culture (like the other factors mentioned) has a great effect on proportion of microcarriers bearing cells and the final cell yield. It has been shown that the efficiency of attachment of cells to microcarriers is similar to the attachment

of these same cells to Petri dish plates. As a guide therefore, microcarrier cultures should be inoculated with the same number of cells/cm² as used when inoculating other types of monolayer cultures (Pharmacia Fine Chemicals, Technical Booklet, 1981). However inoculation densities should also reflect plating efficiencies, as cells with low plating efficiencies require higher inoculation densities to ensure optimum attachment and optimum final cell yield. For many established cell lines, with plating efficiencies greater than 30%, a cell/microcarrier ratio of less than 5 proved optimal, while for cells with a plating efficiency less than 10% (i.e. many primary cell lines) a cell/microcarrier ratio of more than 10 proved optimal (Clark and Hirtenstein, 1981a; Clark et al., 1980). Inoculum condition is also important, the yield from a microcarrier being increased 2 to 3-fold by inoculating with cells taken from exponentially growing cultures rather than confluent cultures (Clark et al., 1980). In studies by these authors, cells at 70 - 80% confluence proved optimal when initiating a microcarrier culture.

Culture media during initial culture phase: For established cell lines additional supplementation to the culture medium normally used, is not required. In contrast, many primary cell lines have been shown to require additional media supplements of normal culture media if maximum cell yields are to be obtained. Many of these additional media supplements have been shown to be of a general nature: pyruvate, non-essential amino acids, adenine, hypoxanthine and thymidine (Clark and Hirtenstein, 1981b), but may be absent from normal culture media. Additional media supplements have

been shown to improve the plating efficiency of many primary cells (Harn and McKeechan, 1979), but many of the mechanisms underlying stimulation of initial attachment and growth of primary cells by these general media components are not known. In the middle or later stages of such cultures, these additional media supplements are not required (Clark and Hirtenstein, 1981b).

Maintaining a microcarrier culture: The main difference between microcarrier and monolayer culture is that microcarrier culture can span a much wider range of cell densities for any single culture. During the microcarrier culture cycle the changes in the density of the cell population are usually greater than 10-fold. Such growth leads to rapid exhaustion of oxygen and medium components and the accumulation of toxic metabolic products. Once large deviations from optimal conditions occur, it is often difficult to return and in many cases permanent damage to the culture is the result. Changes in culture conditions should be anticipated and because of such large changes in cell density in microcarrier cultures, medium changes should be frequent. Another factor in microcarrier culture is the volume of medium. In closed culture vessels, no more than half full proves optimal for gas diffusion (Pharmacia Fine Chemicals, Technical Booklet, 1981). Another key consideration in maintenance of a microcarrier culture is the stirring speed. The rate at which the culture is stirred greatly influences the growth and final cell yield of a culture (Hirtenstein and Clark) and this effect is related to an integrated shear factor. Slow stirring speeds reduce shearing forces on

cells attached to the microcarriers, but too slow a stirring speed results in impaired growth. This effect is due mainly to inadequate gas diffusion and sedimentation and aggregation of microcarriers.

If the stirring speed is too fast then some cells on microcarriers may be detached due to excessive shear forces with impaired growth the result once more. The stirring speed chosen also depends on the design of the spinner flask. The optimal stirring speed when using traditional magnetic spinner vessels is usually 50 - 70 rpm and speeds of 15 - 30 rpm are used with the modified spinner vessels stirred with bulb-shaped rods (Pharmacia Fine Chemicals, Technical Booklet, 1981). A stirring speed sufficient to keep all the microcarriers in suspension with a gradual increase in stirring speed in the latter stages of the culture cycle, has proved optimal for many different cell types in the studies cited above.

CELL FRACTIONATION
PLASMA MEMBRANE ISOLATION

The preparation of the plasma membrane or any other cell organelle in a reasonably pure form may be considered in three stages: (a) disruption and homogenisation of the whole cell/tissue, (b) separation of the particular organelle from the rest of the homogenate in a sufficiently pure form and (c) quantification of purity of a particular organelle.

Disruption

The ultimate aim of any organelle separation is to obtain a highly pure sample of unchanged morphological and metabolic states. Most (if not all) separations fall short of this ideal. In the case of plasma membranes the particular aims in this case can be, to produce large plasma membrane fragments and to minimise the release of intracellular digestive proteases, following disruption of the cells in question. The state in which the plasma membrane is released depends on both the disruption method and the homogenisation conditions. Disruption may be achieved by physical or chemical methods or a mixture of both.

Solid shear methods: The physical stress on the membranes of cells is achieved by the presence of abrasive particles, such as sand or small glass beads, coupled with their use in a mortar and pestle.

Liquid shear methods: In Potter or Dounce homogenisation a pestle is moved or rotated up and down a glass or teflon tube to achieve disruption. The clearance between the wall and the pestle is important as the shearing force depends on the radii of pestle/tube and also on rate of rotation of pestle. Another liquid shear method involving the use of a blender (or liquidiser) has been criticised due to heating effects at high speeds.

Sonication: This method relies on high frequency oscillations to disrupt cells. However the method has serious drawbacks. Heat and free radical production, which cause protein denaturation, are known to occur with sonication, resulting in severe damage to the plasma membrane and other organelles.

Osmotic shock: This method involves incubation of cells in a hypotonic medium which leads to bursting of the cells. Certain cell types may however be more fragile and therefore more susceptible to osmotic shock.

Other procedures: Other alternative disruptive methods include high pressure extrusion in which cells are forced at high pressure through a narrow tube resulting in cell disruption. This method though is poorly understood nor easily controlled. Digestion of cell walls by cellulase or lysosyme and digestion of plasma membranes by trypsin can be used as disrupting methods. Freezing and thawing of cells and treatment of cells with organic solvents are two other disruptive methods.

In all of these disruptive methods, the homogenisation conditions are also important. Due to the relative instability of many biological molecules disruption/homogenisation procedures should be carried out at 4°C. This temperature also reduces the activity of any digestive enzymes that may be released from ruptured lysosomes during the disruptive process. Buffering of the homogenate is also important, since extremes of pH can have detrimental effects on the activities of many enzymes. Trizma base/HCl is a commonly used buffer, which may be used effectively in the range 7.1 - 9.1 at 5°C (this covers the physiological range 7.2 - 7.4).

In summary, disruptive/homogenisation procedures are empirical, the underlying principles not always clearly understood. The exact procedures should be determined for each cell type, with usually physical disruption procedures used in conjunction with mild hypotonic stress.

Isolation

Centrifugation is the most commonly used isolation procedure, in which mixtures are separated on the basis of their densities and/or size. Preparative centrifugation is concerned with the isolation of biological materials of which there are two distinct types; differential and isopycnic centrifugation. Differential centrifugation is essentially kinetic, in which particles move according to their densities and may be pelleted or interrupted at any stage for the removal of the various bands which may have formed. Isopycnic centrifugation is essentially an equilibrium

sedimentation and can be carried out with or without a density gradient. In the latter case, the sample is evenly distributed in a medium of approximate equal density to the specific component to be isolated. Less dense materials will run to the top of the centrifuge tube, while more dense materials will pellet at the bottom. The use of a density gradient allows much finer separation of material on the basis of density. In this procedure, samples are layered on top of a density gradient (continuous or step gradient) and sedimented until the buoyant density of a component is equal to the density of a particular band. Sucrose is the most commonly used density material but has certain disadvantages. Sucrose becomes very viscous at densities greater than 30% (w/w), osmotic effects are large even at low concentrations and sucrose can interfere with certain assays (e.g. Lowry protein assay).

Although centrifugation is the most widely used technique for isolation of organelles, it is not the only one. Plasma membranes may be isolated at the interface of a two-phase polymer system, however the degree of purification obtained by this method may not be very high (Israel, Verjus and Semmel, 1973).

Quantification

Once a cell homogenate has been fractionated, evaluation of purity of the various sub-cellular fractions is required. The evaluation of purity by microscopic examination is very limited. The main approach to this problem is to measure enzymic activities on the basis that many enzymes are restricted to a single site within the cell and thus can be used as marker enzymes to locate

and identify a particular cell site or organelle. The marker enzymes can also be used to assess contamination of organelles. Confirmation of marker enzymes in a specific cellular site is often achieved by histological techniques. Difficulties can arise though, distinguishing between non-specific background staining and the true presence of 'markers' at low concentration in other organelles/sites.

By careful use of known marker enzyme activities the fraction(s) thought to correspond to the isolated plasma membrane may be characterised.

The specific activities of known plasma membrane marker enzymes and known intracellular marker enzymes may be measured, and the specific activities of each particular marker compared to the specific activity of this marker in the whole cell homogenate (i.e. prior to isolation). Quantification of the degree of enrichment and degree of contamination of the plasma membrane preparation can thus be determined in this way. The evidence for the existence of specific plasma membrane marker enzymes is reviewed below.

Na/K ATPase: It is generally accepted that this enzyme is intimately associated with the sodium pump (reviewed elsewhere), and has been shown histochemically on the plasma membrane of a wide variety of cells: de Thé (1968) has shown the Na/K ATPase on the plasma membrane of rat kidney and rat pancreas, Wallach and Ullrey (1962) have demonstrated the presence of Na/K ATPase on the plasma membrane of Ehrlich ascites carcinoma cells. In the analysis of plasma

membranes and smooth membranes of HeLa cells, Bosmann, Hagopian and Eylar (1968) detected no Na/K ATPase activity in the smooth membranes with highly enriched Na/K ATPase specific activity in the plasma membrane fraction. However some reports have claimed that Na/K ATPase activity is not exclusively found in plasma membrane fractions. Glick (1976) has reported that Na/K ATPase activity is found in other cell fractions as well as in the plasma membrane fraction of some plasma membrane preparations.

Adenylate cyclase: Adenylate cyclase has been proposed as one of the best plasma membrane marker enzymes (Solyom and Tram, 1972) and indeed it's presence has been shown histochemically in several rat liver cells (Reik, Petzold, Higgins, Greengard and Barrnett, 1970). However some reports have claimed it's presence in the sarcoplasmic reticulum (Entman, Levey and Epstein, 1969) and in the Golgi apparatus (McKeel and Jarret, 1974).

5'nucleotidase: 5'nucleotidase has been shown to be a plasma membrane marker in many studies (for reviews see De Pierre and Karnovsky, 1974a; De Pierre and Karnovsky, 1974b). However it's validity as a plasma membrane marker in HeLa cells has been called into question by Johnsen, Stokke and Prydz (1974). These workers claimed that 5'nucleotidase did not codistribute with another plasma membrane marker (Na/K ATPase) and on this basis was not exclusively located in the plasma membrane fraction. Brake, Will and Cook (1978) characterised the 5'nucleotidase of HeLa cells and demonstrated that 5'nucleotidase did codistribute with other plasma membrane markers and that little or no 5'nucleotidase activity

could be detected in other cell fractions. The conclusions of these authors, in conflict to those of Johnsen et al. (1974), stated that HeLa 5'nucleotidase was indeed a good plasma membrane marker enzyme.

Intracellular enzyme markers: A set of generally accepted markers for the various cellular organelles has been established (mainly by histochemical methods), although variability between tissues and species does exist. Intact mitochondria can be identified morphologically, but succinate dehydrogenase of the inner mitochondrial membrane is used as an enzyme marker. Lysosomes are detected by assaying for B-hexosaminidase activity or acid phosphatase activity (for review see Wattiaux, 1977). The endoplasmic reticulum is detected by assaying for NADH ferricyanide reductase activity. In liver glucose-6-phosphatase has been shown to be a marker for endoplasmic reticulum, but in other cells and tissues glucose-6-phosphatase is present in low amounts and in these circumstances its validity as a specific marker may be questioned. Lactate dehydrogenase is the marker commonly assayed to show the presence of the soluble cytoplasmic component of the cell.

In summary, specific marker enzymes may be used to detect the presence and enrichment of the plasma membrane but also to determine contamination of the isolated plasma membrane by intracellular components. However there are several misgivings concerning the use of marker enzymes - cell/cell differences in 'marker' enzyme locations can and does exist and also many histochemical methods contain inherent difficulties (e.g. background staining). Furthermore fractionation effects may occur, such

that markers may become associated with other sites following the disruptive/homogenisation procedures. It is worth bearing in mind though, that even though markers may be present in other organelles in small amounts, the marker can still be used, though the error will be proportional to the amount of contamination.

Unlike the intracellular components, the plasma membrane outer surface can be studied without disrupting the cell. Probing of this external surface in intact cells, can reveal plasma membrane markers which avoids many of the ambiguities and problems listed above (e.g. fractionation problems, markers found in more than one cellular location). These external markers are reviewed below.

Ectoenzymes: The plasma membrane may contain enzymes whose active site faces the external medium rather than the cytoplasm. The best example of such an ectoenzyme is acetylcholinesterase which hydrolyses the transmitter substance released into the synaptic cleft of the neuromuscular junction and which is therefore thought to have its active site facing the external medium. An ectopeptidase has been reported in human and dog polymorphonuclear leukocytes (Najjar and Nishioka, 1970) and Agren, Pontén, Ronquist and Westermark (1971) claim the presence of glyceraldehyde-3-phosphate dehydrogenase on the surface of human erythrocytes, Ehrlich ascites carcinoma cells and HeLa cells. In some cells types exo-5'nucleotidase has been reported (Trams and Lauter, 1974). It is worth stating that demonstration that intact cells act on a substrate is hardly sufficient evidence for the presence of an ectoenzyme - the substrate must be shown to be impermeable (other kinds of evidence that must

be gathered for the existence of an ectoenzyme is reviewed by De Pierre and Karnovsky, 1973).

Sialic acid: Removal of a given substance from intact cells by treatment with an enzyme is taken as evidence that this substance is located on the outside of the plasma membrane. This approach has been used with sialic acid. Eylar, Madoff, Brody and Oncley (1962) demonstrated that neuraminidase removes 95 - 100% of sialic acid found in chicken, pig, lamb, calf and human erythrocytes. Treatment of intact guinea pig or human leukocytes with neuraminidase removes 40 - 60% of their total sialic acid (Lichtman and Weed, 1970). Many other examples of sialic acid removal exist, but in all cases one has to be sure that neuraminidase does not enter the cell. This has been accomplished by attaching neuraminidase covalently to a large solid support. De Pierre and Karnovsky (1972) attached neuraminidase to beads of agarose measuring 40 - 210 μm in diameter thus preventing uptake of neuraminidase into the cell.

Hormone binding: The specific binding of hormones (and other ligands) to cell surface receptors can be used successfully as markers for the plasma membrane. The problem of hormone uptake (and also receptor-mediated endocytosis) exists as in the above case, but may be similarly prevented by attaching the hormone to a large solid support as in the case of insulin (Cuatrecasas, 1969).

Nonpenetrating agents: These reagents are often large molecules which react covalently with groups on the cell surface under mild conditions, including neutral pH. Detection of a relatively small number of reagent molecules should also be possible (many probes are fluorescent in nature allowing easy detection of small numbers). As the title suggests, these reagents should be unable to penetrate the plasma membrane. Reagents which have been developed include: 1-anilino-8-naphthalene sulfonate (Romeo, Cramer and Rossi, 1970), formyl methionyl sulphone methyl phosphate, SITS [4-acetamido-4'-isothiocyano-2,2'-stilbine] (Maddy, 1964) and DIDS [4,4'-diisothiocyano-stilbine] (Cabantchik and Rothstein, 1972). These reagents all appear to react with functional groups of surface proteins.

Other approaches in studying the plasma membrane of intact cells include the use of lectins. Lectins are plant proteins which are able to bind to specific carbohydrate groups found in glycoproteins of animal plasma membranes. These lectin proteins, which include wheat germ agglutinin and concanavalin A, can be labelled with ^{125}I to enable detection on the plasma membrane surface. The development of monoclonal antibodies represents a major step forward in the study of plasma membrane structure and function. Monoclonal antibodies can be directed against not only specific receptor proteins but also specific surface antigens, with extremely little non-specific binding. Their remarkable specificity therefore, makes the use of monoclonal antibodies as probes of membrane structures, very attractive indeed.

Plasma membrane isolation on microcarrier culture beads

Membrane isolation procedures that are rapid (e.g. aqueous two-phase polymer separations) also tend to provide minimal purification and many of those that are more reliable (e.g. cell fractionation by isopycnic density gradient centrifugation) can be time consuming. However Cohen, Kallish, Jacobson and Branton (1976, 1977) and Jacobson and Branton (1977) devised a rapid method for the isolation of relatively pure plasma membranes on polylysine-coated acrylamide beads. The method involved attachment of cells to these beads, followed by lysis and shearing away of internal components, leaving plasma membranes attached to the bead surface.

This method takes advantage of the tendency of the negatively charged cell surface to adhere tenaciously to some positively charged surfaces. In the procedures of Cohen et al. (1977) and Jacobson and Branton (1977) polyacrylamide beads were derivatized with poly(L-lysine) to place the positive charge on the beads.

Polylysine derivatized glass beads have also proved suitable in these membrane isolation procedures (Kallish, Cohen, Jacobson and Branton, 1978). To prevent sticking of internal components to the 'bare' regions of beads following cell lysis, Jacobson (1980) suggested neutralisation of the bare regions of the beads immediately following cell attachment, with a polyanion (polyacrylate).

In 1980, Gotlib and Searls utilized commercially available DEAE-Sephadex beads for cell attachment and plasma membrane isolation. The use of these beads did away with prior derivatization with polylysine, as the positive charge was already fixed on the bead surface.

Gotlib (1982) then used microcarrier beads as a solid support for the preparation of plasma membranes of several different cell lines. Furthermore Gotlib showed that the use of buffers of pH 8 (or more) during the membrane isolation procedure, reduced the binding of intracellular contaminants to the beads. The use of commercially available microcarrier beads along with the use of buffers of pH 8 during the membrane isolation process, has eliminated the need for bead derivatization as well as bead neutralization and as a consequence greatly improved the rapidity with which membranes on beads can be isolated. These plasma membrane isolation methods appear to be general, having been used for many different cell lines in the studies cited above.

A potential problem in the isolation of membranes on beads is the possibility of selecting a specific portion of the plasma membrane (i.e. the baso-lateral surface). Jacobson and Branton (1977), Kaulish et al. (1978) and Jacobson (1980) have all addressed this problem by biochemical analysis of the isolated membranes on beads from several different cell types. Jacobson and Branton (1977) and Kaulish et al. (1978) have shown that the protein and phospholipid composition of erythrocyte membranes harvested from beads was indistinguishable from that of erythrocyte ghosts. Gotlib (1982) has shown that the recoveries of various plasma membrane markers was uniform, further suggesting that selectivity of portions of the membrane during the attachment or isolation procedures was unlikely.

Morphological evidence of isolated membranes on beads, show that relatively large intact sheets of membranes are isolated (Cohen et al., 1977; Jacobson and Branton, 1977; Kahlisch et al., 1978), while Gotlib and Searls (1980) and Gotlib (1982) have reported yields of 25% of plasma membrane marker enzyme activities recovered on beads. This latter fact compares favourably with standard membrane isolation procedures.

In all the membrane isolation procedures above, cells were attached acutely to beads, and membranes on beads prepared. The use of microcarrier culture beads allows cultured cells to be grown on beads to a high density, then membranes on beads prepared by the methods outlined above. This chronic attachment of cells to beads during the microcarrier culture however, may lead to differentiation of the plasma membrane surface not observed during acute attachment.

The rapidity with which pure membranes may be obtained (1.5 hr) should make the bead isolation method useful in studies of plasma membranes.

METHODSCell culture

HeLa cells S3 obtained from Flow Laboratories, Irvine, Scotland were grown for 4-6 days in Roux flasks (120 cm²) in Eagle's Basal Medium (BME) supplemented with: 10% (v/v) new-born calf serum, 100 units/ml penicillin/streptomycin and 2 mM glutamine. When the cells approached confluency on the Roux bottle, the culture was treated as follows: the medium was poured off, 5 ml of a 0.25% trypsin solution in Mg/Ca free Earle's salt solution added, Roux bottle shaken until the monolayer of cells detached and the trypsin neutralised by the addition of 45 mls BME (supplemented with 10% serum).

The suspension was then repeatedly passed through a sterile 10 ml syringe and needle until a single cell suspension was obtained. 1 ml of this suspension was used to determine the cell number using a Coulter Counter (model zF) and Coulter Channelyzer (model C 1000).

Usually 5 million cells from the single cell suspension were added to another Roux bottle and made up to approximately 100 ml final volume with fresh complete BME.

HeLa cells were also subcultured onto 5 cm Sterilin plastic petric dish plates in which case 0.4 million cells in a final volume of 4 mls were seeded on each plate, the plates stacked in plastic sandwich boxes and the boxes sealed with plastic tape.

The Roux bottles and sandwich boxes containing plates, were both gassed with a 95% air, 5% CO₂ mixture and kept in a 37° C room.

MDCK cells, obtained from Flow Laboratories, of between 60 and 66 serial passages, were cultured in a similar way to HeLa cells (above) with the following exceptions: All MDCK cells were grown in Minimum Essential Medium with Earle's salts (MEME) supplemented with non-essential amino acids, 2 mM glutamine, 40 µg/ml kanamycin, 5% (v/v) donor horse serum, 5% (v/v) foetal calf serum. The MDCK monolayer was detached from the Roux bottle by incubation for 1 hour in 0.25% trypsin solution in Mg/Ca free, Earle's salt solution containing 2 mM EDTA. Otherwise subculturing and reseeding procedures were similar for MDCK and HeLa cells. All steps were carried out in a laminar flow sterile air cabinet.

Microcarrier cell culture

A Techne (Cambridge) Ltd. MCS-104 stirring system was employed. The 500 ml culture vessel is stirred by a bulb-shaped rod with one end fixed above. The vessel also has a rounded and indented base as well as a 'soft-start' function. Superbead microcarriers were obtained from Flow Laboratories.

Initiating a microcarrier culture

Preliminary experiments were carried out with microcarriers in small petri-dishes. The attachment rate, tendency for cells to clump and optimum cell:microcarrier ratio were estimated in order to optimise initial culture conditions.

Usually 50 ml of an even suspension of sterile 0.5 g superbead microcarriers in phosphate buffered saline (PBS) were washed twice with BME supplemented with 10% new born calf serum and then added to a spinner flask. HeLa cells growing in a large Roux bottle at 70-80% confluence were harvested and 15 million cells added to the spinner flask. The culture volume was made up to 200 ml and the stirring speed was set at 35-40 rpm throughout. The medium was changed every 2 to 3 days; to do this the cells on beads were allowed to sediment, the medium poured off and fresh, warm medium was added to the flask. The flask was then gassed with a 95% air/5% CO₂ mixture. The protocol for initiating a microcarrier culture with MDCK cells were similar except MEME medium containing x2 the usual amount of glucose was used.

Observing cells in a microcarrier culture

Routine observation of growth and the morphology of cells was carried out using a light microscope. A small sample of the culture was placed on a microscope slide and observed under low power.

Counting cells released after trypsinisation

Three 1 ml samples of an evenly suspended culture were placed in small test tubes. The microcarriers were allowed to settle out, the supernatant removed from each tube and the microcarriers washed twice in 2 ml of Ca/Mg-free PBS containing 0.02% (w/v) EDTA, pH 7.4. When the microcarriers had settled out, the wash supernatant from each tube was removed and 1 ml of a 1:1 0.25% (w/v) trypsin in Ca/Mg-free PBS and EDTA 0.02% in Ca/Mg-free PBS was added. The mixture was incubated at 37° C for 15 minutes with occasional agitation or as long as necessary to detach all of the cells from microcarriers (this time taken was determined in a parallel experiment with constant monitoring of detachment of cells from the microcarriers). 2 ml of culture medium was added to each tube to give 3 ml final volume and this suspension filtered through a 100 um nylon mesh under gentle vacuum. The cell suspensions were collected, blasted by passage through a needle and 5 ml syringe, and the single cell suspensions counted in a Coulter Counter (zF) with Channelyzer (C1000) as before.

Measurement of cell viability

Trypan blue solution was prepared in PBS (4 mg/ml) pH 7.4. Approximately 0.9 ml of diluted cell suspension was mixed with 0.1 ml trypan blue solution. After 5 min at room temperature the viable (unstained) and the non-viable (stained) cells were counted in a haemocytometer. (Trypan blue stains the microcarrier beads, therefore cells must be detached from microcarriers beforehand.)

Solutions used

BME, MEME consisted of the following:

Component	BME mg/l	MEME mg/l
CaCl ₂	200	200
D-glucose	1000	1000
MgSO ₄	98	98
KCl	400	400
NaCl	6800	6800
NaH ₂ PO ₄ ·H ₂ O	140	140
NaHCO ₃	2250	2250
phenol red	10	10
L-arginine HCl	21.06	126.40
L-cysteine	12.01	24.02
L-histidine HCl·H ₂ O	10.50	41.90
L-isoleucine	26.23	52.50
L-leucine	26.23	52.50
L-lysine HCl	36.53	73.06
L-methionine	7.46	14.90
L-phenylalanine	16.51	33.02
L-threonine	23.82	47.64
L-tryptophan	4.08	10.20
L-tyrosine	18.11	36.22
L-valine	23.43	49.60
D-biotin	1.00	1.00
D-Ca pantothenate	1.00	1.00
choline chloride	1.00	1.00
folic acid	1.00	1.00
i-inositol	2.00	2.00
nicotinamide	1.00	1.00
pyridoxal.HCl	1.00	1.00
riboflavin	0.10	0.10
thiamin HCl	1.00	1.00

BME was supplemented with 10% (v/v) newborn calf serum, 100 units/ml penicillin/streptomycin and 2 mM glutamine.

MEME was supplemented with 5% (v/v) fetal calf serum, 5% (v/v) donor horse serum, 40 µg/ml kanamycin, 2 mM glutamine and non-essential amino-acids.

Other solutions

The following sterile solutions were obtained from Flow Laboratories (Scotland) or Gibco (UK): Earle's Ca/Mg-free salt solution, new-born calf serum, donor horse serum, fetal calf serum, penicillin/streptomycin, kanamycin, L-glutamine and non-essential amino acids.

Materials

Tissue culture supplies were obtained from Flow Laboratories (Scotland). Microcarrier culture stirring system and spinner flasks MCS-104 were obtained from Techne (Cambridge) Ltd. Superbead microcarriers were obtained from Flow Laboratories (Scotland). Inorganic salts of Analar quality were used.

Plasma membrane isolation on microcarrier culture beads.

This method is adapted from the techniques of Cohen, Kalish, Jacobson and Branton (1977) and Gotlib (1982). A 10 ml sample from a microcarrier culture with cells at or near confluence on the microcarrier beads, was collected into a sterile universal tube (Sterilin). This sample was washed x4 with cold tris buffered saline (TBS; 0.9% NaCl, 5 mM Tris). Between each wash the microcarrier beads were allowed to settle out before the supernatant was removed. Usually almost all of the beads sedimented out in 3 minutes. The sample was then incubated with ice-cold hypotonic solution (1 mM Tris pH 8.0) usually for 30 minutes and then washed twice in ice-cold 10 mM Tris pH 8.0. 10 ml of ice-cold 10 mM Tris pH 8.0 was added to the cells on beads and the suspension vortexed for 10 seconds on a vortex mixer resulting in complete cell lysis. The microcarrier beads were allowed to settle and then washed twice more with ice-cold 10 mM Tris pH 8.0. The supernatant was removed and enough ice-cold 10 mM Tris pH 8.0 was added to give approximately a 50% suspension of beads. This suspension was then sonicated for 10 seconds at 20 W in a Sonifier cell disruptor (model W185) and then washed twice more in ice-cold buffer.

The membrane coated beads were then used for a variety of biochemical assays. All procedures were performed on ice.

Materials

Inorganic salts of Analar quality were used. Microcarrier spinner flasks and stirring system MCS-104 were obtained from Techne (Cambridge) Ltd. The sonicator used was a Sonifier cell disruptor (model W185) from Heat Systems Ultrasonics Inc.

5' nucleotidase (E.C. 3.1.3.5)

This method was adapted from the phosphodiesterase assay of Arch and Newsholme (1976). Enzyme samples of 200 μ l were added to 700 μ l of a solution consisting of: 50 mM Tris, 5 mM $MgCl_2$, 2.5 mM dithiothreitol and 0.23 mg/ml bovine serum albumin (BSA) pH 7.4 in RT 25 assay tubes. The assay tubes were pre-incubated for 3 minutes at 37° C and the reaction initiated by the addition of 100 μ l 10 mM 5'AMP to which 5 μ Ci [3H]-5'AMP was added per ml. 20 minutes later, 200 μ l samples were removed and immediately added to 1 ml of a solution consisting of: 0.6 g anion exchange resin AG1 x2 200-400 mesh Cl^- added to 1 ml H_2O (this gave a 1:1 (v/v) solution). The samples were centrifuged at 9000 g for 1 minute in a Beckman microfuge, then 0.4 mls removed and counted in a liquid scintillation spectrophotometer. Activities were expressed as nmoles 5'AMP hydrolysed per hr per mg protein.

Potassium stimulated p-nitrophenylphosphatase

The ouabain-sensitive component of potassium stimulated p-nitrophenylphosphatase is a function of the plasma membrane marker

enzyme, Na/K ATPase. Activities of potassium stimulated p-nitrophenylphosphatase were determined by the method of Bers (1979) and expressed as μ moles p-nitrophenol produced per hr per mg protein.

Sodium/potassium ATPase (E.C.3.6.1.3)

This enzyme was assayed by an adaptation of the method of Bers (1979).

Samples were incubated with 120mM NaCl, 50mM Tris, 5 mM NaN_3 , 2.5 mM MgCl_2 , 0.4mM ATP, \pm 20 mM KCl and \pm 2 mM ouabain pH 7.4.

The incubation was carried out at 37° C for 15 minutes and stopped by addition of 10% TCA. Phosphate released was measured by the method of Chen, Toribaum and Warner (1956). Activities were expressed as moles phosphate produced per hr per mg protein.

Adenylate cyclase (E.C.4.6.1.1)

This enzyme was assayed in whole cell homogenates or membrane preparations by the following procedure: 40 μ l of regenerating system were added to 1.5 ml microfuge tubes, along with 40 μ l 1BMX/GTP solution, 40 μ l enzyme sample in incubation buffer and 40 μ l of the drug forskolin to give a final concentration of 10^{-4} M forskolin in the total assay volume of 200 μ l. The reaction was initiated by the addition of 40 μ l 5 mM ATP and incubated at 30° C for 10 minutes. The reaction was stopped by addition of 50 μ l 1N HCl, the assay tubes vortex mixed, placed on ice and neutralised with 50 μ l 1N NaOH. Blank tubes were obtained by addition of 50 μ l 1N HCl immediately after addition of 40 μ l 5 mM ATP. Blank tubes were then treated as described for samples. All tubes were centrifuged for 1 minute at 9000 g in a Beckman microfuge to pellet precipitated protein and

microcarrier beads. Duplicate 100 μ l aliquots of the supernatant were assayed for cAMP content. The regenerating system consisted of 100 mM creatine phosphate and 0.2 mg/ml creatine kinase made up in incubation buffer containing 1 mg/ml BSA. The incubation buffer consisted of 80 mM Tris maleate, 4 mM $MgSO_4$ and 0.2 mM EGTA pH 8.1. The IBMX/GTP solution consisted of 5 mM isobutylmethylxanthine and 500 μ M GTP.

Cyclic-AMP production requires an adequate supply of ATP, this is supplied by the regenerating system. The guanine nucleotide GTP is also included in the assay. GTP binds to the regulatory subunit of the adenylate cyclase system and is necessary for the activation of the catalytic subunit. Like ATP, GTP levels are maintained by the regenerating system.

cAMP determination

The 100 μ l supernatant aliquots were assayed in duplicate using a competitive binding protein assay for cAMP (Brown, Albano, Ekins, Sgherzi and Tampion, 1971).

Appropriate controls were always made for non-specific effects of supernatant fluid on the sensitivity of cAMP determination by supplementing standard cAMP curves with appropriate supernatant fluid.

Activities were expressed as pmoles cAMP produced per hour per mg protein.

B-hexosaminidase

This enzyme was assayed according to the method of Barrett (1972). The substrate p-nitrophenyl-N-acetyl-B-glucosaminide was used as substrate. The sample (homogenate or membrane preparation) was placed in a citrate buffer containing 0.3 M NaCl and the reaction stopped by the addition of bicarbonate buffer. Activities were expressed as μ moles p-nitrophenol produced per hr per mg protein.

Succinate dehydrogenase (E.C. 1.3.99.1)

This enzyme was assayed according to the method of Pennington (1961). INT, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (0.1%) was added to assay tubes along with 50 mM sodium succinate, 50 mM potassium phosphate buffer pH 7.4, 25 mM sucrose, and enzyme sample. The reaction was stopped, following incubation for 15 minutes at 37°C, by addition of 10% trichloroacetic acid (TCA). The formazan produced was extracted by ethyl acetate and the O.D. 490 measured. Activities were expressed as nmoles formazan produced per hr per mg protein.

Lactate dehydrogenase (E.C. 1.1.1.27)

This enzyme was assayed according to the method in Boehringer Mannheim Technical Booklet (1973). The enzyme samples were incubated with 0.1 M phosphate buffer, 23 mM sodium pyruvate and 2 mM NADH, pH 7.0. The change in OD 340 nm per unit time is a measure of LDH activity. Usually the O.D. 340 change was read at 1, 2, 3, 4 and 5 minutes following initiation of reaction and was linear over this period. Activities were expressed as units activity per mg protein.

NADH-ferricyanide reductase (E.C. 1.6.99.3)

This enzyme was assayed according to the method of Avruch and Wallach (1971). This enzyme was measured in a 1 ml volume containing: 10 μ moles NADH, 0.66 μ moles $K_3Fe(CN)_6$ and enzyme suspension. The reaction was monitored at $A_{340\text{ nm}}$. Activities were expressed as nmoles NADH oxidised per hr per mg protein.

Protein determinations

The protein content of samples was determined by procedures adapted from the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard.

Materials

General chemicals of Analar quality were used. [3H]-5'AMP (specific activity 12 Ci/mmmole) and [3H]-cyclic AMP (specific activity 40 Ci/mmmole) were obtained from Amersham International, Bucks. The ion exchange resin AGI x2 200-400 mesh, chloride form was obtained from Bio-Rad. Forskolin was obtained from Calbiochem. Creatine kinase, bovine serum albumin (BSA), ouabain and p-nitrophenyl-N-acetyl-B-glucosaminide, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride were all obtained from the Sigma Chemical Company.

Ouabain and digoxin binding

Free sodium pumps were generally measured with [^3H]-ouabain or [^3H]-digoxin at 2×10^{-7} M for 15-20 min. The specific binding was usually taken as the difference between that bound from 0 mM K Krebs and that from 15 mM K Krebs ('OK' and '15K' respectively).

The 15K binding, which was usually less than 10% of the total (for ouabain), was taken as the non-specific binding. Methods above closely follow those of Aiton, Lamb and Ogden, (1981); Lamb and Ogden, (1982) and Baker and Willis, (1970). Another way to measure specific glycoside binding (or free sodium pumps) was employed. ^3H -glycoside was bound in OK Krebs for 20 minutes. The reaction was stopped by washing in cold 5K Krebs and the non-specific component washed out for a further 15 minutes in warm growth medium or warm 5K Krebs. This method, adapted from Griffiths, Lamb and Ogden (1983), was used when specifically binding ^3H -glycoside to cells on beads in a microcarrier spinner culture. Controls were carried out either with cells in spinner culture or with cells on plates in the following way. Washout experiments were carried out using non-radioactive glycoside. At the end of the washout period, the cells were challenged with [^3H]-glycoside in the presence of either warm OK Krebs or warm 15K Krebs, for a further 15 minutes. The results showed no difference in the amount of ^3H -glycoside binding in OK and 15K Krebs strongly suggesting that only further non-specific binding occurs. This and other controls, as carried out by Griffiths, et al., confirm that there is no significant difference between amount of specific glycoside binding obtained by subtracting 15K Krebs binding from OK Krebs binding, or amount of specific glycoside binding obtained by washout of non-specific binding.

Solutions

Glycoside binding experiments were usually carried out in Krebs solution containing: 137 mM NaCl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 0.3 mM NaH₂PO₄, 0.3 mM KH₂PO₄, 12 mM HCl, 14 mM Tris and 11 mM glucose, supplemented with 1% (v/v) new-born calf serum, pH 7.4. K free Krebs (OK) was prepared according to the formula above but omitting KCl and KH₂PO₄; 1% dialysed new-born calf serum was usually added. 15mM K Krebs (15K) was prepared by adding the appropriate amount of 1 mM KCl to OK Krebs solution.

Materials

Ouabain and digoxin were obtained from the Sigma Chemical Company. [³H]-ouabain and [³H]-digoxin (specific activities 30 Ci/mmol) were obtained from Amersham International. Tissue culture supplies were obtained from Flow Laboratories (Irvine). General chemicals of Analar quality were used where possible.

HeLa plasma membrane isolation on discontinuous sucrose gradient

HeLa plasma membranes were isolated by a method adapted from procedures of Atkinson and Summers, (1971) and Brake, Will, and Cook (1978). The method is detailed below.

HeLa S3 cells were grown in a Roux bottle for 4-6 days, washed with 0.9% NaCl, 5 mM Tris pH 7.4 and then removed from the Roux bottle by scraping with a brush, normally used for cleaning test-tubes, into ice cold 6 mls homogenisation buffer: 16 mM NaCl,

1 mM Tris, 0.3mM $MgCl_2$ 0.5 mM KCl adjusted to pH 7.4. After incubation for 30 minutes in the homogenisation buffer the cells were disrupted in two main ways:

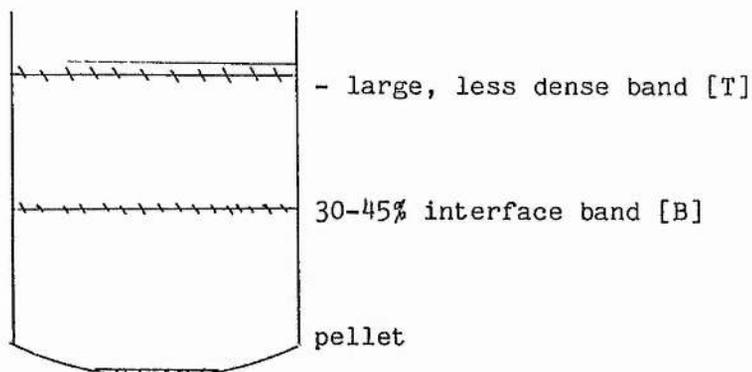
Method (1). The HeLa cell suspension was disrupted by use of a polytron (model PTA 105, Kinematica, Switzerland), setting 6 for 15 seconds and then examined under phase contrast microscopy for the presence of released nuclei, whole plasma membrane ghosts and/or cell debris as a measure of cell disruption.

Method (2). The HeLa cell suspension was disrupted by repeated passage through a 5 ml syringe fitted with a 19 G needle and then examined under phase contrast as method (1) to determine degree of cell disruption.

After homogenisation, 5 ml homogenate was added to 0.5 ml of the following solution : 1.6 M NaCl, 100 mM Tris, 30 mM $MgCl_2$, 50 mM KCl, with 50% (w/w) sucrose in order to restore isotonicity with respect to electrolytes plus 5% sucrose to stabilise cell organelles against further lysis. A low speed spin - to remove whole cells and nuclei was then carried out at 1300g for 30 seconds in a Coolspin centrifuge. Solutions of 30% (w/w) and 45% (w/w) were made up in TBSS (160 mM NaCl, 10 mM Tris, 3 mM $MgCl_2$, 5 mM KCl, pH 7.4). 15 ml of the 30% solution was then layered on top of 15 ml of the 45% sucrose solution in a 38 ml Beckman centrifuge tube. 5 ml of the sample homogenate was placed on top of the gradient.

The tube was placed in an SW 27 rotor and centrifuged in a Beckman L2-65B Ultracentrifuge for 20 minutes at 8000g.

The following bands were obtained:-



According to Brake *et al.* (1978) large plasma membrane fragments appear at the 30-45% interface. Both bands T and B were removed by needle and syringe into separate centrifuge tubes (Beckman 38 ml), diluted 1 in 5 with TBSS to lower the sucrose concentration and then centrifuged in an SW 27 rotor at 8000g for 15 minutes. The supernatants were removed and the pellets resuspended in TBSS. The samples of T and B were assayed for marker enzyme activity.

HeLa plasma membrane isolation on discontinuous sucrose gradient
by method of B. McCaldin

This was an alternative procedure according to the method of B. McCaldin (1978).

The following solutions were made up. Homogenisation buffer: 605 mg Tris, 46 mg $MgCl_2$ and 0.25 ml of 1 M $CaCl_2$. 58% (w/w) sucrose stock solution: 87 g sucrose + 63 ml homogenisation buffer.

HeLa cells were grown for 4-6 days in a Roux bottle, washed x2 in homogenisation buffer then scraped into 50 mls of homogenisation buffer with a brush. The suspension was centrifuged for 10 minutes at 3000g in an MSE Coolspin centrifuge.

The pellet of cells was resuspended in homogenisation buffer to give a final volume of 6 mls and then lysis achieved by repeated passage (x60) through a 19G needle and syringe. Cell disruption was monitored under phase contrast microscopy as above. 3 mls of the homogenate was added to 5 mls of the 58% (w/w) sucrose solution to give a 40% (w/w) final sucrose concentration.

The discontinuous sucrose gradient was prepared in a 38 ml Beckman centrifuge tubes as follows:

2 mls		buffer		
4 mls		25% sucrose solution		
7 mls		30%	"	"
7 mls		35%	"	"
7 mls		40%	"	"
3 mls		50%	"	"
3 mls		55%	"	"
5 mls		58%	"	"

The sample was centrifuged for 75 minutes at 130000g using an SW 27 rotor in a Beckman L2 65B ultracentrifuge. Fraction II, (appearing at the interface between the 25% and 30% sucrose solutions) and fraction III (appearing at the interface between the 30% and 35% sucrose solutions) were collected using a needle

and syringe into another 38 ml centrifuge tube. The volume was made up to 38 mls with homogenisation buffer and the sample centrifuged in an SW 27 rotor at 130000g for 1 hour in a Beckman L2-65B ultracentrifuge. The pellet was collected and resuspended in homogenisation buffer.

Preparation of whole cell homogenates.

(A) HeLa cells growing in a Roux bottle

The two methods used are as described above. In the first method, a suspension of HeLa cells were disrupted by use of a polytron homogeniser at various settings and treatment times. A second method of disrupting HeLa cells was adapted from the procedures of B. McCaldin (1978) and involved passage of HeLa cells through a 19 G hypodermic syringe needle of 0.11 mm diameter. Between 60-100 passages through the needle were tried. In both methods, cellular disruption was monitored under phase contrast microscopy.

(B) HeLa cells growing in microcarrier culture

HeLa cells were grown for 4-6 days in a microcarrier spinner culture and a 10 ml sample was removed into a sterile, universal plastic tube. The cells on beads were allowed to settle out, the supernatant removed and the sample washed x4 with ice-cold 0.9% NaCl, 5 mM Tris, pH 7.4. The sample was then incubated with ice-cold hypotonic solution (1 mM Tris pH 8.0) for at least 30 minutes. After this incubation, the sample was vortex mixed for 10 seconds.

Examination of the sample under phase, revealed no intact cells remaining on beads, released nuclei and much cellular debris.

RESULTSMicrocarrier spinner culture

Microcarrier culture procedures are based on what is already known about the cell type to be cultured. The morphology, plating efficiency and growth properties of a cell type in a traditional monolayer culture procedure, will determine the optimum microcarrier culture procedure. Studies by Clark and Hirtenstein (1981a) and Clark, Hirtenstein and Gebb (1980) have shown that plating efficiency is one of the most useful parameters to consider in this respect. These authors also observed the relationships between plating efficiency and the initial microcarrier culture variables: cell/microcarrier ratio at inoculation, initial culture volume and initial stirring speed, for a wide variety of cell types. HeLa cells and most established cell lines have plating efficiencies greater than 30% and applying the findings of Clark *et al.* (1980), were cultured with a cell/microcarrier ratio of 5, an initial culture volume 100% of final volume and an initial stirring speed of 30-40 rpm (continuous stirring). These values may need to be altered slightly due to other considerations such as: weak or slow attachment to culture surfaces, rounded morphology of cells and a tendency to aggregate upon inoculation. However it was not found to be necessary to alter the above parameter values with HeLa cells.

Preliminary experiments were carried out to assess the attachment of HeLa cells to microcarrier beads in the inoculum conditions stated above. Samples of 1.5×10^4 microcarrier beads/ml were mixed

with 7.5×10^4 HeLa cells/ml (both in BME) in small Petri dishes. The Petri dishes were left for 3 hours and the % of beads having 2 or more cells attached were counted, as well as the % of beads aggregated together. Usually 100 beads were randomly counted. Beads having 2 or more cells attached is a measure of attachment as well as indicative of likely, future cell proliferation. The % aggregation was expressed as the number of beads aggregated per 100 beads counted. The results are shown in Table 1.1.

Table 1.1. HeLa cell/microcarrier association following a 3 hour incubation in a Petri dish

% of beads having 2 or more cells attached	40
% aggregation	30

The association of cells/beads was monitored with a light microscope fitted with a grid lens, following a 3 hour incubation in a static culture. Results are the means of 5 different experiments.

The results in Table 1.1 show that aggregation of beads may be a problem in microcarrier spinner culture.

The attachment and aggregation was then monitored in a micro-carrier spinner culture, inoculated with 7.5×10^4 cells/ml and 1.5×10^4 beads/ml. Samples were removed from the spinner culture 3, 6 and 12 hours after inoculation, then the % of beads having 2 or more cells attached and the % aggregation of beads were determined. The results are shown in Table 1.2

Table 1.2 Cell/microcarrier association in a
microcarrier culture of HeLa cells

	3 hr	6 hr	12 hr
% of beads having 2 or more cells attached	40	75	75
% aggregation	10	15	20

The association of cells/beads was monitored with a light microscope fitted with a grid lens. Results are the means of 3 different experiments.

The results in Table 1.2 show that cell attachment to beads reaches a plateau at about 6 hours and that bead aggregation increases with time, but is much less than in a static culture. In other experiments it was shown that a reduced initial volume (1/3 final volume) did not increase the amount of cell attachment. It was also found that the use of interval stirring or lower stirring speeds (15 rpm) in the first 24 hours, did not increase cell attachment but both slightly increased the degree of aggregation.

Maintaining a microcarrier culture

Once a microcarrier culture has been initiated, certain procedures and precautions are required in order to maintain proliferation of cells and to achieve a good yield from the culture. Optimum conditions were as follows: usually the initial volume was 200 ml, the medium was changed every 2-3 days or as often as necessary as judged by the colour of the medium, the working volume was increased to 300 ml after the 1st medium change and the stirring speed was increased from 30-40 rpm after 2 days and maintained at this speed for the duration of the microcarrier culture. The stirring speed appears critical. In principle, the stirring speed should be just enough to keep the beads in suspension, but if stirring is too slow then excessive aggregation may result and/or a failure to facilitate gaseous exchange. If the stirring speed is too fast, then a general loss of cells from the microcarriers due to excessive shearing forces occurs, and poor cell yields result. The importance of stirring speed has been established in studies by Hirtenstein and Clark (1980).

Plates: HeLa cells growing on microcarrier beads.

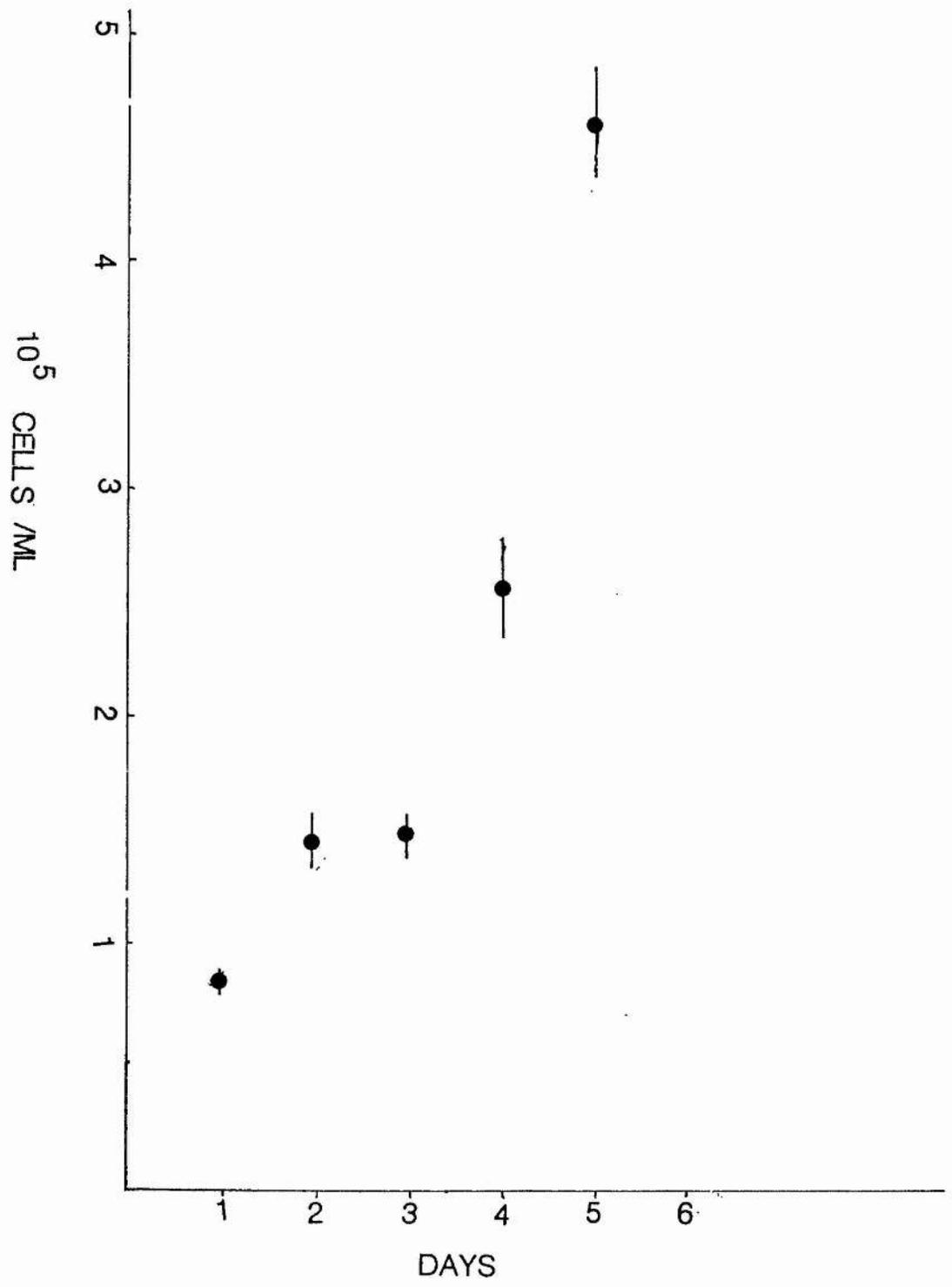
— represents 40 μm in all plates.

Sample slides (wet slide conditions) were photographed using a Biovert Reichert inverted microscope. The microscope had a x10 eyepiece and was set to the x16 objective. Nomarski viewing conditions (interference contrast) were used.





Figure 1a. Growth of HeLa cells in microcarrier spinner culture. The microcarrier spinner culture was inoculated with 7.5×10^5 cells/ml and 1.5×10^4 beads/ml in a volume of 200mls. The medium was changed after 3 days, ensuring that the same final volume (200mls) was always kept constant. Samples (3 x 10mls) were removed each day and washed. The cells were removed from the beads by trypsinisation and the cells and beads separated by filtration through a 100 μ m nylon mesh. The cells were then collected, blasted to form a single cell suspension and each sample counted in a Coulter Counter. Each data point is the mean \pm SEM of 3 observations.



The HeLa cells in the microcarrier culture were harvested after 5-7 days, when optimum cell yield was achieved. Throughout the culture, the HeLa cells had a flattened morphology, indicative of cells in good condition. This was verified by the finding that over 95% of cells removed from beads excluded trypan blue. In all of the different culture conditions tried, some beads with cells attached, did not have a monolayer of cells. Bare patches of various sizes existed on these beads. However it was estimated that 75% of beads with cells attached, did form a monolayer of cells after 5-7 days. The possible reason for existence of these bare patches may be that the microcarrier bead surface may not be uniform. A growth curve of HeLa cells in microcarrier spinner culture is shown in Figure 1a

Microcarrier culture of MDCK cells

MDCK cells are an established cell line with a high plating efficiency. The principles and procedures for the growth of MDCK in microcarrier culture closely followed those for HeLa cells detailed above. The attachment rate and aggregation were monitored for MDCK cells in microcarrier spinner culture at 3, 6 and 12 hrs following inoculation. The inoculum conditions were the same as for HeLa cells: a cell:bead ratio of 5:1, volume 200 ml and a continuous stirring speed of 30 rpm. The results are shown in Table 1.3.

Table 1.3 Cell/microcarrier association in a
microcarrier culture of MDCK cells

	3 hr	6 hr	12 hr
% of beads having 2 or more cells attached	45	75	75
% aggregation	10	15	35

The association of cells and beads was monitored with a light microscope fitted with a grid lens. Results are the means of 3 different experiments.

The results in Table 1.3 show that the attachment rate of MDCK cells to beads is similar to that of HeLa cells. Bead aggregation, (35% after 12 hrs) is much greater than in HeLa cells (20%).

During maintenance of the MDCK culture it was noticed that despite good cell attachment initially, poor cell growth resulted. Doubling the glucose and glutamine concentration of the MEME resulted in a dramatic increase in cell growth, as observed under the microscope. It was concluded that exhaustion of glucose and/or glutamine limited microcarrier culture of MDCK cells. The MDCK cells approached confluency after 5-7 days. During the growth phase, the media became acidic very quickly and so was replaced every 2 days. Aggregation of beads/cells was much more pronounced than with HeLa cells.

This reached 40% beads aggregated at 3 days for MDCK cultures and remained constant thereafter. The stirring speed was increased to as much as 45-50 rpm to reduce aggregation. Similar to HeLa cells, bare regions on beads with cells attached existed.

HeLa PLASMA MEMBRANE ISOLATION ON MICROCARRIER BEADS

HeLa cells grown in a microcarrier culture were harvested after 5-7 days. Plasma membranes were isolated on beads by; lysing, vortexing, washing and sonicating in buffers of pH 8, according to the method of Gotlib (1982). Samples of cells on beads, from the same microcarrier culture used to prepare membranes, were used to prepare whole cell homogenates. The whole cell homogenates were prepared by lysing cells on beads with vortex treatment for 10 seconds, following a 30 minute incubation in 1 mM Tris, pH 8. Membranes on beads and whole cell homogenates were then assayed for various marker enzymes. The enrichment of plasma membrane markers, and the level of various contaminant intracellular markers were determined for membranes on beads relative to whole cell homogenate activities.

Preparation of whole cell homogenates

Cells on beads were homogenised by incubation in hypotonic solution (1 mM Tris pH 8.0) followed by vortex mixing for 10 seconds. Examination of the samples showed no intact cells remaining on the beads, with many released nuclei and much cellular debris in suspension. The attached membranes are not visible under phase, but Cohen et al. (1977) has shown their presence in electron micrographs, as sheets of membrane stuck to the beads.

In experiments where a whole cell homogenate was prepared from HeLa cells growing in a Roux bottle, cells were disrupted by: (a) polytron treatment (b) passage of cells through a 19G needle x 50-100. In the case of (b), the activity of 5'nucleotidase was monitored after various homogenisation procedures (the number of passages of cells through the 19G needle was varied from 50-120). B. McCaldin (1978) reported that 50 or more passages through a 19G needle provided optimum cell disruption. This was confirmed here, since 5'nucleotidase activity was not increased by varying the number of passages of cells through a 19G needle between X 50-100. Examination of whole cell homogenates under phase contrast showed no intact cells, many released nuclei and much cellular debris.

5'nucleotidase

This enzyme has been shown to be a plasma membrane marker in studies by De Pierre and Karnovsky (1973).

The validity of 5'nucleotidase as a plasma membrane marker in HeLa cells has been questioned by Johnsen, Stokke and Prydz (1974). However in a comprehensive study of HeLa 5'nucleotidase by Brake, Will and Cook (1978), it was concluded that HeLa 5'nucleotidase satisfied all the requirements for a good plasma membrane marker in that: 5'nucleotidase codistributed with another plasma membrane marker in discontinuous sucrose gradients, no 5'nucleotidase could be found in other cell organelles and the activity per cell was constant at different cell densities.

The activities of 5' nucleotidase in HeLa membranes on beads and whole cell homogenates are shown in Table 1.4.

Table 1.4. 5' nucleotidase activity in HeLa membranes on beads and whole cell homogenates

	Activity
Whole cell homogenate	1133 ± 110
Membranes on beads	5477 ± 360

Results are expressed as the mean ± SEM of 12 different experiments. Activities are expressed as nmoles 5'AMP hydrolysed per hr per mg protein.

In order to control for the effects of beads on enzyme activities or interference with assays, a whole cell homogenate was prepared from HeLa cells growing as a monolayer in a Roux bottle and then assayed for 5' nucleotidase in the presence of added microcarrier beads or their absence. Sufficient beads were added to give about the same concentration as in a whole cell homogenate prepared from cells on beads. (1.5×10^4 beads/ml). Whole cell homogenates were prepared by passing cells x 75 repeatedly through a 19G hypodermic syringe needle. The results are shown in Table 1.5.

Table 1.5. 5' nucleotidase activity in HeLa whole cell homogenates in the presence or absence of microcarrier beads.

	Activity
Whole cell homogenate + beads	326 ± 20
Whole cell homogenate - beads	307 ± 17

Results are expressed as the mean ± SEM of 3 different experiments.

Activities are expressed as nmoles 5'AMP hydrolysed per hr per mg protein.

The results in Table 1.5 show that beads do not interfere with the 5'nucleotidase assay in whole cell homogenates. The specific activities for this assay are near a factor of 3 less in whole cell homogenates prepared from cells growing in Roux bottles than in whole cell homogenates prepared from cells on beads. This has been repeatedly found throughout many such experiments and is examined and discussed later.

Adenylate cyclase

This plasma membrane marker enzyme was assayed in whole cell homogenates and membranes on beads. The drug forskolin (100uM) which acts directly on the catalytic subunit of the adenylate cyclase system, was used to stimulate cAMP production above basal levels. An ATP regenerating system, GTP and IBMX an inhibitor of phosphodiesterase, were also included in the assay. The results are shown in Table 1.6.

Table 1.6. Stimulation of adenylate cyclase activities in HeLa whole cell homogenates and membranes on beads, by 100uM forskolin

	Activity
Whole cell homogenate	769 ± 44
Membranes on beads	4042 ± 220

Results are expressed as mean ± SEM of 4 different experiments. Activities are expressed as pmoles cAMP produced per hr per mg protein. In both groups, standards and samples were assayed in the presence of the appropriate blank. The whole cell homogenate blank and membrane blank were prepared by terminating without incubation, then adding to standards and samples.

The whole cell homogenates assayed in the above experiments were prepared from cells growing on beads. Whole cell homogenates were also prepared from cells growing as a monolayer in a Roux bottle by passing a prepared suspension of the cells through a 19G hypodermic needle x 75. The specific activities of stimulation of adenylate cyclase by forskolin in whole cell homogenates prepared from cells growing on beads and cells growing in a Roux bottle, are not significantly different. This is in contrast to the 5' nucleotidase specific activities in the two types of HeLa whole cell homogenates used.

Ouabain binding

The specific binding of ouabain to the Na/K ATPase of the plasma membrane was used as another plasma membrane marker. [³H]-ouabain was bound to cells growing on beads in OK Krebs and the non-specific washed off by a 15 minute incubation in warm medium.

The washout of non-specific ouabain bound was tested in the following way. Samples of 10 mls were removed from a microcarrier spinner flask into universal tubes. Half the samples were then incubated with $2 \times 10^{-7} \text{M}$ [³H]-ouabain for 20 minutes in OK Krebs, while the other half of the samples were incubated with $2 \times 10^{-7} \text{M}$ [³H]-ouabain for 20 minutes in 15K Krebs. At the end of the incubations, all cells were washed with cold 5K Krebs, placed on ice and either analysed, or incubated for a further 15 minutes in warm BME and then analysed. The results are shown in Table 1.7.

Table 1.7. Washout of ouabain from HeLa cells

Treatment	20 minutes $2 \times 10^{-7} \text{M}$ [³ H]-ouabain	20 minutes $2 \times 10^{-7} \text{M}$ [³ H]-ouabain + 15 minute washout in warm BME
$2 \times 10^{-7} \text{M}$ [³ H]-ouabain in OK Krebs	2.360 ± 0.113	2.093 ± 0.187
$2 \times 10^{-7} \text{M}$ [³ H]-ouabain in 15K Krebs	0.225 ± 0.041	0.004
Specific activity	2.134 ± 0.113	2.093 ± 0.187

Results are expressed as mean \pm SEM of 3 different experiments. Activities are expressed as pmoles ouabain bound per mg protein.

The results in Table 1.7 show that the 15 minute washout in warm BME removes almost all of the ouabain bound in 15K Krebs and a similar amount from the total ouabain binding in OK Krebs. There is no significant difference between: (a) the specific ouabain binding obtained by subtracting the binding in 15K Krebs (non-specific binding) from the total binding and (b) the specific ouabain binding obtained by a 15 minute washout in warm BME following binding in OK Krebs.

In another set of experiments, the washout of digoxin was investigated. The experimental procedures were exactly the same as those detailed above, except that $2 \times 10^{-7} \text{M}$ [^3H]-digoxin was used instead of ouabain. The results are shown in Table 1.8.

Table 1.8. Washout of digoxin from HeLa cells.

Treatment	20 minutes $2 \times 10^{-7} \text{M}$ [^3H]-digoxin	20 minutes $2 \times 10^{-7} \text{M}$ [^3H]-digoxin + 15 minute washout in warm BME
$2 \times 10^{-7} \text{M}$ [^3H]-digoxin in OK Krebs	2.732 ± 0.061	2.269 ± 0.103
$2 \times 10^{-7} \text{M}$ [^3H]-digoxin in 15K Krebs	0.541 ± 0.026	0.048 ± 0.005
Specific activity	2.189 ± 0.060	2.221 ± 0.104

Results are expressed as mean \pm SEM of 3 different experiments.

Activities are expressed as pmoles digoxin bound per mg protein.

The results in Table 1.8 show that the 15 minute washout in warm BME removes nearly all of the digoxin bound in 15K Krebs. There is no significant difference between: (a) the specific digoxin binding obtained by subtracting the binding in 15K Krebs (non-specific) from the binding in OK Krebs (total binding) and (b) the specific digoxin binding obtained by a 15 minute washout in warm BME following binding in OK Krebs.

HeLa cells with [^3H]-ouabain bound were then used to prepare whole cell homogenates and membranes on beads. Whole cell homogenates were prepared by lysing the cells by 10 second treatment on a vortex mixer following a 30 minute incubation in 1 mM Tris at 4°C. The ouabain bound per mg protein in HeLa whole cell homogenates and membranes on beads is shown in Table 1.9.

Table 1.9. Ouabain binding in HeLa whole cell homogenates and membranes on beads.

	Activity
Whole cell homogenates	2.413 \pm 0.197
Membranes on beads	18.955 \pm 0.537

Results are expressed as the mean \pm SEM of 15 different experiments. Activities are expressed as pmoles ouabain bound per mg protein, i.e. that bound to the Na/K ATPase, the non-specific binding is washed off by a 15 minute incubation in warm BME (37°C) immediately following binding of ouabain ($2 \times 10^{-7}\text{M}$) in OK Krebs for 20 minutes.

In control experiments, blank beads with no cells, were treated in the same way as samples, in order to determine the level of non-specific binding of ouabain to the beads and/or universal tubes.

It was found that non-specific binding of [^3H]-ouabain to beads and/or universal tubes was negligible, giving the same radioactive counts as background. In all ouabain binding experiments, parallel controls were always carried out.

A comparison was made between the values of specific activities for, ouabain binding in whole cell homogenates and for ouabain bound to whole cells, to determine whether any protein is 'lost' in the preparation of a whole cell homogenate. Ouabain was specifically bound to cells growing on beads and samples washed with cold isotonic buffer. Some of the samples were then used to determine the ouabain bound to whole cells, while the remaining samples were used to prepare whole cell homogenates. The results are shown in Table 1.10.

Table 1.10. Ouabain binding in HeLa whole cell homogenates and whole cells.

	Activity
Whole cell homogenates	2.344 \pm 0.150
Whole cells	2.082 \pm 0.1088

Results are expressed as the mean \pm SEM of 17 different experiments. Activities are expressed as pmoles ouabain bound per mg protein.

The results in Table 1.10 show a difference of 11% between the specific activities of ouabain binding to whole cells and ouabain binding in whole cell homogenates. The difference may be accounted for, by the sticking of intracellular components to the sides of the plastic universal tubes in the case of the whole cell homogenate. This would effectively change the concentration of protein in the suspension to be sampled, resulting in differences in the specific activities of ouabain binding in whole cells and in whole cell homogenates. Ouabain bound per mg protein was also determined for cells growing in a Roux bottle. The results obtained for a whole cell homogenate from such cultures were not significantly different from those obtained for a whole cell homogenate prepared from cells growing on beads. Furthermore, in control experiments the addition of blank beads to the binding solution did not alter the amount of ouabain binding. For ouabain binding and adenylate cyclase specific activities, the values obtained for a whole cell homogenate prepared from cells growing on beads in a spinner culture were not significantly different to values obtained for a whole cell homogenate from cells growing in a Roux bottle. However for 5' nucleotidase activity, the whole cell homogenate specific activity is 3-fold higher with cells growing on beads in spinner culture. This finding is discussed later.

Recovery of activity and protein in membranes on beads

[³H]-ouabain was bound to cells growing on beads in a micro-carrier spinner culture, the non-specific washed off and 10ml samples removed. The samples were washed, half of the samples used to determine ouabain binding to whole cells and the remaining half used to determine ouabain binding to prepared membranes on beads. The recovery of activity (ouabain binding) and protein, in membranes on beads as a % of total activity in whole cells, was determined. Usually 30% ouabain binding and 4% protein were recovered in membranes on beads (results are the means of several observations).

Na/K ATPase and p-nitrophenylphosphatase

The direct assay of the Na/K ATPase was carried out in HeLa whole cell homogenates and membranes on beads. Activity is measured as the K-stimulated, ouabain sensitive, phosphate production.

p-nitrophenyl phosphatase activity is a function of the Na/K ATPase, where K-stimulated, ouabain sensitive, production of p-nitrophenol from p-nitrophenylphosphate is monitored at A_{420} on production of the yellow coloured p-nitrophenol product.

Despite many attempts, no K-stimulated and/or ouabain sensitive phosphatase activity was ever found in HeLa whole cell homogenates or membranes on beads. Two possible reasons could be: (a) K-stimulated, ouabain sensitive, phosphatase activity may be such a small part of general phosphatase activity, that it is being lost in the 'noise', (b) Na/K AtPase is highly sensitive to disruption and activity

is lost (due to release of proteolytic enzymes from ruptured lysosomes). The latter possibility (b), was investigated by preparing a large number of HeLa whole cell homogenates at various stages of disruption. However no Na/K ATPase activity could be detected. Lastly both assay procedures (Na/K ATPase and p-nitrophenylphosphatase) were carried out on purified, commercially available, Na/K ATPase and both gave high activity.

In summary, 5'nucleotidase, adenylate cyclase and specific ouabain binding were all used as plasma membrane markers in HeLa whole cell homogenates and membranes on beads. The results are shown in Table 1.11, together with purification factors of enrichment of specific activities in membranes on beads, relative to whole cell homogenates.

Table 1.11. Summary of activities of plasma membrane markers in HeLa whole cell homogenates and membranes on beads.

	Whole cell homogenate	Membranes on beads	Purification factor
5'nucleotidase ^a	1133 ± 110	5477 ± 460	4.8
Adenylate cyclase ^b	769 ± 44	4042 ± 220	5.3
Ouabain binding ^c	2.413 ± 0.197	18.955 ± 0.537	7.9

^aActivities expressed as nmoles 5'AMP hydrolysed per hr per mg protein.

^bActivities expressed as pmoles cAMP produced per hr per mg protein.

^cActivities expressed as pmoles ouabain bound per mg protein.

The results in Table 1.11 show 5-8fold enrichment of plasma membrane markers. This represents favourable purification of HeLa plasma membranes compared to other methods of HeLa plasma membrane isolation described below.

Intracellular marker enzymes

The results obtained from the 3 plasma membrane markers assayed are described in detail above. To determine the degree of intracellular contamination of the membranes on beads, (caused by sticking of internal components following lysis) various intracellular marker enzymes were assayed. The specific activity of these marker enzymes was determined in membranes on beads and in whole cell homogenates. The purification factors, of the specific activities in membranes on beads relative to specific activities in whole cell homogenates, were determined. The marker enzymes assayed were: lactate dehydrogenase (cytosol marker), succinate dehydrogenase (mitochondria marker), β -hexosaminidase (lysosome marker) and NADH-ferricyanide reductase (endoplasmic reticulum marker). Whole cell homogenates and membranes on beads were prepared from cells growing on beads in a microcarrier culture and the specific activities of the enzymes above, determined. The results are shown in Table 1.12.

Table 1.12. Activities of intracellular marker enzymes in HeLa whole cell homogenates and membranes on beads.

	Whole cell homogenate	Membranes on beads	Purification factor
Lactate dehydrogenase ^a	0.0013 ± 0.0001	*	-
β hexosaminidase ^b	3.9 ± 0.08	2.8 ± 0.02	0.7
Succinate dehydrogenase ^c	80 ± 4.3	*	-
NADH-ferricyanide reductase ^d	703 ± 107	*	-

All results are the mean ± SEM of at least 3 different experiments.

^a Activities expressed as U per mg protein.

^b Activities expressed as μmoles p-nitrophenol produced per hr per mg protein.

^c Activities expressed as pmoles formazan produced per hr per mg protein.

^d Activities expressed as nmoles NADH oxidised per hr per mg protein.

* No detectable activity.

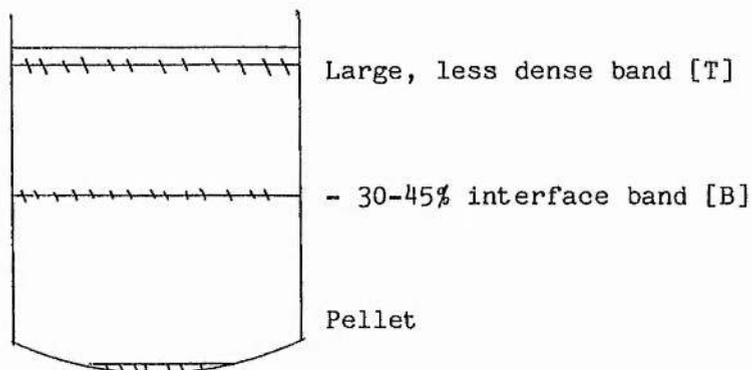
The results show that little contamination of membranes on beads from internal cell components, occurs. All the intracellular markers are depleted in the membranes on beads, most to the point

where no activity can be detected. The lysosomal marker is depleted by a factor of 1.4, which represents slight contamination of the membranes. As for the plasma membrane markers, controls were carried out to determine whether beads interfered with the enzyme assays. Whole cell homogenates were prepared from cells growing in a Roux bottle, blank beads added to part of the homogenate to give a concentration of 2×10^4 beads/ml and the enzymes assayed in the presence or absence of beads. No interference of the enzyme assays by beads occurred.

HeLa PLASMA MEMBRANE ISOLATION ON DISCONTINUOUS SUCROSE GRADIENTS

The aims of this work were to consider other HeLa plasma membrane preparations and compare the enrichment of membrane markers, with those found with HeLa plasma membranes isolated on beads. Brake et al (1978) have isolated HeLa plasma membranes, by methods adapted from those of Atkinson and Summers, (1971). These involve plasma membrane isolation on a 2-step, discontinuous sucrose gradient. B. McCaldin (1978) also developed a method for HeLa plasma membrane isolation, involving an 8-step discontinuous sucrose gradient as shown in the Methods section. The procedure of Brake et al. (1978) was tried first. HeLa cells (50 million) were incubated for 30 minutes in 4ml hypotonic buffer, following removal of cells from a Roux bottle. The cells were then homogenised by passing the cells x 75 through a 19G hypodermic needle. The degree of homogenisation was monitored under phase contrast. Some of the whole cell homogeneate was sampled for enzyme activity, while the remainder was placed on top of the 2-step gradient (30%, 45% w/w sucrose). This was centrifuged at 8000g for 20 minutes. The results are shown in Figure 1b.

Figure 1b. Result of centrifugation of HeLa whole cell homogenate on a 2-step discontinuous sucrose gradient.



Both the T and B bands were removed by syringe and needle, washed and assayed separately for 5' nucleotidase activity. Activity was found in both bands. The results are shown in Table 1.13.

Table 1.13. 5' nucleotidase activity in HeLa whole cell homogenates and the T and B bands from the sucrose gradients.

	Activity	Purification factor
Whole cell homogenate	310 ± 23	-
T band	746 ± 80	2.5
B band	677 ± 20	2.2

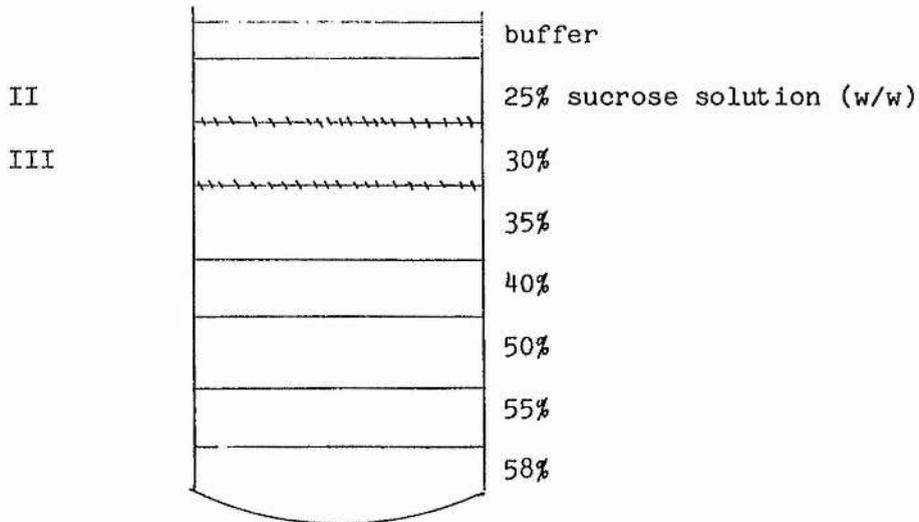
Results are expressed as the mean ± SEM of 3 different experiments. Activities are expressed as nmoles 5'AMP hydrolysed per hr per mg protein. Purification factors are enrichment of specific activities in the T and B bands relative to whole cell homogenate activity.

The results show that the plasma membrane marker 5'nucleotidase is enriched in both sucrose gradient bands. Brake et al. (1978) reported that large plasma membrane aggregates appeared at the 30-45% interface of the sucrose gradient and that the purification of 5'nucleotidase in the membranes was 10-12fold relative to the whole cell homogenate. This high purification of plasma membranes was never found. The work of B. McCaldin (1978) confirmed these findings. Using the procedures of Atkinson and Summers (1971), he could only ever achieve about 2-fold purification of 5'nucleotidase activity relative to the whole cell homogenate.

The discontinuous sucrose gradient method of B. McCaldin, for isolation of HeLa plasma membrane was also tried. HeLa cells (50 million) were removed from a Roux bottle and incubated for 30 minutes in 4 ml hypotonic buffer. The cells were then homogenised by passing the cells x 75 through a 19G hypodermic needle. The degree of homogenisation was monitored under phase contrast. Some of the whole cell homogenate was sampled for enzyme activity, while the remainder was placed on top of the 8 step sucrose gradient.

This was centrifuged at 130000g for 75 minutes, fractions II and III collected (results shown in Figure 1c) and centrifuged for 1 hour at 130000g in buffer. The pellets were collected and assayed for 5'nucleotidase activity.

Figure 1c. Result of centrifugation of HeLa whole cell homogenate on an 8-step discontinuous sucrose gradient.



Fraction II (appearing at the interface between the 25% and 30% sucrose solutions) and fraction III (appearing at the interface between the 30% and 35% sucrose solutions) were collected with a needle and syringe, washed and assayed for 5'nucleotidase activity.

Other bands, not shown in Figure 1c, appear at the interfaces of other sucrose concentrations.

The other bands (not shown in Figure 1c) contain various intracellular components. These have been categorised by B. McCaldin (1978) according to their marker enzyme activities. The plasma membrane appears, almost exclusively in bands II and III. The results of 5'nucleotidase activities in whole cell homogenates and bands II and III are shown in Table 1.14.

Table 1.14. 5'nucleotidase activity in HeLa whole cell homogenate and band II and III from the sucrose gradient.

	Activity	Purification factor
Whole cell homogenate	295 ± 31	-
Band II	1545 ± 23	5.2
Band III	1815 ± 29	6.2

Results are expressed as the mean ± SEM of 3 different experiments.

Activities are expressed as nmoles 5'AMP hydrolysed per hr per mg protein. Purification factors are enrichment of specific activities relative to whole cell homogenate activity.

The results in Table 1.14 show that enrichment of plasma membranes relative to whole cell homogenates is much greater using the method of B. McCaldin, than using the method of Brake et al. (1978). These results confirm the findings of B. McCaldin, he found 6-fold purification of both 5'nucleotidase and Na/K ATPase markers in band III. In band III he also found some acid phosphatase activity and NADH-ferricyanide reductase activity, both with purification factors just less than 1 (acid phosphatase is a lysosomal marker, NADH-ferricyanide reductase is an endoplasmic reticulum marker).

The aims of this section of work have been to compare the purity of HeLa plasma membranes on beads with HeLa plasma membranes prepared on discontinuous sucrose gradients. The methods of Brake et al. (1978) showed no more than 2.5-fold enrichment of the plasma

membrane marker 5'nucleotidase and was not examined further. The methods of B. McCaldin (1978) gave much greater enrichment of plasma membranes. 5'nucleotidase activity was enriched 6-fold in isolated plasma membranes. This result confirms the findings of B. McCaldin.

He obtained other marker enzyme activities for isolated plasma membranes and found that Na/K ATPase was also enriched 6-fold but intracellular markers could not be detected, except for acid phosphatase (lysosome) and NADH-ferricyanide reductase (endoplasmic reticulum) which both gave purification factors of just under 1.

DISCUSSIONMicrocarrier spinner culture

The results in Tables 1.1 and 1.2 show the initial attachment rate of HeLa cells to microcarrier beads and the % aggregation in both static and microcarrier spinner culture. HeLa cells have a plating efficiency of about 50% indicating that 50% of cells attached will proliferate. A measure therefore of the proportion of beads with 2 or more cells attached was taken as a measure of likely future proliferation of cells on these beads. The attachment rate in a microcarrier culture (Table 1.2) did not increase over the period 6 to 12 hours. This may reflect the findings of Clark and Hirtenstein (1981a), who showed that the ability of anchorage dependent cells to attach, decreases the longer the cells are kept in suspension.

Clark and Hirtenstein (1981a) and Clark, Hirtenstein and Gebb (1980) have defined optimum initial culture conditions for both primary and established cell lines in microcarrier culture. They have shown that for established cell lines (which have plating efficiencies of greater than 30%) the optimum initial culture conditions are: low cell: microcarrier ratio <5, initial culture volume 100% of final volume, continuous initial stirring speed and no extra medium supplements. These findings were largely confirmed for microcarrier culture of HeLa cells; reducing the initial culture volume to 30% of final level and/or the use of interval stirring, did not increase either initial attachment or final cell yield. The above authors have also defined optimum initial culture conditions for primary cell lines (which have a low plating efficiency - less than 30%):

high cell:microcarrier ratio >5, initial culture volume 30% of final volume, interval stirring speed and extra medium supplements. Presumably these culture conditions for primary cells allow cells and beads to come into frequent contact in the first few hours following inoculation of culture thus facilitating the intrinsic poor attachment properties of primary cell lines. Unlike many established cell lines, primary cells generally do not survive in suspension for very long.

Maintenance of the microcarrier culture

Hirtenstein and Clark (1980) have shown that stirring speed is important in the maintenance of a microcarrier; too fast a stirring speed and attached cells may fall off the beads, while too slow a stirring speed and excessive aggregation may occur. It was found that if the stirring speed was increased from 30 rpm to 40 rpm after the initial culture phase, then aggregation of HeLa cells was reduced, but that this increase in stirring speed did not affect final cell yield. The growth curve (Figure 1a) shows that HeLa cell numbers increase logarithmically with a generation time similar to that found in traditional monolayer culture, suggesting that the conditions used are favourable for the growth culture of HeLa cells. This is supported by the fact that the HeLa cells in a microcarrier culture were observed to have a flattened morphology on the beads and that a viability test showed 95% cell viability.

The beads used, 'superbeads' obtained from Flow Laboratories (Scotland) have a maximum cultivatable surface area for cell growth of $0.24 \text{ m}^2/0.5 \text{ g}$ superbeads. HeLa have an approximate attachment

surface area of about $600 \mu\text{m}^2$ and so the theoretical, maximum cell yield should be about 400 million cells/microcarrier culture (each microcarrier culture was inoculated with 0.5 g beads - about 3 million beads, and 15 million HeLa cells). However the observed final cell yield from such a microcarrier culture at 6 or 7 days was only about 150 million cells. Inoculation of a culture resulted in only 75% of the beads having 2 or more cells attached, while only 75% of these beads at the end of the culture appeared to form a continuous monolayer. Bare patches of various sizes existed on many beads in the culture. Beads which ultimately formed a continuous monolayer of cells, therefore accounted for only about 50% of the total number of beads present. With such a heterogeneous mixture of beads having varying quantities of cells attached, it proved difficult to quantify 'partially' covered or 'sparsely' covered beads. Another problem arose in trying to decide whether the beads which appeared to form a monolayer of cells, really represented the theoretical maximum number of cells able to cover the beads at confluence. In theory, each microcarrier bead (of mean size - an additional problem is that the beads are not of uniform size but cover a range of sizes) should, at confluence, be covered with about 120-150 HeLa cells.

Attempts to count the cells on one bead proved difficult if not impossible because of focussing problems in a large depth of field due to the spherical-shaped beads. Comparisons between HeLa cells growing on beads and HeLa cells in traditional monolayers, suggested though, that the observed monolayer did not represent maximum covering of beads.

The bare regions on beads observed in the microcarrier culture of HeLa cells, has been reported in other studies. Cohen, Kalish,

Jacobson and Branton (1977) using polylysine-coated polyacrylamide beads, Kahlisch, Cohen, Jacobson and Branton (1978) using polylysine-coated glass beads and Gotlib (1982) using commercially available microcarrier beads (obtained from Pharmacia and the Biorad Corporation), have all reported the existence of such bare regions following the acute attachment of different cells to each bead-type used. Kahlisch et al. (1978) have suggested that the bare regions exist because the polylysine coating may not be uniform, and thus unable to bind cells. It is possible that commercially available microcarriers (including superbeads) may have a non-uniform charge distribution, resulting in cells unable to bind or proliferate over such 'non-uniform' areas. However it is worth pointing out that the manufacturers do claim that a uniform charge distribution exists on the beads. This possible suggestion apart, the reason for the existence of such bare regions on microcarrier beads, when the cells are present in such a large excess, is not known.

In summary, good cell growth (and good cell health) is obtained with the microcarrier culture of HeLa cells under the culture conditions stated. However the yield obtained is about 30-40% of the maximum theoretical yield. Alteration of the culture conditions shown to be optimal in other studies did not improve this yield and offered no suggestions as to how this yield may be improved.

Preliminary experiments investigating the microcarrier culture of MDCK cells showed that following initial attachment, subsequent poor growth resulted. Doubling both the glutamine and glucose concentration, dramatically improved the growth characteristics of this culture, suggesting that these media components were limiting.

This observation may serve to emphasise the potential of microcarrier culture in the production of a large number of cells per unit volume; the growth of MDCK cells in microcarrier culture has quickly exhausted medium components, normally in sufficient quantity for growth of MDCK cells in traditional monolayer culture.

Isolation of HeLa plasma membranes on microcarrier beads

The ultimate aim of the growth of HeLa cells in microcarrier culture was to develop a method for the rapid isolation of HeLa plasma membranes on microcarrier beads. The principle of isolation of plasma membranes on solid supports was already established by Cohen *et al.* (1977), Gotlib and Searls (1980) and Gotlib (1982).

These authors attached cells to beads 'acutely' over a period of hours, then isolated the plasma membranes on beads. The methods presented in this present study involved obtaining firstly a high yield of HeLa cells by growth in microcarrier culture, followed by the preparation of plasma membranes on beads. The results are discussed below.

In order to characterise prepared plasma membranes on beads, marker enzymes were assayed in both whole cell homogenates and in isolated membranes. Tables 1.4 and 1.5 show that 5'nucleotidase specific activity in a whole cell homogenate prepared from HeLa cells growing on beads is 3-fold higher than that from HeLa cells growing in a Roux culture. This phenomenon was found many times and was not the result of activation of 5'nucleotidase activity by microcarrier beads (Table 1.5). A possible explanation may be

that the 5'nucleotidase active site may be on the inner face of the plasma membrane of HeLa cells, in which case the cell homogenate obtained from cells on beads will have its cytoplasmic surface fully exposed and therefore the 5'nucleotidase active site fully activated; this would be in contrast to the active site in the other type of whole cell homogenate (obtained from cells growing in a Roux) which will presumably be largely occluded inside resealed plasma membrane vesicles. Another possible reason for the observed differences in 5'nucleotidase activity may be that preparation of a whole cell homogenate from cells growing on beads unmasks latent 5'nucleotidase sites (perhaps by exposure of the cytoplasmic face of the plasma membrane?), but does not happen in the preparation of the whole cell homogenate from cells growing in a Roux. A third possibility remains however, that growth of HeLa cells on beads (for some reason) results in a 3-fold increase in the levels of 5'nucleotidase compared to that found in HeLa cells growing in a Roux. Such differences in specific activities between the 2 types of HeLa whole cell homogenates were not found with the other 2 plasma membrane marker enzymes employed, suggesting that it is a specific effect for 5'nucleotidase.

Ouabain binding to the sodium pump was also used as a plasma membrane marker. In order to check that only specific ouabain binding was being measured, the 15 minute washout of the non-specific binding used in the assay was compared with the total and non-specific binding levels. Table 1.8 confirmed that the 15 minute washout did actually remove all of the non-specific portion. This is important because if all the non-specific binding had not been removed, then incorrect

purification factors may have been obtained as a result of the non-specific binding dissociating during the membrane isolation procedure. It is worth noting that the preparation time for plasma membrane isolation on beads is about 1.5 hours and at the temperature used (4°C) essentially no specifically bound ouabain will be lost from the plasma membrane during this time. In fact only about 14% of specifically bound ouabain is lost from the plasma membrane at 4°C after 24 hours. The demonstration that no non-specifically bound ouabain remains after a 15 minute washout, is also important in experiments in which the rate of loss of ouabain from the plasma membrane is being measured (Chapter 2); any non-specific ouabain bound would be lost at a different rate to specific (sodium pump bound) ouabain and thus may affect the overall rate.

No K-stimulated, ouabain sensitive activity could be detected when the enzyme Na/K ATPase was assayed directly. The assay procedures used for Na/K ATPase and p-nitrophenylphosphatase, were shown to measure Na/K ATPase activity in other cell types in the lab (ventricular myocytes) and in commercially available Na/K ATPase, suggesting that the assay procedure was not at fault. A possible reason for not detecting such activity in HeLa cells could be that this enzyme is highly sensitive to disruption of the plasma membrane and/or sensitive to released proteases from lysosomes ruptured during the homogenisation procedure. This possibility was investigated by altering the homogenisation procedure (i.e. in many cases milder homogenisation was used). However no K-stimulated, ouabain sensitive activity could be detected under different homogenisation conditions. Another possibility for not being able to detect Na/K ATPase activity in HeLa cells, is that K-stimulated, ouabain sensitive phosphatase

activity is such a small part of general phosphatase activity, that it is being lost in the 'noise'. To test this possibility, the Na/K ATPase was assayed in a concentrated sample of a prepared whole cell homogenate from 2 Roux bottles, but still no K-stimulated, ouabain sensitive activity could be detected. Furthermore, no Na/K ATPase activity was detected in prepared HeLa plasma membranes, even though the enzyme is present in a more purified form than in cell homogenates. The reasons for not being able to detect Na/K ATPase activity in HeLa cells (homogenates or membranes) are still not clear.

The amount of ouabain binding in whole cell homogenates is 11% less than in whole cells (Table 1/10). This perhaps arises due to the sticking of intracellular protein to the sides of the assay tubes during homogenisation, so effectively reducing the protein concentration of the sample and thus resulting in a reduced specific activity for whole cell homogenates. The value obtained for specific activity of ouabain binding in whole cell homogenates was the value used to calculate enrichment of specific activity in prepared plasma membranes. This was carried out in order to obtain comparability with that of the other plasma membrane markers, for which the specific activities in whole cell homogenates were compared to the specific activities in membranes on beads.

Table 1.11 shows the specific activities of the plasma membrane markers in whole cell homogenates and membranes on beads. The purification factors for 5'nucleotidase, adenylate cyclase and ouabain binding were 4.8, 5.3 and 7.9 respectively. This enrichment of HeLa plasma membranes compares favourably with HeLa plasma membranes

prepared on discontinuous sucrose gradients by the methods of Brake, Will and Cook (1978) and McCaldin (1978). Table 1.13 and 1.14 show the enrichment of 5'nucleotidase obtained using the methods of Brake et al. (1978) and McCaldin (1978). The results in Table 1.14 confirm the purification of plasma membrane found by McCaldin, but the high purification (10-12fold) reported by Brake et al. (1978) was never found; only 2-fold purification was obtained (Table 1.13). This value of 2-fold purification was also reported by McCaldin (1978) using the procedure of Brake et al.

The degree of membrane purification must be weighed against operation time; the total time including centrifugation time and time for preparation of samples. The method of Brake et al. (1978) had an operation time of about 2.5 hours, while that of McCaldin (1978) had an operation time of about 3.5 hours. Johnsen, Stokke and Prydz (1974) used a method for the isolation of HeLa plasma membranes which had an operation time of about 20 hours. The plasma membranes obtained were enriched 7-fold in 5'nucleotidase activity and 19-fold in Na/K ATPase activity. Bosmann, Hagopian and Eylar (1968) claimed 120-fold enrichment in 5'nucleotidase activity and 30-fold enrichment in Na/K ATPase activity using a procedure with an operation time of about 33 hours. Such a massive enrichment of 5'nucleotidase while theoretically possible, is unlikely in practice. Isolation of HeLa plasma membranes on beads has an operation time of only 1.5 hours whilst obtaining comparable enrichment of membranes to several of the more time consuming methods above. It is likely that short operation times are important in time course studies in which the ouabain bound to plasma membranes is determined at various time intervals (results, Chapter 2); as very long operation

times, with many handling steps, may result in dissociation of ouabain during the isolation procedure and therefore give spurious results.

A potential criticism of the isolation of membranes on beads is that a specialised membrane region may be selected on the bead.

This may be a problem in both acute experiments (in which cells are attached to beads over a period of several hours and then membranes isolated) and also in chronic experiments (in which cells are grown on beads for 5-7 days and then membranes isolated). In such acute experiments, with different cell types used, it has been shown however, by gel electrophoresis studies that the total complement of membrane is isolated and not a selected region (Jacobson and Branton, 1977; Kalish *et al.*, 1978; Jacobson, 1980). Gotlib, in studies with several different cultured cells, has shown that plasma membrane markers are uniformly enriched, suggesting that selectivity of membrane regions does not occur. The possibility of selectivity in chronic experiments (in which cells are grown on beads for 5-7 days and then membranes isolated) is perhaps much more likely than in acute experiments, due to differentiation of attached cells over several generations. The results in Table 1.11 however, show that the enzymic markers (5'nucleotidase and adenylate cyclase) are enriched by the same amount. The higher enrichment of the chemical marker ouabain, may be due to the fact that a certain amount of activity loss occurs for the enzymic markers (but not for the chemical marker ouabain) during the preparation of membranes on beads. This data is not conclusive as to whether selectivity is occurring during the isolation of HeLa plasma membranes on beads. However, even if selectivity does occur, it is very unlikely that

it will affect any of the measured rate losses of ouabain from the plasma membrane (results, Chapter 2), as sodium pumps (ouabain binding sites) have been shown to be uniformly distributed over the surface of HeLa cells (Al Gharably, Lamb, Ogden, Owler and Tenang, 1985), with no evidence that the sodium pumps on one side of the plasma membrane behave any differently to those on the other side of the plasma membrane. Selectivity of plasma membrane regions is not only a potential criticism of the method used to prepare membranes on beads, but also occurs with membranes isolated on sucrose gradients. Often, 2 or more bands are obtained on sucrose gradients each containing enriched plasma membrane marker activity (see Tables 1.13 and 1.14). These bands represent isolated regions of the plasma membrane with slightly different densities and perhaps slightly different protein composition; in short, selected regions of the plasma membrane are obtained, perhaps due to breakage of the plasma membrane at specific stress regions during homogenisation.

The relative amount of membrane isolated is another aspect of membrane isolation procedures worth consideration. The results in Chapter 1 (p.62) show that about 30% of the total ouabain initially bound to cells, is recovered, while 4% of the total protein is recovered. This recovery of plasma membrane compares favourably with the reported recoveries of marker enzyme activities in plasma membranes isolated on sucrose gradients.

The level of intracellular contamination found in isolated HeLa plasma membranes on beads also compares favourably with other isolation procedures. B. McCaldin (1978) reported acid phosphatase and NADH ferricyanide reductase activities (both with a purification

factor of just less than 1) in the same fraction as purified HeLa plasma membranes. Isolated HeLa plasma membranes on beads show only slight lysosomal contamination, with other intracellular markers unable to be detected (Table 1.12). Assuming that these lysosomes contaminating the plasma membrane contain ouabain after a certain period of time, the levels of lysosomal contamination (purification factor of just less than 1) is not sufficient to significantly affect the calculated rate losses of ouabain from the plasma membrane (results, Chapter 2).

Conclusions

Microcarrier cell culture can be used successfully to grow HeLa cells (and MDCK cells) to a fairly high density per unit volume of the medium used. The HeLa cells grow logarithmically, with about the same generation time as found in traditional monolayer culture and were shown to possess greater than 95% cell viability. The reasons for not obtaining maximum theoretical cell yield from the microcarrier culture are not known. HeLa plasma membranes may then be isolated on beads, obtaining membranes of comparable purity as more traditional membrane isolation procedures. The rapidity (time: 1.5 hours) with which membranes can be isolated on beads and the high yield obtained per unit volume, makes this technique of preparing membranes on beads a very useful method for time course experiments carried out in the present study. Selectivity of specific membrane domains were not thought to be a problem within the context of the present investigations; obtaining measurements of the rate loss of cardiac glycosides from HeLa plasma membranes.

CHAPTER 2

UPTAKE AND EXCRETION OF CARDIAC GLYCOSIDES IN HeLa CELLS

INTERNALISATION OF OUABAIN DURING LOW K STRESS

INTRODUCTIONRECEPTOR-MEDIATED ENDOCYTOSIS

Endocytosis is a general term often used to describe ingestion of extracellular materials by protozoa, or digestion of foreign materials in the extracellular fluid by phagocytic cells. Receptor-mediated endocytosis, unlike many general endocytic events, is highly specific. The receptors are integral membrane proteins each of which has a binding site that fits a particular ligand. Ligands (such as large proteins, hormones etc.) sometimes present in the extracellular medium in very low concentrations (and with a vast excess of unrelated molecules) are able to bind with very high affinity to receptors of target cells and be subsequently internalised. Receptor-mediated endocytosis thus provides a mechanism whereby different cells of multicellular organisms can extract from the extracellular medium the specific substances they require, rejecting the rest. Understanding of the process of receptor-mediated endocytosis and of the importance of receptor-mediated endocytosis to cell function have increased dramatically over the last few years; recent progress is reviewed below.

The first example of receptor-mediated endocytosis was by Roth and Porter in 1964 who demonstrated how insect and bird eggs acquire the large amount of protein stores in the yolk body to nourish the embryo - proteins are synthesised elsewhere and imported into the developing oocyte. They showed in mosquito that the precursor protein vitellogenin is synthesised in the female's liver, secreted into the blood and carried into the ovary where it binds to 300,000 receptors (in tiny pits) on the surface of the oocyte. The pits are

internalised as vesicles, the vesicles fuse and vitellogenin cleaved to release 2 essential yolk proteins - lipovitellin and phosphovitin.

Later in the early 1970's, elegant work by Goldstein and Brown provided much more information on the mechanism of receptor-mediated endocytosis (for review see Goldstein and Brown, 1977). These authors investigated binding and uptake of LDL (low density lipoprotein) in human fibroblasts. The LDL complex contains a binding protein apo-b, which binds LDL to specific receptors. Using electron microscopic and autoradiographic techniques with the conjugated ligands ferritin-LDL and ^{125}I -LDL, they showed that 50-80% of all the LDL receptors were clustered over the 2% of the cell surface represented by structures called coated pits, at 4°C. These pits were so called because they appeared to be coated with a 'bristle' region on the cytoplasmic side. When the temperature was raised from 4°C to 37°C the coated pits were observed to invaginate and coated endocytic vesicles were formed. After 5 to 10 minutes the ferritin-LDL was observed in the lysosome as a result of fusion of lysosomes with incoming vesicles. Biochemical stripping techniques were then used to distinguish between surface and cellular associated ^{125}I -LDL and the rates of uptake of LDL measured (Brown and Goldstein, 1976). This biochemical data paralleled the electron microscopic evidence for the rapid uptake, and degradation of LDL within the lysosome compartment of the cell. Proteases in the lysosome have been shown to cleave the protein component of LDL, while cholesteryl esters are cleaved by an acid lipase yielding free cholesterol. Genetic studies have yielded important information on the process of receptor-mediated endocytosis; with mutants responsible for the genetic disease

called familiar hypercholesterolaemia having been characterised (Goldstein, Brown and Stone, 1977). Perhaps the most informative is a rare mutation called R^{d+10} in which receptors bind LDL but do not allow the LDL to be internalised or even incorporated into coated pits. This may suggest that an LDL receptor has in addition to an LDL binding site, another active site, the 'internalisation site', which is necessary for the receptor to be recognised as a component of a coated pit (and thus internalised). This model implies that coated pit components specifically select receptors for internalisation.

The model of LDL uptake in fibroblasts, describing ligand binding, coated pit occupancy, endocytic vesicle formation and transfer of ligand to lysosomes, has been shown to occur in many other cell types (Brown and Goldstein, 1976; Goldstein and Brown, 1977).

Coated pit formation

Coated pits derive their name from their indented shape and fuzzy cytoplasmic coat, as observed with the electron microscope. Freeze fracture studies have suggested that coated pits contain a unique set of transmembrane proteins (Orci, Carpentier, Perrelet, Anderson, Goldstein and Brown, 1978). Coated vesicles (derived from coated pits) have been isolated by Pearse from a variety of cell types and characterised (Pearse, 1975; Pearse, 1976; Pearse, 1978). Pearse found that the coated vesicles were composed predominantly of a single protein which she named clathrin. It was not known if receptors were always found in pits or if occupancy was ligand induced, however Anderson, Goldstein and Brown (1976) have shown that LDL receptors are found in coated pits, whether ligand

is bound or not. Furthermore, subsequent internalisation of the LDL receptor takes place in the absence of ligand (Anderson, Brown and Goldstein, 1977a; Anderson *et al*, 1976; Anderson, Goldstein and Brown, 1977b). These authors have calculated that an LDL receptor spends on average 9 minutes on the cell surface, about 3 minutes randomly distributed and 6 minutes clustered in coated pits. The receptors for $\alpha 2$ macroglobin, insulin and EGF (epidermal growth factor) in 3T3 cells, have been shown to occupy coated pits only in the presence of ligand (Maxfield, Schlessinger, Schechter, Pastan and Willingham, 1978). Other examples of both constitutive (independent of ligand binding) and ligand induced events are reviewed below.

The mechanisms by which ligands induce receptors to occupy coated pits are largely unknown, although information on the LDL receptor mutation (above) suggests the involvement of highly specific signalling.

Another unanswered question is whether different receptor:ligands complexes enter the cell via the same coated pit?

Do Ligands and receptors enter cells together?

The pathway of receptor-mediated endocytosis has been largely investigated by binding of a labelled ligand at 4°C (stops internalisation) and following the ligand stepwise through each cell compartment when the temperature is raised to 37°C. It is often assumed that receptors and ligands enter cells together as a complex and are separated at some intracellular site (lysosomes). However, experimental evidence has been presented which indeed shows that receptors are internalised. Wall and Hubbard (1981) have shown (by use of specific electron dense probes) that the hepatocyte asialoglycoprotein receptor is found inside the cell. Mellman, Steinman, Unkeless

and Cohn (1980) have labelled the M₆Fc receptor and found this receptor localised in intracellular vesicles. The LDL receptor has been shown to be internalised in the absence of ligand (Anderson et al., 1977a; Anderson et al., 1976; Anderson et al., 1977b). A model for separation of ligand and receptor inside the cell is based on the fact that many ligands dissociate from their receptors at low pH. The best example is the demonstration that the mannose-6-phosphate glycoprotein receptor does not bind ligands at low pH in intact cells (Gonzalez-Noriega, Grubb, Talkad and Sly, 1980), or when the receptor is purified (Sahagian, Distler and Jourdian, 1981). Iron (Fe) has also been shown to separate from its carrier protein transferrin at low pH in vitro and in vivo (Karin and Mintz, 1981). Geuze, Slot, Straus, Lodish and Schwarz (1983) used double-labelling immunoelectron microscopy on ultra-thin sections of rat liver to show that the asialoglycoprotein receptor dissociates from its ligand in intracellular vesicles; these vesicles were named CURLs (compartment of uncoupling of receptor and ligand). Evans and co-workers (Evans, Dix and Cooke, 1984; Saermark, Flint and Evans, 1985; Evans, 1985) have isolated various endocytic vesicles from rat liver in nycodenz density gradients - 2 'light' endocytic vesicle fractions and a heavier receptor-containing fraction have been characterised. These authors have also identified an ionophore-activated Mg²⁺-ATPase functioning as an ATP driven proton pump, in these endocytic vesicles. Another interesting fact which has emerged from the work of Evans and co-workers is that the protein composition of these endocytic vesicles is different from the plasma membrane, suggesting that either specialised plasma membrane proteins are selectively transferred to endocytic membrane networks or the endocytic vesicles are separate entities from the plasma membrane.

The morphological and biochemical data of Geuze et al. and Evans provides evidence for the intracellular uncoupling of receptors and ligands within intracellular endocytic vesicles.

Receptor recycling

A body of evidence now exists that in many receptor-mediated endocytic processes, the internalised ligand may be degraded while the receptor recycled. Such a scheme may be for conservation purposes or indeed regulatory. Kinetic experiments by Goldstein, Basu, Brunschede and Brown (1976) and Anderson et al. (1977_b) have suggested that LDL receptors are recycled. This recycling can account for the fact that fibroblasts continue ingesting saturating levels of LDL at a uniform rate for more than 6 hours even when the synthesis of new receptors is blocked by cycloheximide. If recycling did not occur, all of the receptors would be consumed within the first 10 minutes after exposure to LDL. Furthermore, reserve pools of LDL receptors have not been found inside the fibroblast cells (Basu, Goldstein and Brown, 1978) - further evidence that true recycling is taking place. It has already been shown that LDL receptors are internalised in association with LDL (see above). Recycling of other receptors internalised in endocytic processes, has also been demonstrated in various cell types for: the transcobalamin II receptor (Ascoli and Puett, 1978), the asialoglycoprotein receptor (Tanabe, Pricer and Ashwell, 1979) the mannose/N acetylglucosamine receptor (Stahl, Schlesinger, Sigardson, Rodman and Lee, 1980) and several other receptors.

Some receptors, however, do not recycle; the EGF (epidermal growth factor) receptor is a well documented example of this. When EGF is incubated with fibroblasts, the initial wave of receptor-mediated endocytosis leads to a rapid 80% depletion in the number of surface receptors (Carpenter and Cohen, 1976; Das and Fox, 1978). This reduction has been attributed to a degradation of the internalised EGF:receptor complex, which is not accompanied by the reappearance of new receptors. In the continued presence of epidermal growth factor, cells establish a new steady state in which they bind, internalise and degrade the hormone continuously at about 20% of the initial rate. Whether receptor recycling occurs during this steady state is not clear. Gardner and Fambrough (1977) have shown that following ligand binding to the acetylcholine receptors of muscle cells, regeneration of receptor activity requires de novo synthesis and insertion of new receptors i.e. no recycling of receptors takes place.

There is a body of evidence which shows that many different receptors recycle continuously at the same rate in the presence or absence of any ligand. Such events are described as constitutive, as opposed to ligand induced internalisation (e.g. EGF). Furthermore when receptors recycle in the absence of ligand, it is likely that they follow the same path for receptor-mediated endocytosis as for various internalised ligands. Anderson, Brown, Beisiegel and Goldstein (1982) followed the path of LDL receptor recycling in the absence of ligand by using antibodies raised against the receptor, they found that the LDL receptor was internalised along the same pathway as ligand and at the same rate. Hopkins and Trowbridge (1983) using

monoclonal antibodies specific for the transferrin receptor, have shown that the transferrin receptor is found internalised in endocytic vesicles. The work of Geuze et al. (1983) and Evans and co-authors (above) also supports the idea that receptors are internalised along the same pathway as ligand.

It is possible therefore that turnover of all receptors or proteins in the plasma membrane follows the same pathway described for receptor-mediated endocytosis i.e. via coated pits, endocytic vesicles etc. Experimental evidence for this hypothesis has still to be presented.

Endocytic vesicles

Much work on receptor-mediated endocytosis has concentrated on events inside the cell; on how endocytic vesicles (as described by Brown and Goldstein) form, and then deliver the ligand/receptor to specific intracellular sites.

Endocytic vesicles were described by Brown and Goldstein (1976) and isolated from various cell types by Pearse (1975, 1976, 1978). Endocytic vesicles were thought to bud off from invaginating coated pits and subsequently fuse with lysosomes. Furthermore when the endocytic vesicles budded off from coated pits, the clathrin protein was thought to recycle back to form new coated pits - clathrin was shown in vitro to be able to spontaneously assemble into a basket like structure thought to be the building block of coated pits

(Ungewickell and Branton, 1981). Evidence has been presented against this model of clathrin recycling; antibodies raised against

clathrin and injected inside cells did not arrest receptor-mediated endocytosis or precipitate intracellular clathrin (Willingham, Dickson and Pastan, 1981). Petersen and van Deurs (1983) performed serial section analysis which showed unequivocally that endocytic vesicles were independent organelles.

Helenius, Mellman, Wall and Hubbard (1983) have called these endocytic vesicles endosomes and have suggested that these endosomes provide an 'acid bath' through which plasma membrane components continually pass during recycling. These authors have also suggested that endosomes may either: (a) mature into pre-lysosomal structures which may then fuse directly with lysosomes, or (b) act as a vesicle shuttle, with the contents transferred from one endosome population to the next. Helenius et al. have presented very little experimental evidence for or against such models. However it has been established that endocytic vesicles contain an H^+ -ATPase and are acidic in nature (Evans et al., 1984; Saermark et al., 1985; Evans, 1985).

Willingham, Pastan and co-authors have presented a detailed and conflicting model to the endosome model. In their model, the endocytic vesicles are named receptosomes - to state their exclusive role in receptor-mediated endocytosis. Based on morphological evidence receptosomes are thought to be connected by a 'neck' to the plasma membrane. Willingham (1981) has claimed that this connection allows receptosomes to be labelled by impermeant probes. Receptosomes like endosomes are not thought to form by coated pits budding off from the plasma membrane, but may contain an energy dependent ion pump which drives receptosome formation (Dickson, Schlegel, Willingham and Pastan, 1982). The major difference between the endosome model

and the receptosome model however, is the site of delivery inside the cell. Willingham and Pastan (1982) have claimed that receptosomes do not fuse with lysosomes, rather ligands such as EGF and β galactosidase (both labelled with peroxidase) have been observed to be transported from receptosomes and then into the Golgi before final transfer of ligand to the lysosomes. Willingham and Pastan (1984) have identified the delivery of ligand (by receptosomes) to the trans-reticular portion of the Golgi. The trans-reticular region of the Golgi is a tubular array attached to the (normally described) Golgi stacks. Coated pit structures (different to those described on the plasma membrane) have been identified in studies following EGF uptake by cells (Willingham and Pastan, 1982).

It is thought that these coated pits of the trans-reticular Golgi may mirror in function those at the cell surface by accumulating ligands in some sort of receptor specific process. Ligands may then be delivered to lysosomes. The role of the trans-reticular Golgi may be that of sorting ligands destined for either recycling or transfer to the lysosomes. Willingham and Pastan (1984) have shown differences between EGF and transferrin in this respect; EGF which is degraded in the lysosomes, is found concentrated in these Golgi coated pits, while transferrin, which is recycled, is not concentrated in these Golgi coated pits. These authors have also suggested that receptor:ligand dissociation takes place in receptosomes prior to delivery of ligand to the trans-reticular Golgi, although they have no evidence for this. This model of receptor-mediated endocytosis presented by Willingham and Pastan, involving transfer

to the trans-reticular Golgi of (perhaps) separated ligand and receptor has several attractions. Firstly, it is clear that specific signals are involved in the sorting of specific ligands and receptors (directing ligands or receptors destined for degradation or recycling) and the Golgi may provide such a locus for sorting of incoming components. Secondly the recycling of internalised receptors may proceed by the same pathway as has been shown to operate for either newly synthesised proteins destined for insertion in the plasma membrane or proteins destined for secretion from the cell. Blobel and Dobberstein (1975) have shown that such newly synthesised proteins are synthesised in rough endoplasmic reticulum, transferred to the Golgi where processing (e.g. glycosylation, selective proteolysis) occurs, then finally transported to either the plasma membrane or the extracellular fluid. Blobel has shown the importance of an N-terminal signal sequence in directing proteins along this route (this signal peptide is cleaved off in the process). It seems reasonable to suggest that internalised proteins (receptors) might also be recycled back to the plasma membrane along this route.

The exocytic pathway for many ligands is largely unresolved, but probably proceeds along a separate pathway to that presented above.

Inhibitor studies

Several important inhibitors have been used to inhibit key steps of the receptor-mediated endocytic pathway and thus yield information on the biochemical mechanisms underlying these steps.

Weak bases: Weak bases such as chloroquine and ammonium chloride have been reported to block the internalisation step and the lysosomal processing step. These weak bases are thought to act by raising the pH of acidic endocytic vesicles or lysosomes, thus inhibiting their normal functioning. Anderson et al. (1982) have shown that LDL receptors become trapped inside cells in the presence of chloroquine, while Fitzgerald, Morris and Saelinger (1980) have shown that chloroquine inhibits degradation of internalised insulin within lysosomes. An example of inhibition of internalisation is the demonstration by Fitzgerald et al. (1980) that chloroquine stops internalisation of *Pseudomonas* toxin in mouse LM cells. Many of the other studies cited above have also used chloroquine in their investigations to block receptor-mediated endocytosis.

Monodansylcadaverine: Monodansylcadaverine is a member of a group of compounds shown to inhibit receptor-mediated endocytosis of $\alpha 2$ macroglobulin, EGF, vesicular stomatitis virus (Dickson et al., 1982) and various other ligands. Monodansylcadaverine and the other compounds are thought to act by inhibition of the enzyme transglutaminase - an enzyme thought to be essential for coated pit formation (Goldstein et al., 1979; Davies, Davies, Levitzki, Maxfield, Milhaud, Willingham and Pastan, 1980). Dickson et al. (1982) have shown that monodansylcadaverine blocks the transfer of $\alpha 2$ M/receptor complexes into coated pits.

Monensin: Various reports have demonstrated that proton ionophores such as monensin, block internalisation of various ligands in receptor-mediated endocytic processes. Dickson et al. (1982) demonstrated that monensin would allow the accumulation of $\alpha 2$ M/receptor complexes

in coated pits (unlike monodansylcadaverine), but would inhibit their subsequent transfer from coated pits to receptosomes. Schlegel, Willingham and Pastan (1981) have also demonstrated similar effects by monensin on EGF entry to cells.

Other inhibitors: Several other inhibitors or conditions have also been shown to inhibit receptor-mediated endocytosis of various ligands. Korc, Matrisian and Magun (1984) have reported inhibition of EGF receptor-mediated endocytosis by altering intracellular Ca. Fitzgerald Morris and Saelinger (1982) have reported that no external calcium arrests the internalisation of pseudomonas toxin. Microtubular antagonists - cytochalasin B and nocodazole have been shown to affect endocytic processes (White and Hines, 1984; Thyberg and Stenseth, 1981). Larkin, Brown, Goldstein and Anderson (1983) have shown that depletion of intracellular K levels arrested both coated pit formation and the rate of receptor-mediated endocytosis of both LDL and EGF in fibroblasts. This inhibitory effects were reversible by addition of KCl. These authors have suggested that K may mediate intracellular signalling of receptor-mediated endocytosis.

Conclusions

Although much progress has been made in study of receptor-mediated endocytosis in the last four years, many of the biochemical mechanisms remain unresolved. In particular the specific signals which are involved in the sorting and trafficking of internalised ligand and receptor. Another interesting aspect is why cells need receptor-mediated endocytosis? Certain materials are required to be brought into the cell e.g. transferrin/iron, LDL, yolk proteins etc., however receptor-mediated endocytosis may also be involved in regulation of many hormonal ligands. Down regulation of EGF receptors has been demonstrated in the presence of ligand (Carpenter and Cohen, 1976). Another example of down regulation is in β receptor desensitisation induced by the presence of catecholamine (Evans et al. 1984). In many cases (e.g. insulin receptor) the regulatory role of internalisation (if any) is not clear.

There is much evidence (presented above) which suggests that receptors are continually being internalised and re-inserted back into the plasma membrane in the absence of ligand. It seems reasonable to suggest therefore that the pathway of receptor-mediated endocytosis is the route by which all membrane proteins are continually turned over and replaced.

MODULATION OF SODIUM PUMP NUMBERS IN HeLa PLASMA MEMBRANESSodium pump

The Na/K ATPase of animal cell membranes has been purified from various sources, reconstituted into lipid vesicles and observed to catalyse the different modes of Na, K transport at rates similar to those observed in intact cells (Hokin, 1981). This represents convincing evidence that the integral protein Na/K ATPase is identical to the sodium pump.

The number of Na/K ATPase molecules in HeLa plasma membranes may be measured by the number of specific ouabain binding sites. Experimental evidence has shown that ouabain and K^+ ions compete for a single binding site on each sodium pump. Furthermore the amount of ouabain binding correlates with the degree of inhibition of pump activity (Baker and Willis, 1970; Baker and Willis, 1972; Boardman, Lamb and McCall, 1972; Lamb and Ogden, 1982).

On the basis of this evidence, the terms: sodium pump numbers, ouabain binding sites and Na/K ATPase molecules are often used in the same context throughout this present study. In some instances however, this relationship may be tenuous, in which case further clarification is stated.

Modulation of sodium pump numbers

Virtually all components of cells are in a state of molecular flux - being continually synthesised and degraded (or turned over).

The advantages of this continuous biosynthesis and turnover (despite the expenditure of energy required) may be for repair/replacement or modulation of component numbers in response to a changing environment (induction of many metabolic enzymes in response to stress conditions is a widely known phenomenon). The Na/K ATPase is no exception to this, having been shown to exist in such a dynamic state in many different cell types.

Regulation of the Na/K ATPase may be either short term or long term. The former in HeLa cells occurs within the time-scale seconds to minutes and results in increased rate of pumping in response to $[Na]_i$. The K_m for K uptake is 0.8 mM, so the sodium pump is normally maximally stimulated with respect to this ion in normal medium (5 mM K). On the other hand, the K_m for Na efflux is about 10 - 15mM and this appears to be about the level of $[Na]_i$. This means that small changes in $[Na]_i$ may stimulate or depress ion pumping activity (Skou, 1965; Glynn, 1968). Long term regulation of the Na/K ATPase occurs over a period of hours and involves modulation of numbers of copies of this enzyme. This present study is concerned with this type of regulation of the sodium pump.

Various studies have shown modulation of sodium pump numbers in several tissues. Hanwell and Peaker (1975) have shown both an increase in activity and numbers of sodium pumps in duck salt glands, in response to a high salt environment. Increases in erythrocyte, myocardial and renal Na/K ATPase V_{max} activity have been reported to occur in rats chronically maintained on low K diets (Chan and Sanslone, 1969; Erdmann, Bolte and Luderitz, 1971), or in rats chronically maintained on low levels of glycosides (Lindsay and Parker,

1976). Lo and Edelman (1976) have shown an increase in the number of Na/K ATPase molecules in thyroid hormone (T_3) treated rats. In an elegant study, Lo and Edelman (1976) examined the nature of this induction and showed that this increase in Na/K ATPase number was not due to changes in the turnover (or degradation) rate, but due to changes in the biosynthetic rate of this enzyme. The remainder of this review is concerned with the modulation of sodium pump numbers in HeLa cells.

Uptake of ouabain in HeLa cells

Various experiments were carried out around 1972 investigating various aspects of ouabain binding to HeLa cells. Boardman *et al.* (1972) showed that if HeLa cells were incubated for 24 hours in low concentrations of ouabain, the cells were subsequently able to bind more ouabain. Total binding represented twice that found in fresh (control) cells. Around the same time, Vaughan and Cook (1972) carried out other ouabain binding experiments. They specifically labelled all the sodium pump sites of HeLa cells, washed the cells and returned them to normal growth medium, and found that after 5 to 8 hours the HeLa cells were able to bind an additional amount of ouabain equal to the initial saturating pulse, despite the fact that most of the ouabain bound in this first pulse was still cell associated. Vaughan and Cook also demonstrated that true cell recovery was taking place despite the continual presence of the glycoside; pump activity (^{86}Rb influx) measured after the initial saturating pulse, returned to normal 5 to 8 hours later. The $[K]_i$ levels also returned to normal (control) levels by the 8 hour time point.

At this time (1972), what was happening to the pulse bound ouabain and how recovery was taking place in its continued presence, was not clear. Boardman et al. (1972) suggested that the accumulation of ouabain may be due to increased production of sodium pumps in response to chronic glycoside stress.

Internalisation of ouabain

Studies carried out to explain the above observation have shown that ouabain specifically bound to HeLa cells is internalised (Cook, Will, Proctor and Brake, 1976; Cook and Brake, 1978; Cook, Tate and Shaffer, 1982). In their experiments HeLa cells were initially labelled with [^3H]-ouabain, the cells fractionated at various time intervals following the pulse and the homogenate run on a continuous sucrose gradient. Initially the [^3H]-ouabain codistributed with the plasma membrane marker 5' nucleotidase with no [^3H]-ouabain able to be detected inside the cell. However 40 hours following the initial pulse, [^3H]-ouabain was found codistributed with a lysosomal marker β hexosaminidase. Furthermore, shear sensitivity showed that internalised ouabain was associated with a particulate fraction and that β hexosaminidase and ouabain were released from this particulate fraction (by osmotic shock), at the same rate. At no time interval could ouabain be detected free in the cytoplasmic portion of the cell. This pathway for uptake of ouabain in HeLa cells resembles in many respects the pathway of receptor-mediated endocytosis of many other ligands: i.e. ouabain enters the cell via a receptor, is transported to the lysosomes, where the ligand is (probably) separated from its receptor and released (or exocytosed) from the

cell. However morphological evidence identifying several of the individual steps of a receptor-mediated endocytic process such as endocytic vesicles and separation of ligand:receptor, has not yet been presented.

Ouabain is eventually released unmetabolised from HeLa cells at a rate of about $3.5\% \text{ hr}^{-1}$ (Boardman et al., 1972; Griffiths, Lamb and Ogden, 1983). Griffiths et al. (1983) have shown that the rate of release (exocytosis?) of ouabain is much slower than that of other glycosides, digoxin and digitoxin (10% per hour). Furthermore these authors have also shown that weak bases greatly slow (x 3) the rate of excretion of digoxin and digitoxin, but do not alter that of ouabain. A model which explains this lack of effect on ouabain excretion is to suppose that lysosomal activity releases digoxin and ouabain from the sodium pump and that both then diffuse across the lysosome membrane. Digoxin and digitoxin have high lipid solubilities and so the main determinant of excretion rate will be the rate of lysosomal activity; once this is inhibited, the overall rate is decreased. Ouabain on the other hand, has a low lipid solubility and so the main determinant of the excretion rate is the rate of diffusion out of the cell; inhibiting lysosomal activity therefore does not affect the overall excretion rate. Al Gharably (1985) has shown that ouabain is found in the lysosomal interior, while digoxin is probably associated with the lysosomal membrane - findings consistent with the above model.

The early observations of Boardman et al. (1972) and Vaughan and Cook (1972) describing ouabain accumulation by HeLa cells can be accounted for by internalisation of ouabain bound to sodium pumps

and insertion of fresh sodium pumps in the plasma membrane; with a slow rate of release of internalised ouabain from the cell.

Effect of serum concentration on sodium pump numbers

Aiton and Lamb (1984) showed that if HeLa cells are grown in media containing increasing concentrations of serum for 24 hours, this leads to an increase in the sodium pump site numbers. These authors have demonstrated that the chronic effect of increased serum concentration does in fact produce a real increase in sodium pump numbers (as measured by ouabain binding) by demonstrating firstly that the affinity of sodium pumps in high serum for ouabain is unchanged (same association and dissociation rates for ouabain binding and release) and secondly that the increase in sodium pumps correlates with increased pump activity (as measured by ouabain sensitive ^{86}Rb influx).

In time course studies following transfer of cells to medium containing elevated serum concentrations, Aiton and Lamb (1984) have shown that there is a lag period of 3 - 6 hours before any increase in sodium pump numbers is detected. The increase in sodium pump site numbers then proceeds at a rate of $13\% \text{ hr}^{-1}$ until a new steady state is reached at 16 - 24 hours. It was then shown by these authors that the serum stimulated increase in sodium pump numbers could be abolished by protein synthesis inhibitors. Cycloheximide, which inhibits mRNA translation, not only inhibits the serum stimulated increase in sodium pump numbers, but caused a $2\% \text{ hr}^{-1}$ decrease of pump site numbers in cells in high or low serum. Actinomycin D

which inhibits transcription, caused only cessation of the serum stimulated effect with no decrease in pump site numbers. Aiton and Lamb (1984) have suggested that this difference between cycloheximide action and actinomycin D action is due to stores of preformed sodium pump mRNA in HeLa cells.

Aiton and Lamb (1984) have shown that the component of serum responsible for stimulation of sodium pump site numbers in HeLa cells is found in the high molecular weight ($> 50,000$) serum fraction. Furthermore they have also shown that the active component is exhaustible; repeated use of the same medium containing serum diminishes the serum-stimulated response (this is not due to exhaustion of other medium components).

Effect of low K medium on sodium pump numbers

Boardman et al. (1972) and Boardman Huett, Lamb, Newton and Polson (1974) have shown that if HeLa cells were stressed with respect to their normal cation metabolism either by chronic incubation in sub-lethal concentrations of ouabain or by chronic incubation in low K medium (0.2 - 0.4 mM K), then the cells respond over a period of hours by increasing their sodium pump sites. Boardman et al. (1974) showed that the rates of ouabain association and dissociation in low K stressed cells was no different to that found in controls, suggesting a real increase in sodium pump numbers rather than a change in the affinity of ouabain for the sodium pump. Pollack, Tate and Cook (1981a) have confirmed the above observations of Boardman et al. (1974) and also demonstrated that a real increase in sodium pump numbers was occurring in HeLa cells in response to low K stress,

by showing an increase in Na dependent, K sensitive phosphorylation of the Na/K ATPase in isolated membranes, as well as an increase in Vmax of Na/K ATPase activity in isolated membranes. Both of these measurements correlated with an increase in ouabain binding. Pollack et al. (1981a) also showed that the effects of low K medium on HeLa cells were specific, as 5' nucleotidase activity or K insensitive phosphorylation were not found to have increased in isolated membranes from stressed cells.

Induction of sodium pump numbers in HeLa cells as a result of prolonged growth in low concentrations of ouabain, was often found to be x 2 or greater (Boardman et al., 1974), however the induction of sodium pump numbers in the plasma membrane was found to be only a 20% increase (Pollack et al., 1981a). The effects on ion gradients by chronic low K stress and low concentrations of ouabain are similar, and the cells should perceive these two stress conditions as identical (not proved though), but the response to chronic glycoside stress is not easily controlled because of a very steep dose response curve. Furthermore the use of ouabain as both a probe and as a stress introduces additional complications; for this reason investigation of induction of sodium pump numbers has largely utilised low K stress conditions. Gargus, Miller, Slayman and Adelberg (1978) have shown that if the medium K concentration is too low (< 0.2 mM) then cultured cells do not survive well, so for this reason the low K medium often used in the above studies has $[K]_o$ usually between 0.2 - 0.5 mM.

In time course studies following the response to low K stress, Pollack et al. (1981a) have shown that sodium pump numbers increase

steadily and reach a near steady state with often double the number of pump sites after 24 hours. The nature of this induction is reviewed below.

Direct measurement of sodium pump turnover

All of the above experiments have relied on ouabain as a label for the sodium pump, however it is possible that many of the above observations, may be ligand induced events (i.e. internalisation of sodium pumps may be ligand induced, as is found with EGF receptors). Pollack, Tate and Cook (1981b) have examined this possibility by measuring the turnover of the native sodium pump. In their experiments, Pollack et al. (1981b) employed a density label technique used by Devreotes and Fambrough (1976) and Gardner and Fambrough (1977): (a) HeLa cells were grown for several generations in ^{13}C amino acids and then returned to normal medium, (b) plasma membranes were isolated at various time intervals and the Na/K ATPase specifically phosphorylated with ^{32}P by a Na dependent, K sensitive reaction (the specifically labelled protein was previously identified on SDS gels as corresponding to the catalytic subunit of the Na/K ATPase), (c) isolated membranes were mixed with control membranes in which the Na/K ATPase was specifically labelled (as above) with ^{33}P (the control membranes act as internal standard), (d) the mixed membranes were solubilised in SDS and analysed in velocity gradients. The results of such experiments showed that the density shift between the ^{13}C -labelled and control enzyme becomes smaller with time until after 24 hours the density shift is negligible - the ^{13}C -labelled enzyme has been replaced by enzyme of control density. Pollack et al. (1981b) have calculated the turnover rate from the density

shift and found it to be $t_{1/2}$ 5.4 hours or approximately $12\% \text{ hr}^{-1}$. Pollack et al. (1981b) also observed an initial lag period of about 6 hours, before the onset of decrease in the density label in the above experiment. Devreotes and Fambrough (1976) have called this lag period the transit time, which represents the time taken for transport of newly synthesised sodium pumps (presumably) in the endoplasmic reticulum, through the Golgi apparatus to eventual insertion in the plasma membrane.

Cook and Brake (1978) have estimated that the internalisation of ouabain from the HeLa plasma membrane proceeds at a rate of $t_{1/2}$ 5 hours - a value very close to the turnover rate of the native sodium pump. The inference from this is that ligand (ouabain) binding does not induce sodium pump internalisation, rather sodium pump internalisation is a constitutive mechanism and therefore independent of ligand binding.

Turnover of the sodium pump during low K stress

The level of the sodium pump (or any other enzyme) in the plasma membrane may be described by the expression $[E] = \frac{k_{\text{syn}}}{k_{\text{tur}}}$, where $[E]$ is the concentration of enzyme, k_{syn} the enzyme synthetic rate and k_{tur} the turnover rate of the enzyme. In the steady state condition $k_{\text{syn}} = k_{\text{tur}}$; however changes in either the synthetic rate or turnover rate of an enzyme in response to external stimuli can result in alteration of the number of functional molecules in the plasma membrane. The possible role of changes in the turnover rate of the sodium pump during chronic low K stress was investigated by Pollack et al. (1981b). They measured the turnover rate of the sodium pump in

low K stressed cells using the procedures reviewed immediately above, and found that the turnover rate decreased from 3.1/generation in controls to 1.3/generation in low K stressed cells.

Pollack et al. (1981b) have suggested, that the halving of the turnover rate of the sodium pump can account solely for a doubling of pump sites during low K stress assuming that K_{syn} remains constant, and it will only be when there are twice the number of sodium pump sites in the cell surface that a new steady state will be achieved. Evidence supporting this model (in addition to the measured turnover rate) comes from examining the reversal of the low K stress. If cells were modulating the sodium pump site numbers by changes in the synthetic rate of this enzyme, then on restoration to normal K, there would still be in transit within the cell, sodium pumps synthesised at twice the control rate which would insert into the plasma membrane - in short, a lag period due to this transit time would be observed on restoration of normal K medium. Pollack et al. (1981b) found no lag period but a prompt return of sodium pump numbers to control levels. This finding supports the model above, that sodium pump numbers during low K stress are modulated by changes in turnover rate and not synthetic rate. Pollack et al. (1981b) also noted that the cells return to the control state at a rate of about $12\% \text{ hr}^{-1}$, which is the measured turnover rate of sodium pumps in HeLa cells. Pollack et al. (1981b) have suggested that modulating sodium pump numbers by alteration of the turnover rate allows an immediate and rapid response to stress conditions.

Boardmann et al. (1974) have shown that during low K stress conditions, HeLa cells may respond in fact to increases in intracellular

sodium, rather than lowered external K in the medium. This was demonstrated in experiments in which the $[K]_o$ was lowered, without raising $[Na]_i$; achieved by substituting external Na with sorbitol. This treatment resulted in no increase in sodium pump site numbers after 24 hours incubation, despite the low K medium condition.

Boardman et al. (1974) also showed that the protein synthesis inhibitor cycloheximide abolished the increase of sodium pump numbers during low K stress - a result apparently inconsistent with the model of Pollack et al. (1981b).

Interactive effects

Modulation of sodium pump numbers has been shown to occur in response to high serum and low K stress conditions. High serum is thought to result in increased synthesis of the sodium pump, while low K medium, is thought to cause a decrease in sodium pump turnover rate (with no change in synthetic rate), thus increasing sodium pump sites in the membrane. Aiton and Lamb (1984) have shown however that the ability of low K medium to increase sodium pump sites in the membrane is dependent upon the serum concentration of the medium; the increase in numbers is diminished with increased concentrations of serum. These results show that the serum and low K effects are interactive. Boardman, Hume, Lamb and Polson (1975) have shown that growth of HeLa cells in Li (as a substitute for Na) medium can also cause an increase in sodium pump site numbers, but that this effect is independent of the serum concentration of the medium. These results show that there are clear differences in the way that K and Li interact with serum.

Hume and Lamb (1976) have shown that a 10 minute exposure of HeLa cells to 1 mM ATP caused a delayed increase in sodium pump numbers and that this increase could be abolished by increased serum concentration.

Aiton and Lamb (1984) have suggested that all of the interactive effects (above) observed in HeLa cells, are consistent with different control pathways which interact to modulate sodium pump site numbers.

Effects of cycloheximide

Modulation of sodium pump numbers by the conditions reviewed above, has been investigated using the protein synthesis inhibitor cycloheximide (cycloheximide inhibits the translation process).

Aiton and Lamb (1984) have shown that the serum stimulated increase in sodium pump numbers is abolished by cycloheximide; resulting in a $2\% \text{ hr}^{-1}$ decline in pump numbers following a 3 hour lag, for cells incubated in high serum. Control cells treated with cycloheximide also show a $2\% \text{ hr}^{-1}$ decline in sodium pump site numbers following a 3 hour lag period. Griffiths et al. (1983) and Lamb and Ogden (1982) have proposed that this rate of decline in pump site numbers is a measure of the sodium pump turnover rate, although this does not agree with the direct measure of the rate ($12\% \text{ hr}^{-1}$ found by Pollack et al., 1981b). Another conflicting finding by Boardman et al. (1974) is that cycloheximide abolished the increase in sodium pump site numbers caused by low K stress and thereafter caused a $2\% \text{ hr}^{-1}$ decline in sodium pump site numbers (no indication of a lag period was given). The model proposed by Pollack et al. (1981b) (that modulation of sodium pump numbers occurs by alteration of

turnover and not synthetic rate of sodium pumps) may suggest that differences between low K stressed cells and controls, both in the presence of cycloheximide, may have been expected. This is discussed more fully elsewhere.

Aiton, Lamb and Ogden (1981) have shown that if HeLa cells are grown in low concentrations of ouabain and then returned to fresh (normal) medium, recovery of sodium pump site numbers is cycloheximide sensitive indicative of the requirement for de novo protein synthesis. Furthermore, when recovery is allowed to proceed (in the absence of cycloheximide), there is a 6 hour lag period before any increase in pump sites can be detected. Recovery of pump sites then proceeds at a rate of approximately $12\% \text{ hr}^{-1}$. The lag period observed is very similar to the transit time for synthesis reported by Pollack et al. (1981b), while the rate of recovery of pump sites $12\% \text{ hr}^{-1}$ is very similar to the estimated synthetic rate (the synthetic rate is equal to the calculated turnover rate $12\% \text{ hr}^{-1}$ in the steady state condition). Aiton and Lamb (1984) have calculated that the cycloheximide sensitive increase in sodium pump numbers in medium containing a high concentration of serum, proceeds at a rate of about $13\% \text{ hr}^{-1}$.

Conclusions

The binding of ouabain to sodium pumps (Na/K ATPase) of HeLa cells and subsequent internalisation to the lysosomal compartment of the cell probably proceeds along a pathway similar to that for receptor-mediated endocytosis of other well studied ligands. This pathway is of interest in it's own right i.e. the mechanisms of release of ouabain from receptors, the pathway of release of ouabain (and digoxin) from the cell, the possible role of receptor recycling are all important features of ouabain uptake still to be elucidated. Sodium pumps are also subject to long term regulation over a period of hours - days, in response to various stress conditions (reviewed above). The mechanisms and signals underlying this long term regulation of the number of sodium pump sites in the plasma membrane is also of great interest and importance.

METHODSBiochemical stripping using pronase.

This method is adapted from the procedures of Karin and Mintz (1981). Internalised ligand may be distinguished from surface bound ligand by the resistance of internalised ligand to treatment with pronase.

HeLa cells grown for 3-5 days on 5 cm plastic Petri dish plates, were pulsed with [³H]-ouabain and the non-specific binding washed off as described previously. Pronase solution was prepared: 0.25% (w/v) in Earle's balanced salt solution (Ca/Mg free). Following specific binding of ouabain, plates were incubated at 37° C with 4 mls warm BME supplemented with 10% new-born calf serum, for various times; 0, 2, 4, 6 hours. At the end of the incubation periods, the medium was removed from the plates, the plates washed with cold 5K Krebs x4 and the plates placed on ice. 750 µl aliquots on ice-cold 0.25% pronase solution were added to the plates and the plates incubated for up to 1 hour at 4° C. At the end of this incubation period, the pronase was neutralised by the addition of 750 µl warm BME medium supplemented with 10% new-born calf serum. The suspension was blasted as described previously to yield a single cell suspension. A 500 µl aliquot was removed and then centrifuged through oil in the following way: a 200 µl mixture of 1 part di-iso-octyl-phthalate oil (specific gravity 0.98) 4 parts dibutyl phthalate (specific gravity 1.045), was placed in a 1.5 ml microfuge tube and the 500 µl aliquot from the single

cell suspension layered on top of the oil. The sample was centrifuged at 9000g in a Beckman microfuge for 1 minute. Three distinct layers resulted, from top to bottom: the supernatant, the oil and the cellular pellet. The supernatant was removed and counted for radioactivity, then the microfuge tube dropped into liquid nitrogen. The cellular pellet was then cut from the frozen microfuge tube, placed in a vial and counted for radioactivity.

Biochemical stripping using acetic acid

This method was adapted from the procedures of Haigler, Maxfield, Willingham and Pastan (1980). In this method internalised ligand is distinguished from surface bound ligand by the resistance of internalised ligand to treatment with acetic acid.

HeLa cells were grown for 3-5 days on plates and [³H]-ouabain bound as described previously. Plates were incubated at 37° C with warm BME supplemented with 10% new-born calf serum for various times; 0, 2, 4, 6 hours. At the end of the incubation periods, the medium was removed from the plates, the plates washed with cold 5K Krebs x4 and the plates placed on ice. Plates were incubated with 1 ml of acetic acid (0.3 M, pH 2.5) containing 0.5 M NaCl for 6 minutes at 4° C. The acetic acid was removed from each plate into vials, each plate washed with 0.5 mls acetic acid solution, the 0.5 ml wash solution pooled with the 1 ml aliquot to give a final sample volume of 1.5 mls from each plate. The radioactivity removed by acetic acid treatment was counted. This corresponded to surface bound ligand. The remaining cell associated

radioactivity was removed by incubating the cells on the plates with 1 N NaOH (1 ml) for 1 hour. The 1 N NaOH was then neutralised by the addition of 0.2 ml 5 N HCl to give a final volume of 1.2 mls. This volume was counted for radioactivity.

Measurement of cardiac glycoside loss from the plasma membrane of HeLa cells

HeLa cells were grown for 4-6 days in a microcarrier spinner culture then [^3H]glycoside (ouabain or digoxin) was bound to the cells and the non-specific binding washed out as previously described.

Immediately following this washout, 100mls fresh warm, BME was added to the spinner flask, then two 40 ml samples of an even suspension were removed from the spinner flask and transferred into two other spinner flasks. The volume in each spinner flask was made up to 200 mls with fresh, warm BME ensuring that each flask contained approximately the same concentration of cells on beads. Two 10 ml samples and two 5 ml samples were removed from each spinner flask into universal tubes and placed on ice.

These samples were used to determine the zero time values of (a) membrane bound ligand and (b) cell associated ligand by methods previously described. One of the 3 spinner flasks was designated as control while a variety of drugs and or different media conditions were routinely tested in the two other, experimental spinner flasks. If the effect of a drug was being tested, then enough of a standard stock solution of the drug was added to the spinner flask to give the required final concentration. If the effects of media conditions were being tested then after the zero time

samples had been removed from the spinner flask, cells on beads were allowed to settle out, the medium removed and fresh 'experimental' medium added to the spinner flask. The cells on beads were washed a further two times and the final volume adjusted, with experimental medium, to approximately 175 mls.

The 3 spinner flasks (one control, two experimental) were incubated in a 37° C room with the cultures being stirred at the same speed as was used for growth. 3 x 10 ml samples and 3 x 5 ml samples were withdrawn from each spinner flask into sterile universal tubes at various time intervals following labelling with [³H]-glycoside (usually, 6, 12, 24 hr time points were measured). The 10 ml samples were used to determine membrane bound ligand, the 5 ml samples were used to determine cell associated ligand by methods previously described. Plots of loss of membrane bound glycoside against time and loss of cell associated glycoside against time were made for controls and the various experimental treatments

Experimental treatments

The possible effects of various drugs or chemicals on, the loss of glycoside from the cell membrane and on the washout of glycoside, were investigated by the procedures described above. Stock solutions of each chemical/drug were prepared and enough added to the experimental flasks to give the required final concentration of the particular drug/chemical. The following drugs/chemicals were tested: vinblastine, cytochalasin B, nocodazole, chlorpromazine, monodansylcadaverine, trifluoroperizene, cycloheximide and monensin.

The possible effects of low extracellular calcium and low intracellular calcium on loss of glycoside from the cell membrane and washout of glycoside from the cell were also studied. Calcium-free BME containing 2 mM EGTA was prepared according to the formula given by Flow Laboratories for BME, omitting calcium salts. To deplete extracellular Ca, cells growing in a microcarrier culture were washed x2 with Ca-free BME supplemented with 10% dialysed new-born calf serum and 2 mM EGTA, then incubated with this medium and the indicated rate measurements made. To deplete intracellular Ca, cells growing in a microcarrier culture were washed x2 and then incubated with warm, Ca-free BME supplemented with 10% dialysed new-born calf serum, 2 mM EGTA and 10 μ M of the calcium ionophore A23187. The indicated rate measurements were then made. In all above experiments cell numbers/protein were closely monitored to evaluate cell 'health' in medium containing the various drugs/chemicals.

Measurement of ouabain loss from the plasma membrane of HeLa cells during low potassium (low K) stress

HeLa cells were grown for 4-6 days in a microcarrier spinner culture, [3 H]-ouabain bound to the cells and the non specific binding washed out. Experimental flasks were washed with fresh, warm, low K BME medium (0.2 - 0.4 mM K) and the rate of loss of glycoside from the plasma membrane measured. (All of these methods are previously described in detail).

At some time point, the low K BME was removed from the spinner flask and replaced with fresh warm, normal BME (contains 5 mM K).

The ouabain bound to the plasma membrane was periodically determined prior to, and immediately after, removal of the low K stress.

These measurements showed any change in the rate of loss of glycoside following removal of the low K stress.

In other experiments cells grown in a microcarrier spinner culture for 4-6 days were incubated in low K BME for 48 hrs. The specific binding and rate of loss of glycoside from the plasma membrane was then determined as previously described.

Usually, low K BME was supplemented with 2% (v/v) dialysed, new-born calf serum. In another set of experiments, the rate of loss of glycoside from the plasma membrane was measured in cells incubated in low K BME supplemented with various concentrations of dialysed serum.

The effect of chronic growth of HeLa cells in sublethal concentrations of ouabain

HeLa cells were grown for 4-6 days in a microcarrier spinner culture and then subdivided into 3 flasks, A, B, C. Flask A was incubated with BME containing 16 nM [³H]-ouabain, flask B incubated with BME containing 16 nM non radioactive ouabain and flask C, the control, incubated with BME. (16 nM ouabain is close to the experimentally determined EC₅₀ value, Griffiths unpublished data). The three flasks were then incubated for 48 hrs. At the end of

this time, flasks B and C were pulse labelled with 2×10^{-7} M [^3H]-ouabain and the non specific binding washed out by methods previously described. 3×10 ml samples were removed from each flask (A, B, C), plasma membranes prepared and the ouabain bound to the plasma membrane determined.

If any induction of ouabain binding sites has taken place then the sum of the specific activities A + B should be significantly greater than the value obtained for C.

Materials

Pronase, chloroquine, nocodazole, cytochalasin B, monodansyl-cadaverine, ionophore A23187, cycloheximide and monensin were obtained from the Sigma Chemical Company.

Chlorpromazine was obtained from May and Baker Ltd. and trifluoroperizene was obtained from Smith, Klyne and French Ltd. Tissue culture materials were obtained from Flow Laboratories (Irvine). General chemicals of Analar quality were used where possible.

Solutions

Sterile Low K BME was prepared according to the formula given by Flow Laboratories for BME, omitting KCl. Dialysed serum (2% v/v) was usually added to the BME and the K content adjusted to 0.2 - 0.4 mM by adding the appropriate amount of 1 M KCl. Samples were removed from the experimental flasks and the K concentration in the experiential medium determined with a EEL 450 flame

photometer. The K concentration of the BME was usually set to 0.2-0.4 mM.

RESULTS

CARDIAC GLYCOSIDE LOSS FROM HeLa PLASMA MEMBRANES AND HeLa CELLS

The aims of the following experiments were to investigate further the uptake (and excretion) of cardiac glycosides in HeLa cells, with the use of specific inhibitors and/or different media conditions. HeLa cells in a microcarrier culture were labelled with ouabain, the non-specific binding washed off and the cells on beads returned to normal growth medium. At various time intervals, membranes on beads were prepared and the ouabain bound to the plasma membrane determined. Typical results are shown in Table 2.1.

Table 2.1. Ouabain loss from HeLa plasma membranes

Time (hours)	% initial value
0	100
6	59.1 ± 2.5
12	29.9 ± 1.8
24	10.0 ± 0.8

Results are expressed as mean ± SEM, n = 12.

Data from 6 different experiments.

It is now well established that ouabain and other cardiac glycosides are internalised following binding to the sodium pump of HeLa cells and, with time, are transported to the lysosome compartment of the cells (Cook and Brake, 1978; Cook, Tate and Shaffer, 1982). In the lysosome, it is envisaged that separation of the pump:glycoside complex occurs although no direct evidence for this is available. Ouabain and other cardiac glycosides are internalised by receptor-mediated endocytosis, released in the lysosomes and then lost from the cells by apparently exocytosis. These mechanisms are discussed in depth, elsewhere.

Griffiths, Lamb and Ogden (1983) have fitted ouabain accumulation data on 2 models of the uptake process. Model 1 assumed that the glycoside bound to the sodium pump on the cell surface and eventually dissociated i.e. no internalisation. Model 2 assumed that the initial step involved a similar surface binding but that the glycoside/pump complex was then internalised and some replacement of the surface pump occurred. Model 1: $O + E \rightleftharpoons OE$, where O is the cardiac glycoside, E the sodium pump and OE the pump/glycoside complex.

Model 2: $O + E \rightarrow OE$ internalised

E ← pump insertion

The accumulation data of Griffiths *et al.* (1983) fitted model 2 more closely. It is likely that digoxin and digitoxin also fit this model in HeLa cells.

In other experiments, Pollack, Tate and Cook (1981b) measured the turnover of the sodium pump of HeLa cells directly, by a ^{13}C

density label technique. In this method, HeLa cells were grown in heavy (^{13}C) amino acids, then returned to normal growth medium containing ^{12}C amino acids. At time intervals, HeLa plasma membranes were isolated, the sodium pump specifically phosphorylated, the plasma membranes solubilised in SDS and centrifuged in density gradients. The density shift of the ^{13}C -labelled subunit of the sodium pump became smaller with time and eventually approached the control density. From this data, a turnover rate for the sodium pump was calculated and found to be $t_{1/2} = 5.4$ hour ($12.8\% \text{ hr}^{-1}$). This value is close to the value obtained for ouabain loss from the plasma membrane 9.7 ± 0.5 (Table 2.1), and strongly suggests that glycoside loss from the HeLa plasma membrane is achieved solely by internalisation (and that the binding of glycoside to the sodium pump does not induce internalisation).

In summary, the above data suggests that: (a) the rate of loss of ouabain from the HeLa plasma membrane is due to internalisation and not dissociation into the external medium (b) ouabain binding to the sodium pump does not induce or stimulate pump internalisation, sodium pumps turnover at the same rate whether ouabain is bound or not and (c) a measure of loss of glycoside from the plasma membrane essentially reflects turnover of the native sodium pump.

It seems reasonable therefore, to treat ouabain loss from HeLa plasma membranes (or HeLa cells) as a single exponential process. If data from experiments measuring loss of glycoside from HeLa plasma membranes (or HeLa cells) is expressed as a single exponential and the data fitted by a log plot to a straight

line (best fit) by the Glim package, then the rate of loss of glycoside from the plasma membrane can be calculated from the slope of the line. Results for ouabain and digoxin are shown below in Table 2.2 and Table 2.3.

Table 2.2. Rate of loss of glycoside from HeLa plasma membranes

	Rate loss
Ouabain	9.7 ± 0.4
Digoxin	9.9 ± 0.4

Results are expressed as % loss.hr⁻¹ ± SEM. For ouabain n = 46, data from 5 separate experiments and for digoxin n = 32, data from 4 separate experiments.

Table 2.3. Rate of loss of glycoside from HeLa cells.

	Rate loss
Ouabain	4.5 ± 0.5
Digoxin	9.5 ± 0.3

Results are expressed as % loss.hr⁻¹ ± SEM.

For ouabain n = 28, data from 4 separate experiments and for digoxin n = 22, data from 4 separate experiments.

Figures 2a and 2b show the loss of glycoside (ouabain and digoxin) from the HeLa plasma membrane and from whole cells.

The rate of loss of ouabain from whole cells is calculated to be $4.5\% \text{ hr}^{-1}$ a value much less than the rate of loss of ouabain from the plasma membrane $9.7\% \text{ hr}^{-1}$. The rate of loss of digoxin from whole cells $9.5\% \text{ hr}^{-1}$, is similar to that found for the rate of loss from the plasma membrane $9.9\% \text{ hr}^{-1}$. The difference in the rates of loss from whole cells (washout rate) of ouabain and digoxin is thought to be due to differences in lipid solubility. Digoxin, which has a high lipid solubility, is released from the lysosomes much more slowly than ouabain which has a low lipid solubility. The rate determining step for ouabain washout is thought to be its rate of diffusion out of the cell. These processes have been investigated by Griffiths *et al.* (1983) and Al Gharably (1985) and are discussed later.

Figure 2a also shows the loss of ouabain from the plasma membrane at 4°C . 88% of the ouabain bound initially still remains on the plasma membrane after 24 hours in growth medium at 4°C . This result is consistent with the findings of others, that receptor-mediated endocytosis can be inhibited by energy inhibitors or low temperature (4°C or less). (Brown and Goldstein, 1976; Larkin, Brown, Goldstein and Anderson, 1983; Dickson, Schlegel, Willingham and Pastan, 1982). Low temperature studies (or the use of energy inhibitors) have enabled the separation of ligand binding from subsequent ligand/receptor internalisation. It is worth pointing out that in the case of glycoside binding, all the receptor sites can be saturated using 2×10^{-7} M glycoside, within 20 minutes.

Figure 2a: Rate of loss of ouabain from HeLa plasma membranes and HeLa cells. Ordinate: % of amount of ouabain bound initially. Abscissa: time (hours). HeLa cells in a microcarrier culture were labelled with [³H]-ouabain, the non-specific binding washed off, the culture divided and then returned to growth medium at 4 C or 37 C. At various time intervals: (a) ouabain bound to the plasma membrane (□, 37 C incubation), (b) cell associated ouabain (○, 37 C incubation) and (c) ouabain bound to the plasma membrane (●, 4 C incubation) were all determined. For (a) and (b) each data point is the mean ± SEM, n=5. Data from 2 separate experiments. For (c) each data point is the mean ± SEM, n=4. data from 1 experiment.

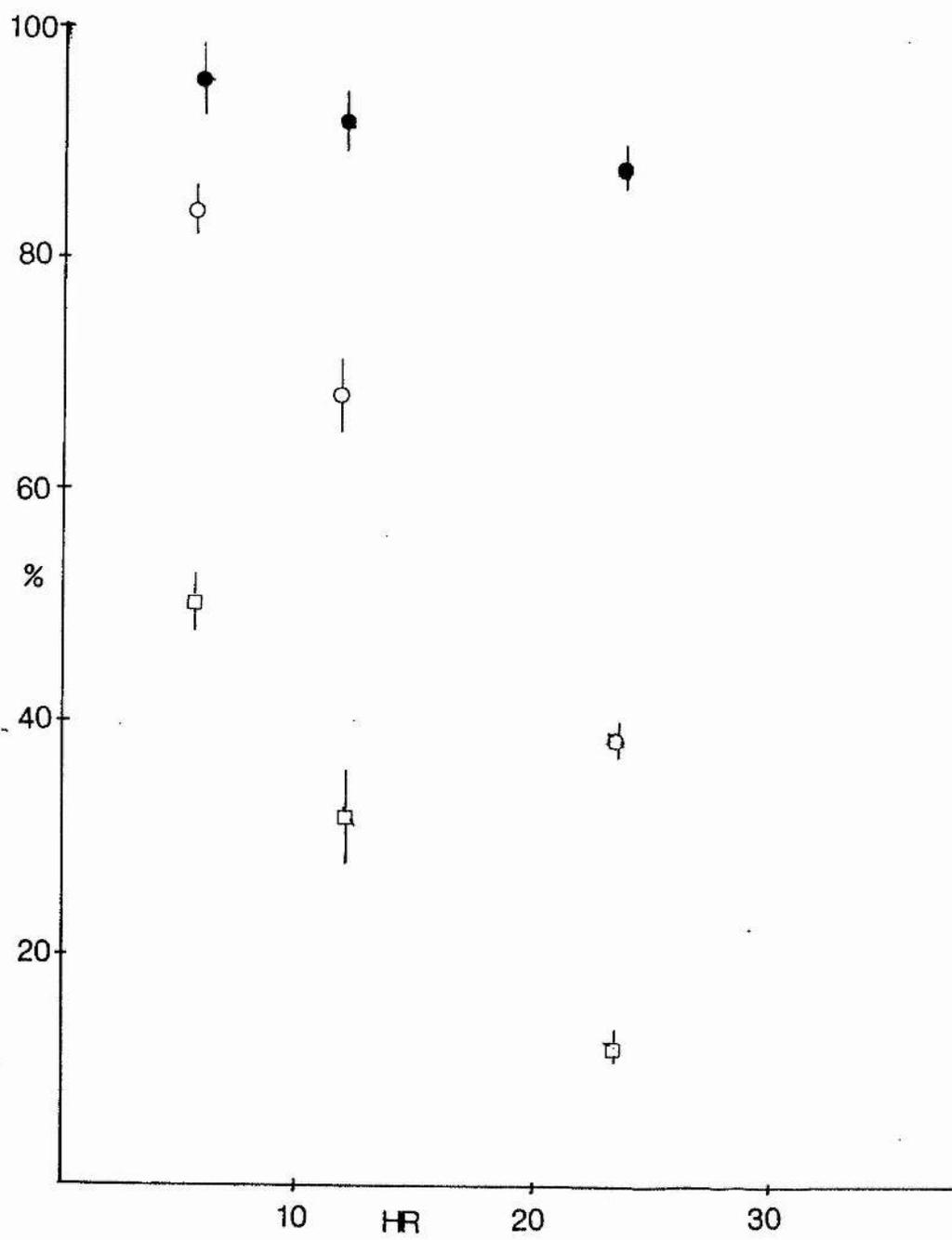
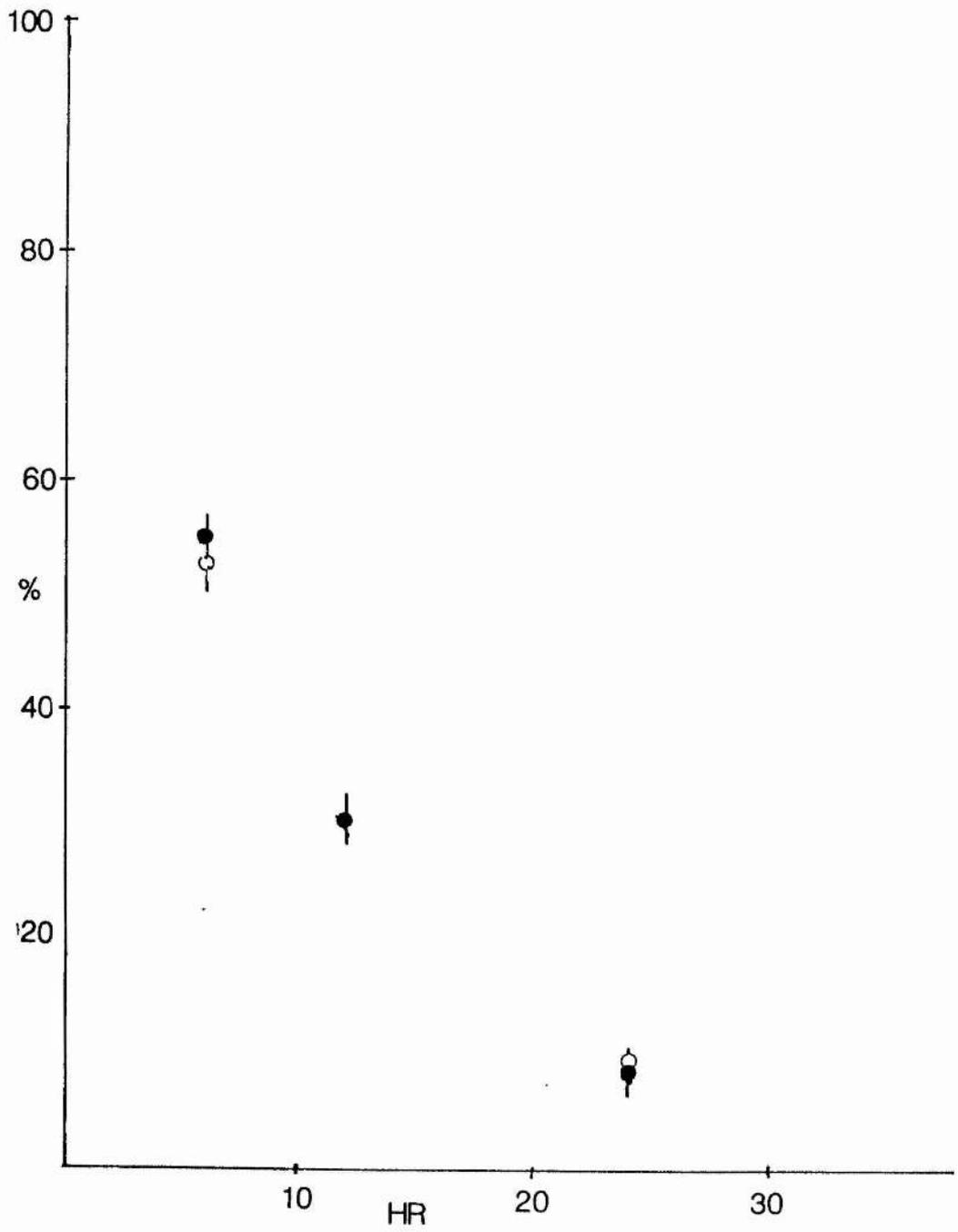


Figure 2b: Rate of loss of digoxin from HeLa plasma membranes and HeLa cells. Ordinate: % of amount of digoxin bound initially. Abscissa: time (hours). HeLa cells in a microcarrier culture were labelled with [³H]-digoxin, the non-specific binding washed off, the culture divided and then returned to growth medium at 37 C. At various time intervals: (a) the digoxin bound to the plasma membranes (●, 37 C incubation) and (b) the cell-associated digoxin (○, 37 C incubation) were determined. Each data point is the mean ± SEM, n=9-12. Data from 4 separate experiments.



During this time, it is envisaged that internalisation of receptors ($12\% \text{ hr}^{-1}$) will have little effect on the determination of the number of surface receptor sites.

Another approach to measure internalisation of glycoside (sodium pump) was tried. Many workers have used biochemical or acid stripping, to separate surface bound and internalised ligand (Carpenter and Cohen, 1976; Haigler, Maxfield, Willingham and Pastan, 1980). These methods were applied to HeLa cells to investigate ouabain uptake. [^3H]-ouabain, 2×10^{-7} M, was bound to HeLa cells on 5 cm plates for 20 minutes and the non-specific binding washed off. The plates were immediately washed again with ice-cold buffer then: (a) incubated for 30 minutes or 60 minutes with 0.25% pronase at 4°C , centrifuged through oil (1 part di-iso-octyl phthalate : 4 parts dibutyl phthalate), the cell pellet and supernatant collected and counted for radioactivity, (b) incubated for 6 minutes at 4°C with acetic acid (0.2 M, pH 2.5) containing 0.5 M NaCl, the supernatant removed for radioactive counting, the cells on plates washed, dissolved in 1 M NaOH and also counted for radioactivity. In both cases (a) and (b) the supernatant should contain surface bound ouabain and the cells, internalised ouabain. The results are shown in Tables 2.4 and 2.5.

Table 2.4. Effect of pronase treatment on cell surface ouabain

Length of incubation with pronase at 4°C	Radioactivity		
	Supernatant	Cell pellet	Released
30 minutes	590 ± 53	488 ± 42	45%
60 minutes	360 ± 29	286 ± 39	44%

Results are expressed as mean ± SEM, n = 7. Data from 2 separate experiments. Activities are expressed as cpm radioactivity per plate.

The results in Table 2.4 suggest that 0.25% pronase is not effective in removing all (or most) of the surface bound ouabain. Incubating with pronase for 60 minutes instead of 30 minutes did not increase the amount of surface bound ouabain released. The possibility remains however, that ouabain on binding, becomes occluded and so insensitive to external attack by pronase.

Table 2.5. Effect of acetic acid on cell surface ouabain.

Length of incubation with acetic acid at 4°C	Radioactivity		
	Supernatant	Cell pellet	Released
6 minutes	4945 ± 130	394 ± 56	93%

Results are expressed as mean ± SEM, n = 10. Data from 2 separate experiments. Activities are expressed as cpm radioactivity per plate.

The results in Table 2.5 suggest that unlike pronase treatment, acetic acid treatment for 6 minutes at 4°C is effective at removing most (93%) of the surface bound ouabain. The next experiment carried out was to incubate cells on plates with [³H]-ouabain bound, in warm BME at 37°C for several hours. Since sodium pumps/ouabain are internalised at a rate of approximately 10% hr⁻¹, then after 5 hours about half of the ouabain initially bound, should be acetic acid insensitive (i.e. internalised). In such experiments, acetic acid treatment after 5 hours incubation of cells in growth medium, did not release half of the ouabain initially bound. It was found that between 85-95% of ouabain initially bound was still releasable after 5 hours, results very different from the measured loss of ouabain from the plasma membrane. Acetic acid treatment was found to drastically alter the ion contents of HeLa cells, suggesting that the plasma membrane structure is disrupted.

Investigation of cardiac glycoside uptake and excretion in HeLa cells

The aim of the following series of experiments was to investigate the biochemistry of receptor-mediated endocytosis and exocytosis of cardiac glycosides in HeLa cells, by measuring glycoside uptake and excretion in the presence of specific inhibitors or different media conditions. The inhibitors and media conditions used, have been reported to affect the rate of receptor-mediated endocytosis, by inhibition of a specific component of the receptor-mediated endocytic pathway. In all experiments the conditions and concentrations of drug were as reported in the literature,

but several different concentrations were often used in addition. The experimental procedures were as follows; HeLa cells in a micro-carrier culture were labelled with [^3H]-glycoside, the non-specific binding washed off and the cells returned to normal growth medium. The cell suspension was immediately divided into, a control and experimental flask(s). The experimental flask(s) contained the drugs and/or conditions being tested. At various time intervals, the glycoside bound to prepared plasma membranes and the glycoside associated with cells, were determined. The rates of loss of glycoside from HeLa plasma membranes and from HeLa cells were determined by expressing the results as a single exponential and obtaining the rate loss from the slope of the fitted line (log plot) computed by the Glim package. In all the experiments, cell conditions and cell protein were carefully monitored over the course of the experiment, in order to assess cell death caused by any of the experimental conditions. No drug or condition used, reduced cell protein by more than 5% over the time-course of any experiment. Experimental data and control data were fitted to a straight line (log plot) by the Glim package, various error structures examined and a t-test used to test for possible significance of experimental line plot from control line plot. Furthermore the control and experimental lines of all experimental conditions below were subjected to a test of linearity i.e. a drug may be altering the pattern of glycoside release from the membrane or cell. Data was fitted to a linear (log plot) and cubic model and an f-test used to test goodness of fit to the linear model. All experimental conditions (and controls) fitted a linear model (log plot), consistent with a single exponential decay of glycoside loss.

Chloroquine

Chloroquine and other weak bases such as amantadine and ammonium chloride have been reported widely as effecting receptor-mediated endocytotic processes. They do so by blocking the internalisation step (Sando, Titus-Dillon, Hall and Neufield, 1979; Fitzgerald, Morris and Saelinger, 1980) or the lysosomal processing step (Goldstein, Anderson and Brown, 1979; Helenius, Kartenback, Simons and Fries, 1980).

In a series of experiments on the effects of chloroquine and other weak bases on the accumulation and efflux of digoxin and ouabain in HeLa cells, Griffiths et al. (1983) demonstrated that: (a) the weak bases greatly slow (x 3) the rate of excretion (washout rate) of digoxin but do not alter that of ouabain, (b) the weak bases increase the accumulation of digoxin (and digitoxin) but may decrease that of ouabain. The site of action of the weak bases on glycoside action was not known, it could be at the internalisation or at the lysosomal step of the process. These possibilities were tested by measuring the rate of loss of glycoside from the plasma membrane (internalisation) and the rate of loss of glycoside from whole cells (washout rate), in the presence and absence of 100 μ M chloroquine.

The results are shown in Tables 2.6 and 2.7.

Table 2.6. Effect of chloroquine on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.7 ± 0.3	4.8 ± 0.4
100 μM chloroquine	9.5 ± 0.5 ^{n.s.}	4.7 ± 0.4 ^{n.s.}

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 12 (controls) and n = 14 (chloroquine), with data from 2 separate experiments. For cell rate loss, n = 14 (controls) and n = 16 (chloroquine), with data from 2 separate experiments. n.s. = not significantly different from controls.

Table 2.7. Effect of chloroquine on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.3 ± 0.3	9.2 ± 0.4
100 μM chloroquine	9.3 ± 0.5 ^{n.s.}	6.3 ± 0.4 [*]

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 18 (chloroquine), with data from 2 separate experiments. For cell rate loss, n = 20 (controls) and n = 20 (chloroquine), with data from 2 separate experiments. n.s. = not significantly different from controls.

* = P < 0.01. Significantly different from control values.

The results in Tables 2.6 and 2.7 show that chloroquine does not significantly affect the rate of loss of ouabain or digoxin from the plasma membrane of HeLa cells. These results strongly support the idea that, the increase in accumulation of digoxin in HeLa cells caused by weak bases is not due to action of the weak bases on the initial internalisation step of digoxin. Al Gharably (1985) has presented evidence which shows that weak bases increase the amount of digoxin in the lysosomal fraction of HeLa cells from that found in controls. Taken together, the results presented above suggest that the site of action of weak bases on the handling of digoxin by HeLa cells, takes place at the lysosomal step of the process and not at the initial internalisation step. The above results also show that chloroquine appears to have no effect on the internalisation or washout rate of ouabain.

Chlorpromazine

This drug has been reported to alter the fluidity of the plasma membrane (Clausen, Harving and Dahl-Hansen, 1973) and has been shown by Lamb and Ogden (1982) to influence the reappearance of fresh sodium pump sites in HeLa cells following acute glycoside block. The possible effects of chlorpromazine on the rate of loss of ouabain and digoxin from HeLa plasma membranes and HeLa cells, were investigated.

The results are shown in Tables 2.8 and 2.9.

Table 2.8. Effect of chlorpromazine on the rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	8.9 ± 0.4	3.8 ± 0.5
20 μM chlorpromazine	8.6 ± 0.5 n.s.	3.6 ± 0.6 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (chlorpromazine), with data from 2 separate experiments. For cell rate loss, n = 20 (controls) and n = 20 (chlorpromazine), with data from 2 separate experiments. n.s. = not significantly different from controls.

Table 2.9. Effect of chlorpromazine on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.4 ± 0.5	9.8 ± 0.3
20 μM chlorpromazine	9.8 ± 0.6 n.s.	9.5 ± 0.6 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 17 (controls) and n = 15 (chlorpromazine), data from 2 separate experiments. For cell rate loss, n = 16 (controls) and n = 17 (chlorpromazine), data from 2 separate experiments. n.s. = not significantly different from controls.

The results in Tables 2.8 and 2.9 show that 2 μM chlorpromazine has no effect on either rate of loss of glycoside from HeLa cells or rate of loss of glycoside from HeLa plasma membranes. In other experiments (data not shown) up to 100 μM chlorpromazine was used. This concentration of chlorpromazine caused a rapid loss of glycoside from HeLa plasma membranes and HeLa cells. This concentration of chlorpromazine also caused cell death (indicated by a decrease in cell protein over the course of the experiments) and general cell fragility. In summary therefore, too high a concentration of chlorpromazine causes cell fragility and eventually cell death, probably through puncturing of the plasma membrane.

Cycloheximide

Lamb and Ogden (1982) and Lamb and Aiton (1984) have shown that the protein synthesis inhibitor cycloheximide causes about a 2% hr^{-1} decrease of pump site numbers and causes the complete abolition of the serum stimulated increase in sodium pump sites. The possible effects of cycloheximide on rate of loss of glycoside from HeLa cells and HeLa plasma membranes, were investigated.

The results are shown in Tables 2.10 and 2.11.

Table 2.10 Effect of cycloheximide on the rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.5 ± 0.5	4.4 ± 0.5
100 µg/ml cycloheximide	9.3 ± 0.4 n.s.	4.2 ± 0.3 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (cycloheximide), data from 2 separate experiments. For cell rate loss, n = 10 (controls) and n = 10 (cycloheximide), data from 1 experiment. ns = not significantly different from controls.

Table 2.11. Effect of cycloheximide on the rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.5 ± 0.4	9.4 ± 0.5
100 µg/ml cycloheximide	9.4 ± 0.3 n.s.	8.0 ± 0.4 **

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (cycloheximide), data from 2 separate experiments. For cell rate loss, n = 10 (controls) and n = 10 (cycloheximide), data from 1 experiments. n.s. = not significantly different from controls.

** P < 0.05. Significantly different from controls.

The results in Tables 2.10 and 2.11 show that cycloheximide does not affect the rates of glycoside loss from HeLa plasma membranes or the rate of loss of ouabain from HeLa cells but slightly slows the rate of loss of digoxin from HeLa cells. A possible reason for the latter observation, put forward by Griffiths et al. (1983), is that cycloheximide reduces the supply of lysosomal enzymes - the lysosomal handling step being the rate limiting step for digoxin excretion, but not for ouabain. This explanation is supported by the fact that in the presence of chloroquine, (which inhibits lysosomal activity), cycloheximide has no effect (Griffiths et al., 1983). The results in Tables 2.10 and 2.11 also suggest that protein synthesis is not required for internalisation of ouabain and digoxin, but in the presence of cycloheximide, sodium pump sites decrease at a rate of $2\% \cdot \text{hr}^{-1}$ (Lamb and Ogden, 1982). The results presented above however, suggest that this decrease in pump sites is not due to changes in the rate of receptor-mediated endocytosis of cardiac glycosides, but due to changes in the insertion rate of new pump sites in the plasma membrane. The latter process being dependent on protein synthesis, must also be independent of internalisation or excretion of glycoside.

Monodansylcadaverine

Receptor-mediated endocytosis of many ligands is inhibited by the reagent monodansylcadaverine and other related alkylamines. Several studies have shown that there is a striking correlation between the ability of a range of compounds to inhibit receptor: ligand internalisation and their ability to inhibit the enzyme transglutaminase (Davies, Davies, Levitzki, Maxfield, Willingham and Pastan, 1980; Levitzki, Willingham and Pastan, 1980; Baldwin, Prince, Marshall, Davies and Olefsky, 1980). Levitzki et al. (1980) have observed that inhibitors of transglutaminase block the clustering of ligand:receptor complexes in clathrin-coated pits - part of the endocytotic pathway. Transglutaminase catalyses the formation of E-(γ -glutamyl)-lysine between protein molecules, and also catalyses the coupling of amines and diamines to the γ -carboxyl residue of glutamine. The proposed mechanism is that transglutaminase is a plasma membrane enzyme that may crosslink receptors in the area of coated pits, thereby facilitating aggregation and internalisation. The possible effects of monodansylcadaverine on the rate of loss of ouabain and digoxin from HeLa plasma membranes and HeLa cells, were investigated. Various concentrations of monodansylcadaverine were tried.

The results are shown in Tables 2.12 and 2.13.

Table 2.12. Effect of monodansylcadaverine on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	8.5 ± 0.5	4.9 ± 0.2
50 μM monodansylcadaverine	8.1 ± 0.5 n.s.	4.9 ± 0.3 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (monodansylcadaverine), data from 2 separate experiments. For cell rate loss, n = 20 (controls) and n = 20 (monodansylcadaverine), data from 2 separate experiments. n.s. = not significantly different from controls.

Table 2.13. Effect of monodansylcadaverine on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.3 ± 0.7	9.4 ± 0.4
50 μM monodansylcadaverine	9.0 ± 0.5 n.s.	9.2 ± 0.4 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 10 (controls) and n = 10 (monodansylcadaverine), data from 1 experiment. For cell rate loss, n = 10 (controls) and n = 10 (monodansylcadaverine), data from 1 experiment. n.s. = not significantly different from controls.

The results in Tables 2.12 and 2.13 show that 50 μM monodansylcadaverine does not affect the rate of loss of glycoside from HeLa plasma membranes and HeLa cells. In other experiments, (data not shown) the rate of loss of ouabain from HeLa plasma membranes and HeLa cells was unaffected by the presence of 100 μM monodansylcadaverine. These results may suggest that receptor-mediated endocytosis of cardiac glycosides is not dependent on the enzyme transglutaminase, however further information such as: the degree of inhibition of transglutaminase, the levels of this enzyme in HeLa cells and if cardiac glycosides cluster in clathrin-coated pits, is required before additional conclusions on the role of transglutaminase in cardiac glycoside internalisation in HeLa cells can be made.

Calcium

Calcium (Ca^{2+}) has many diverse cellular effects including a key role as a second messenger. Recent studies have shown that Ca plays a role in the process of receptor-mediated endocytosis of several different ligands (Korc, Matrisian and Magun, 1984; Sundan, Sandvig and Olsnes, 1984; Sandvig and Olsnes, 1982; Fitzgerald, Morris and Saelinger, 1982; Tupper and Bodine, 1983). The possible effects of Ca on the rate of loss of ouabain and digoxin from HeLa plasma membranes and HeLa cells were investigated in 3 ways: (a) measuring rates of loss of glycoside from HeLa plasma membranes and HeLa cells in Ca-free BME containing 2 mM EGTA, (b) measuring rates of loss of glycoside from HeLa plasma membranes and HeLa cells in Ca-free BME containing 2 mM EGTA and 10 μM A23187, thus depleting

cellular Ca, (c) measuring rates of loss of glycoside from HeLa plasma membranes and HeLa cells in normal BME in the presence of 10 μM trifluoroperizene (TFP). In all cases, cell protein was carefully monitored in order to assess cell death. In no instance was cell protein depleted by more than 5%. The results are shown in Tables 2.14, 2.15, 2.16, 2.17, 2.18 and 2.19.

Table 2.14. Effect of reduced external Ca on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	8.8 \pm 0.4	4.8 \pm 0.3
Ca-free media + 2mM EGTA	8.5 \pm 0.6 n.s.	4.6 \pm 0.5 n.s.

Results are expressed as % loss.hr⁻¹ \pm SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (Ca-free media + 2 mM EGTA), data from 2 separate experiments. For cell rate loss, n = 20 (controls) and n = 20 Ca-free media + 2 mM EGTA), data from 2 separate experiments. n.s. = not significantly different from controls.

Table 2.15. Effect of reduced external Ca on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	10.3 ± 0.9	9.8 ± 0.4
Ca-free media + 2 mM EGTA	10.0 ± 0.6 n.s.	9.7 ± 0.4 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 10 (controls) and n = 9 (Ca-free media + 2 mM EGTA), data from 1 experiment. For cell rate loss, n = 10 (controls) and n = 10 (Ca-free media + 2 mM EGTA), data from 1 experiment. n.s. = not significantly different from controls.

Table 2.16. Effect of reduced internal Ca on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	8.8 ± 0.4	4.8 ± 0.3
Ca-free media + 2 mM EGTA + 10 μM A23187	8.5 ± 0.6 n.s.	4.6 ± 0.6 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 10 (controls) and n = 10 (Ca-free media + 2 mM EGTA + 10 μM A23187), data from 1 experiment. For cell rate loss, n = 10 (controls) and n = 9 (Ca-free media + 2 mM EGTA + 10 μM A23187), data from 1 experiment. n.s. = not significantly different from controls.

Table 2.17. Effect of reduced internal Ca on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.8 ± 0.5	9.6 ± 0.4
Ca-free media + 2 mM EGTA + 10 μM A23187	9.7 ± 0.4 n.s.	9.6 ± 0.5 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 10 (controls) and n = 10 (Ca-free media + 2 mM EGTA + 10 μM A23187), data from 1 experiment. For cell rate loss, n = 10 (controls) and n = 9 (Ca-free media + 2 mM EGTA + 10 μM A23187), data from 1 experiment. n.s. = not significantly different from controls.

Table 2.18. Effect of TFP on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	10.3 ± 0.4	3.3 ± 0.5
Normal media + 10 μM TFP	10.5 ± 0.4 n.s.	3.6 ± 0.5 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 25 (controls) and n = 28 (10 μM TFP), data from 3 separate experiments. For cell rate loss, n = 29 (controls) and n = 28 (10 μM TFP), data from 3 separate experiments. n.s. = not significantly different from controls.

Table 2.19. Effect of TFP on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.4 ± 0.4	9.4 ± 0.3
Normal media + 10 µM TFP	9.2 ± 0.5 n.s.	9.3 ± 0.2 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 10 (controls) and n = 10 (normal media + 10 µM TFP), data from 1 experiment. For cell rate loss, n = 10 (controls) and n = 10 (normal media + 10 µM TFP), data from 1 experiment.

n.s. = not significantly different from controls.

Calcium has been reported as being implicated in the process of receptor-mediated endocytosis of several different ligands (Korc et al., 1984; Sundan et al., 1982; Tupper and Bodine, 1983).

It has also been reported that the enzyme transglutaminase, an enzyme implicated in receptor-mediated endocytosis, is Ca-dependent (Levitzki et al., 1980).

Whether the involvement of Ca in receptor-mediated endocytosis of the ligands reported above is through transglutaminase or by another mechanism is not clear. The results in Tables 2.14, 2.15, 2.16, 2.17, 2.18 and 2.19 show that the rates of loss of glycoside from HeLa plasma membranes and HeLa cells is unaffected by any of the different 'calcium conditions' above.

Monensin

Weak bases such as chloroquine and ammonium chloride have been reported as affecting receptor-mediated endocytosis of many ligands by: (a) blocking the internalisation step (Sando et al., 1979; Fitzgerald et al., 1979) (b) the lysosomal processing step (Goldstein et al., 1979; Helenius et al., 1980). The results presented above suggest that weak bases do not affect the internalisation step of ouabain or digoxin, but affect the lysosomal processing step which slows the excretion rate of digoxin but not that of ouabain. (The ouabain excretion rate is not slowed because the rate determining step is the diffusion rate of ouabain out of the cell). Monensin is an ionophore that collapses proton gradients by the electroneutral exchange of a proton for a monovalent cation (preferably sodium) across a membrane (Pressman, 1976). This drug, although unrelated to lysosomotropic weak bases, should function in a similar way by dissipating the acidic environment of lysosomes. The effect of monensin, on rates of loss of glycoside from HeLa plasma membrane and HeLa cells, are shown below in Tables 2.20 and 2.21.

Table 2.20. Effect of monensin on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	8.9 ± 0.6	4.0 ± 0.3
50 μM monensin	8.6 ± 0.7 ^{n.s.}	4.2 ± 0.6 ^{n.s.}

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (monensin), data from 2 separate experiments. For cell rate loss, n = 19 (controls) and n = 20 (monensin), data from 2 separate experiments. n.s. = not significantly different from controls.

Table 2.21. Effect of monensin on rate of loss of digoxin from HeLa plasma membranes and HeLa cells

Condition	Rate loss	
	plasma membrane	cell
Control	9.4 ± 0.6	9.9 ± 0.4
50 μM monensin	9.6 ± 0.2 ^{n.s.}	6.7 ± 0.4 [*]

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (monensin), data from 2 separate experiments. For cell rate loss, n = 30 (controls) and n = 30 (monensin), data from 3 separate experiments. n.s. = not significantly different from controls.

* P < 0.01. Significantly different from controls.

The results in Tables 2.20 and 2.21 show that monensin does not significantly affect the rate of loss of ouabain or digoxin from the plasma membrane of HeLa cells. Monensin has no effect on the excretion rate of ouabain, but slows the excretion rate of digoxin from HeLa cells. These results are qualitatively identical to those for the effect of weak bases on glycoside handling by HeLa cells and support the model that weak bases and the proton ionophore monensin inhibit lysosomal function by dissipating the acidic environment of lysosomes. It is worth emphasising that this is achieved by different chemical mechanisms: weak bases may accumulate in the lysosomes as non-ionised molecules, while monensin causes equilibration of protons across the lysosome.

In all the above experiments, 50 μM monensin did not reduce cell ATP levels by more than 5%.

Cytochalasin B and nocodazole

A number of studies have shown that cytoskeletal-disrupting agents inhibit receptor-mediated endocytosis and exocytosis of several ligands (White and Hines, 1984; Kull and Cuatrecasas, 1981; Thyberg and Stenseth, 1981). The cytoskeleton may be important in the transport of receptosomes inside the cell (discussed later). The possible effect of the cytoskeletal-disrupting agents cytochalasin B and nocodazole, on the rates of glycoside loss from HeLa plasma membranes and HeLa cells were investigated. The results are shown in Tables 2.22 and 2.23.

Table 2.22. Effect of cytochalasin B on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	8.8 ± 0.4	4.6 ± 0.4
10 µg/ml cytochalasin B	8.6 ± 0.7 n.s.	5.1 ± 0.6 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 18 (controls) and n = 19 (cytochalasin B), data from 2 different experiments. For cell rate loss, n = 20 (controls) and n = 18 (cytochalasin B), data from 1 experiment. n.s. = not significantly different from controls.

Table 2.23. Effect of cytochalasin B on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.6 ± 0.3	9.2 ± 0.4
10 µg/ml cytochalasin B	9.9 ± 0.6 n.s.	8.9 ± 0.4 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 10 (controls) and n = 10 (cytochalasin B), data from 1 experiment. For cell rate loss, n = 10 (controls) and n = 10 (cytochalasin B), data from 1 experiment. n.s. = not significantly different from controls.

Table 2.24. Effect of nocodazole on rate of loss of ouabain from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.8 ± 0.6	4.6 ± 0.4
10 µg/ml nocodazole	10.1 ± 0.7 n.s.	4.8 ± 0.4 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 18 (nocodazole), data from 2 separate experiments. For cell rate loss, n = 20 (controls) and n = 20 (nocodazole), data from 2 separate experiments. n.s. = not significantly different from controls.

Table 2.25. Effect of nocodazole on rate of loss of digoxin from HeLa plasma membranes and HeLa cells.

Condition	Rate loss	
	plasma membrane	cell
Control	9.6 ± 0.2	8.8 ± 0.4
10 µg/ml nocodazole	9.8 ± 0.3 n.s.	8.6 ± 0.2 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM. For plasma membrane rate loss, n = 20 (controls) and n = 20 (nocodazole), data from 2 separate experiments. For cell rate loss n = 20 (controls) and n = 15 (nocodazole), data from 2 separate experiments. n.s. = not significantly different from controls.

The results in Tables 2.22, 2.23, 2.24 and 2.25 show that the cytoskeletal-disrupting agents do not affect the rate of loss of glycoside from the HeLa plasma membrane or the rate of loss of glycoside from HeLa cells.

MODULATION OF SODIUM PUMP NUMBERS IN HeLa PLASMA MEMBRANES

It has been shown that if HeLa cells and other cultured cells are stressed by either chronic growth in low K medium (0.2 mM - 0.4 mM) or chronic growth in sublethal concentrations of cardiac glycosides, then the cells respond by increasing their ouabain binding capacity and transport capacity (Lamb and McCall, 1972; Boardman, Lamb, and McCall, 1972). These observations have been confirmed by Cook, Will, Proctor and Brake (1976), Pollack, Tate and Cook (1981a) and Pollack, Tate and Cook (1981b). The increases in ouabain binding found in such chronic stress experiments above, are paralleled by increases in ouabain-sensitive V_{max} for ^{86}Rb uptake, increases in K-sensitive phosphorylation of isolated membranes and increases in Na/K ATPase activity (Boardman et al., 1972; Pollack et al., 1981a). Furthermore these responses appear specific in that 5'nucleotidase and K-insensitive phosphorylation are not increased in isolated membranes from stressed cells (Pollack et al., 1981a). All the results above are consistent with the idea that in HeLa cells grown chronically in low K media or in sublethal concentrations of glycoside, the amount of Na/K ATPase is increased.

The aim of the following series of experiments was to try to investigate the nature of the up-regulation of HeLa Na/K ATPase by measuring the internalisation rate of the ligand ouabain in stressed conditions. The serum stimulated increase in ouabain

binding sites in HeLa cells was also investigated. The experimental procedures were as follows:- HeLa cells were grown in microcarrier culture for 4 days, then incubated for a further 24 - 48 hours in 'stressed conditions' (i.e. low K media, 10^{-8} M glycoside, high K media etc.), or normal medium. At the end of this period, the cells on beads were labelled with [3 H]-ouabain, the non-specific binding washed off and the cells returned to the appropriate medium.

The cell suspension was then immediately divided into a control flask and experimental flask(s). The experimental flask(s) contained the 'stressed media condition' being tested, the control flask normal BME. At various time intervals, the ouabain bound to prepared plasma membranes and the ouabain associated with cells, were determined. The rates of loss of ouabain from HeLa plasma membranes and from HeLa cells, were determined by expressing the results as a single exponential and obtaining the rate loss from the slope of the fitted line (log plot) computed by the Glim package.

In all the experiments described below, cell protein was carefully monitored over the course of any experiment, in order to assess cell death. No chronic stress condition reduced cell protein by more than 5% over the time-course of any experiment. As before, experimental data and control data were fitted to a straight line (log plot) by the Glim package, various error structures examined and a t-test used to test for possible significance of experimental line plot from control line plot. Furthermore the control and experimental lines of all experimental conditions below, were subjected to a test of linearity. Data was fitted

to a linear (log plot) and cubic model, and an f-test used to test goodness of fit to the linear model. All experimental conditions (and controls) fitted a linear model (log plot), consistent with a single exponential decay of glycoside loss.

Serum

It has been shown by Aiton and Lamb (1984) that HeLa cells grown for 24 hours or more in medium containing high serum concentrations (20% (v/v)), increase their sodium pump sites by up to 50% compared to HeLa cells in 1% (v/v) serum. In experiments following the adaptation of HeLa cells in high serum conditions, there was found to be a lag period of 6 hours, followed by a steady increase in pump site numbers until a new steady state is achieved.

Serum stimulation of pump site numbers was abolished by cycloheximide and actinomycin D.

The nature of this serum stimulation was examined by measuring the rate of loss of ouabain from HeLa plasma membranes in medium containing high (20%) and low (1%) serum following 24 hours incubation in these media. The results are shown in Table 2.26.

Table 2.26. Effect of 1% and 20% serum on rate of loss of ouabain from HeLa plasma membranes

Condition	Rate loss
Control	9.3 ± 0.4
Medium containing 1% serum	9.2 ± 0.4 n.s.
Medium containing 20% serum	9.2 ± 0.3 n.s.

Results are expressed as % loss.hr⁻¹ ± SEM, n = 18 (controls), n = 19 (1% serum) and n = 19 (20% serum). Data from 2 separate experiments. n.s. = not significantly different from control.

The results in Table 2.26 show that the rate of loss of ouabain from HeLa plasma membranes was the same in high (20%) and low (1%) serum concentrations. This result suggests that the increase in pump site numbers in high serum is due to an increase in the biosynthetic rate or insertion rate of pumps and not due to changes in the turnover (or internalisation rate).

Low K medium

Lamb and McCall (1972), Boardman et al., (1972), Cook et al. (1976) and Pollack et al. (1981a, 1981b) have shown that if HeLa and other cultured cells are grown in low K medium (0.2 - 0.4 mM K) then the number of sodium pump sites increases in the membrane over a period of hours until a new steady state is reached. Aiton and Lamb (1984), Boardman, Huett, Lamb, Newton and Polson (1974) have shown that the increase in sodium pump numbers during

low K stress is modulated by other factors: $[Na]_i$, $[Li]_o$, $[serum]_o$ which can all interact with the low K response. In the low K experiments below, $[K]_o$ was kept at 0.2 - 0.4 mM, and the $[serum]_o$ was 2% (v/v) a value which ensured a large increase of sodium pump numbers in low K medium. Higher concentrations of serum, used in conjunction with low K medium, were found to diminish the low K stimulated increase in sodium pump numbers (Aiton and Lamb, 1984).

Rate of loss of ouabain from HeLa plasma membranes during low K stress (medium containing 0.2 - 0.4 mM K, 2% serum) following a 24 hour incubation in low K medium, is shown in Table 2.27. The results are also plotted in Figure 2c.

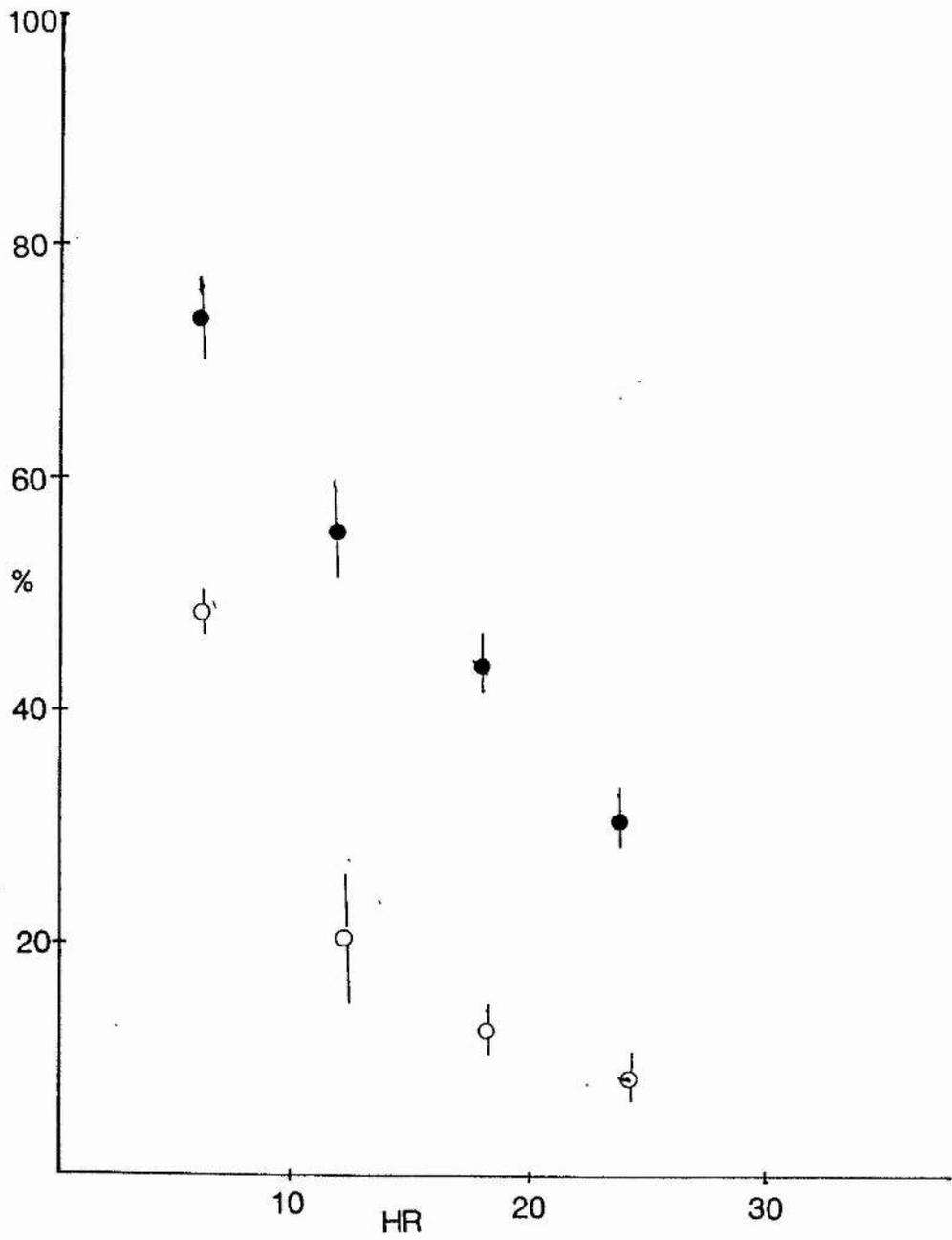
Table 2.27. Rate of loss of ouabain from HeLa plasma membranes during low K stress.

Condition	Rate loss
Control	9.8 ± 0.7
Low K stress	$3.9 \pm 0.5^*$

Results are expressed as $\% \text{ loss} \cdot \text{hr}^{-1} \pm \text{SEM}$, $n = 28$ (controls) and $n = 25$ (low K stress). Data from 3 separate experiments.

* $P < 0.01$. Significantly different from controls.

Figure 2c: Rate of loss of ouabain from HeLa plasma membranes following 24 hours incubation in low K medium. Ordinate: % of amount of ouabain bound initially. HeLa cells in a microcarrier culture were incubated in low K medium (0.2-0.4mM K, 2% serum) for 24 hours. At the end of this incubation period, the cells on beads were labelled with [³H]-ouabain, the non-specific binding washed off, the culture divided and then the cells returned to low K medium or normal medium (5 mM K, 10% serum). At various time intervals, the ouabain bound to the plasma membrane was determined for cells in low K medium (●) and normal medium (○). Each data point is the mean±SEM, n=6-9. Data from 3 separate experiments.



The results (data not shown) confirmed that an induction of pump site numbers occurred, due to 24 hour incubation in low K medium. (144% of control values). Figure 2c shows that the rate of loss of ouabain is less in low K medium than in normal medium. The results in Table 2.27 give the measured rate losses in normal and low K medium. The results show that the rate of loss of ouabain from HeLa plasma membranes is slower during low K stress than in normal medium. These results are very similar to the findings of Pollack et al. (1981b) in which the internalisation rate of the native sodium pump, was measured during low K stress using a density labelling technique. A model was proposed by these authors, to account for the observed results: In the steady state the level of sodium pumps is described by $[E] = \frac{k_{syn}}{k_{tur}}$, where $[E]$ is the sodium pump concentration, k_{syn} is the synthetic rate and k_{tur} the turnover rate of fresh pumps into the membrane. The increase in sodium pump numbers can be accounted for by a decrease in the turnover rate with no change in the synthetic rate. When the number of sodium pumps turned over again matches the number of sodium pumps being synthesised, then a new steady state level is achieved. It is clear that such a model can also account for the results observed in Table 2.27 and Figure 2c in which the internalisation rate of the ligand ouabain is measured. These results presented above again suggest that, even in low K stress conditions, the internalisation rate of the native sodium pump is the same as that when the ligand ouabain is bound, with ouabain and the sodium pump being intrinsically associated throughout the internalisation process.

In another experiment, the rate of loss of ouabain from HeLa plasma membranes was measured immediately following transfer of HeLa cells into low K medium (0.2 - 0.4 mM K, 2% serum). The results are shown in Table 2.28 and Figure 2d.

Table 2.28. Rate of loss of ouabain from HeLa plasma membranes immediately following application of a low K stress.

Condition	Rate loss
Control	8.8 ± 0.5
Low K stress	4.6 ± 0.5*

Results are expressed as % loss.hr⁻¹ ± SEM, n = 20 (controls) and n = 18 (low K stress). Data from 2 separate experiments.

*P < 0.01. Significantly different from controls.

The results in Figure 2d show the change to low K stress of the loss of ouabain from HeLa plasma membranes. The measured rate losses of ouabain from HeLa plasma membranes, in normal (control) and low K medium, are given in Table 2.28. The results suggest an immediate change to the low K stress (i.e. no lag period). The response of HeLa cells, incubated for 42 hours in low K media and then returned to normal medium, was examined. The results are shown in Figure 2e and Table 2.29.

Figure 2d: Rate of loss of ouabain from HeLa plasma membranes immediately following application of a low K stress Ordinate: % of amount of ouabain bound initially. Abscissa: time (hours). HeLa cells in a microcarrier culture were labelled with [³H]-ouabain, the non-specific binding washed off, the culture divided and the cells returned to low K medium (0.2-0.4 mM K, 2% serum) or normal medium (5 mM K, 10% serum). At various time intervals, the ouabain bound to the plasma membranes was determined for cells in low K medium (●) and normal medium (○). Each data point is the mean ± SEM, n=4-6. Data from 2 separate experiments.

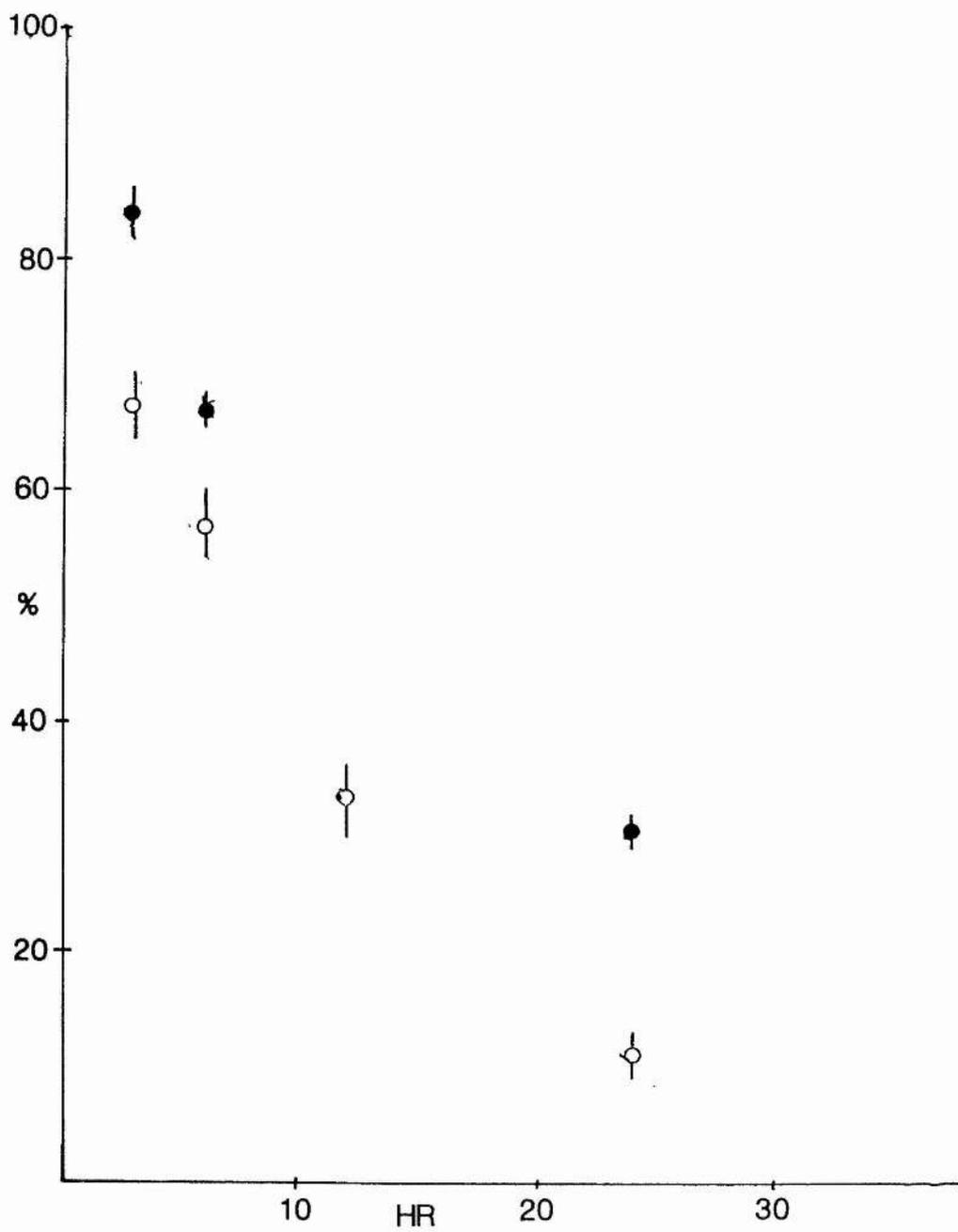


Figure 2e: Rate of loss of ouabain from HeLa plasma membranes in normal medium following 42 hours incubation in low K medium. Ordinate: % of amount of ouabain bound initially. Abscissa: time (hours). HeLa cells in a microcarrier culture were divided and then incubated in normal medium (controls) or low K medium for 42 hours. At the end of this incubation period, the cells on beads were labelled with [³H]-ouabain, the non-specific binding washed off and then each group both returned to normal medium. At various time intervals, the ouabain bound to the plasma membrane was determined for cells pre-incubated in low K medium (●) and cells pre-incubated in normal medium (○). Each data point is the mean[±]SEM, n=4-6. Data from 2 separate experiments.

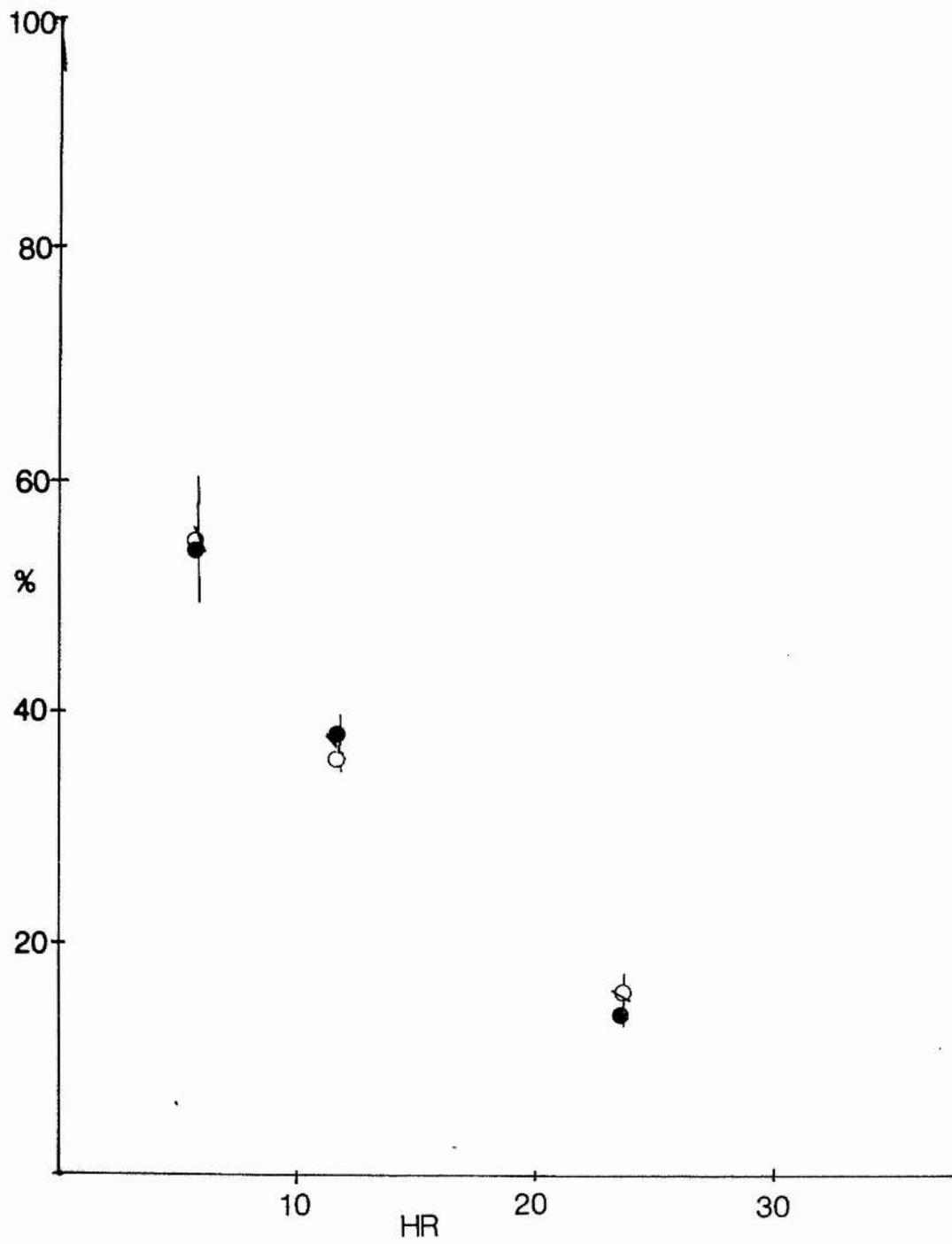


Table 2.29. Rate of loss of ouabain from HeLa plasma membranes in normal (5 K) medium following 42 hours incubation in low K medium.

Condition	Rate loss
Control	9.2 ± 0.4
42 hours in low K medium then returned to normal medium	9.1 ± 0.3 ^{n.s.}

Results are expressed as % loss.hr⁻¹ ± SEM, n = 20 (controls) and

n = 19 (low K incubation, then returned to normal medium).

Data from 2 separate experiments. n.s. = not significantly different from control.

The results in Figure 2e indicate no difference between the control and experimental conditions, on return to normal growth medium. This is consistent with the idea that the rate of loss of ouabain from HeLa plasma membranes of cells pre-incubated in low K medium, immediately returns to the control rate when placed back in normal medium. Pollack et al. (1981b) have shown that an elevated number of sodium pump sites induced by incubation in low K medium, promptly returns to control levels, when cells in low K are returned to normal medium. These results also suggest that no lag time exists for the return of sodium pump site numbers to control levels. Taken together, the results in Table 2.29, Figure 2e and the results of Pollack et al. (1981b) presented above, are consistent with a model, whereby when a low K stress is removed from HeLa cells, the cells respond immediately, by

altering the turnover or internalisation rate of sodium pumps. The modulation of sodium pump sites in HeLa cells during low K stress is discussed more fully elsewhere.

As mentioned above, modulation of sodium pump sites in HeLa cells is complex, with perhaps several interactive factors involved at any one time. Aiton and Lamb (1984) have shown that the increase in sodium pump numbers observed during low K stress is diminished by increased serum concentrations. This finding was further investigated, by measuring the rate of loss of ouabain from HeLa plasma membranes in low K media containing different serum concentrations. HeLa cells growing in normal medium, were labelled with [³H]-ouabain, the non-specific binding washed off, the cell suspension divided and incubated in: normal medium (5 mM K, 10% serum); 0.2 - 0.4 mM K, 2% serum; 0.2 - 0.4 mM K, 10% serum; or 0.2 - 0.4 mM K 20% serum. The rate of loss of ouabain from HeLa plasma membranes was determined for each media condition. The results are shown in Table 2.30 and Figure 2f.

Table 2.30. Rate of loss of ouabain from HeLa plasma membranes in controls and low K media containing different serum concentrations.

Condition	Rate loss
Controls	9.1 ± 0.3
Low K medium + 2% serum	4.6 ± 0.4*
Low K medium + 10% serum	9.3 ± 0.5 n.s.
Low K medium + 20% serum	9.8 ± 0.7 n.s.

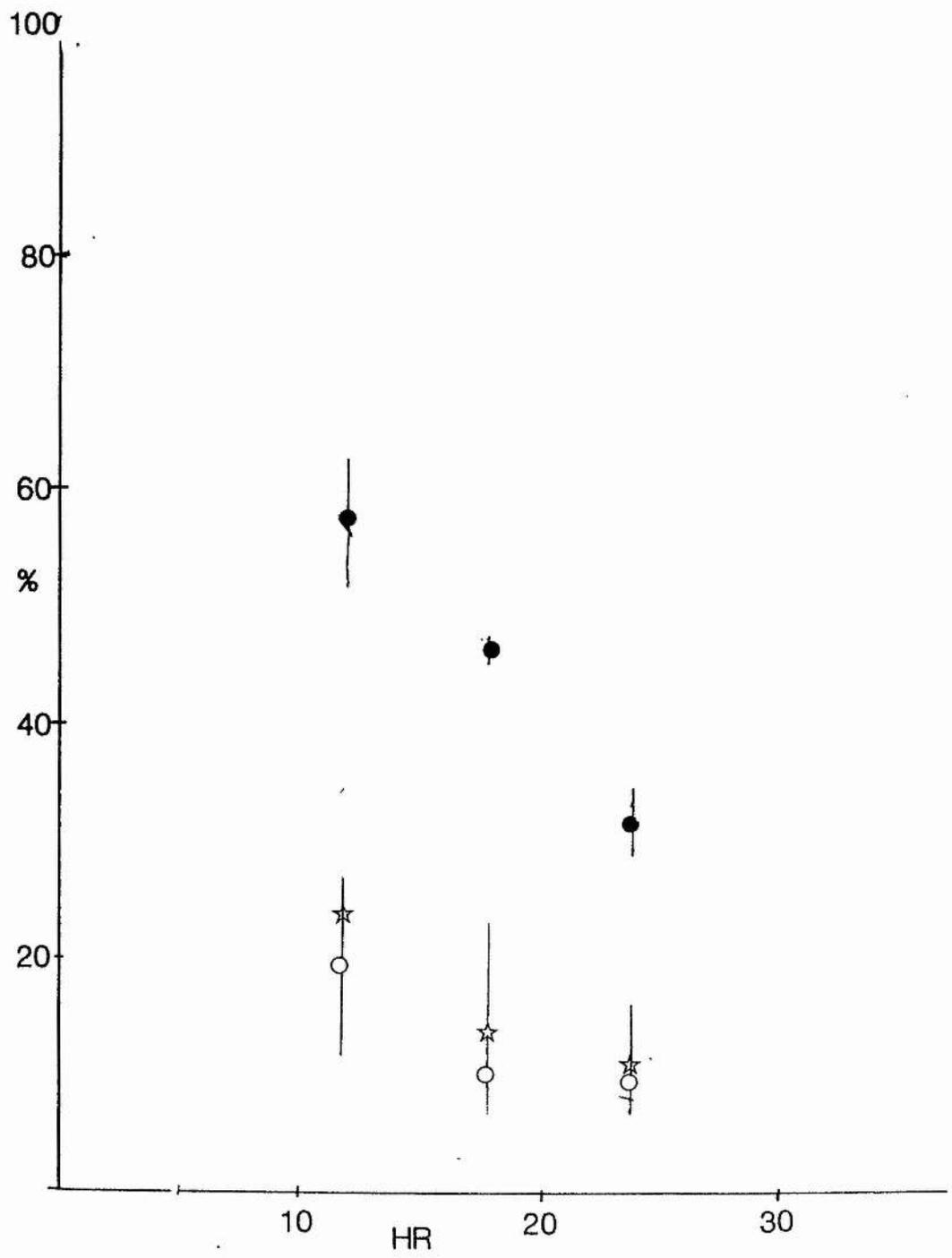
Results are expressed $\% \text{ loss} \cdot \text{hr}^{-1} \pm \text{SEM}$, $n = 10$ (controls), $n = 10$ (low K medium + 2% serum), $n = 10$ (low K medium + 10% serum) and $n = 10$ (low K medium + 20% serum). Data from 1 experiment.

n.s. = not significantly different from controls.

* $P < 0.01$. Significantly different from controls.

The results in Table 2.30 and Figure 2f show that increased concentrations of serum (10%, 20%) in low K medium, causes no change in the rate of loss of ouabain from HeLa plasma membranes from that of controls (normal medium 10% serum), while a 2% serum concentration of serum in low K medium, causes a reduction in the rate of loss of ouabain from HeLa plasma membranes (also found previously, above). These results can account for the findings of Aiton and Lamb (1984). In their studies, they found that increased serum concentrations diminishes the increase in sodium pump numbers induced by low K stress. The increased sodium pump numbers during low K stress can be explained by a reduction in the internalisation rate of the sodium pump (see above), then if increased serum concentrations causes no such reduction in internalisation rate of the sodium pump (as is suggested by the data in Table 2.30), no increase in sodium pump numbers will be observed (assuming of course that the insertion rate of fresh pumps in the plasma membrane remains constant throughout). These ideas are discussed more fully elsewhere.

Figure 2f: Rate of loss of ouabain from HeLa plasma membranes in low K media containing 2%, 10% and 20% serum. Ordinate: % of amount of ouabain bound initially. Abscissa: time (hours). HeLa cells in a microcarrier culture were labelled with [³H]-ouabain, the non-specific binding washed off, the culture divided and then returned to (a) low K medium containing 2% serum (●), (b) low K medium containing 10% serum (☆) or (c) low K medium containing 20% serum (○). At various time intervals, the ouabain bound to the plasma membrane was determined for cells in the 3 conditions. Each data point is the mean ± SEM, n=3. Data from 1 experiment.



The excretion rate of ouabain during low K stress was investigated. HeLa cells growing in normal medium, were labelled with [³H]-ouabain, the non-specific binding washed off and the cells returned to normal growth medium. The cell suspension was divided and incubated in normal medium (control) and low K medium (containing 0.2 - 0.4 mM K, 2% serum). The rate of loss of ouabain from HeLa cells was then determined. The results are shown in Table 2.31.

Table 2.31. Rate of loss of ouabain from HeLa cells immediately following application of a low K stress.

Condition	Rate loss
Control	4.2 ± 0.1
Low K stress	4.3 ± 0.3 ^{n.s.}

Results are expressed as % loss.hr⁻¹ ± SEM, n = 20 (controls) and n = 20 (low K stress). Data from 2 separate experiments. n.s. = not significantly different from controls.

High K effects

Larkin, Brown, Goldstein and Anderson (1983) observed that depletion of intracellular potassium by incubation of cells in K-free medium, resulted in the cessation of both coated pit formation and receptor-mediated endocytosis in fibroblasts. These authors also showed that such effects were reversible on addition of KCl, restoring normal levels of $[K]_i$. It was thought possible that the cessation of receptor-mediated endocytosis and coated pit formation may perhaps be due to changes in the membrane potential rather than a response to lowered $[K]_i$. The possible effects of membrane potential on the rate of loss of ouabain from the plasma membrane, were investigated in HeLa cells. Preliminary experiments were carried out in which the membrane potential was drastically altered; high K medium was made up according to the recipe for BME medium except that sodium salts were substituted with potassium salts and vice-versa. The measured potassium and sodium concentrations of this medium were 135 mM and 12 mM respectively (2% dialysed serum was added). HeLa cells growing in normal medium were labelled with $[^3H]$ -ouabain, the non-specific binding washed off and the cells returned to normal medium. The cell suspension was divided and incubated in normal medium (control) and high K medium (135 mM K, 12 mM Na, 2% dialysed serum). The rate of loss of ouabain from HeLa plasma membranes was then determined. The results are shown in Table 2.32 and Figure 2g.

Table 2.32. Rate of loss of ouabain from HeLa plasma membranes immediately following application of a high K stress.

Condition	Rate loss
Control	9.2 ± 0.5
High K stress	5.3 ± 0.5*

Results are expressed as % loss.hr⁻¹ ± SEM, n = 20 (controls) and n = 20 (high K stress). Data from 2 separate experiments.

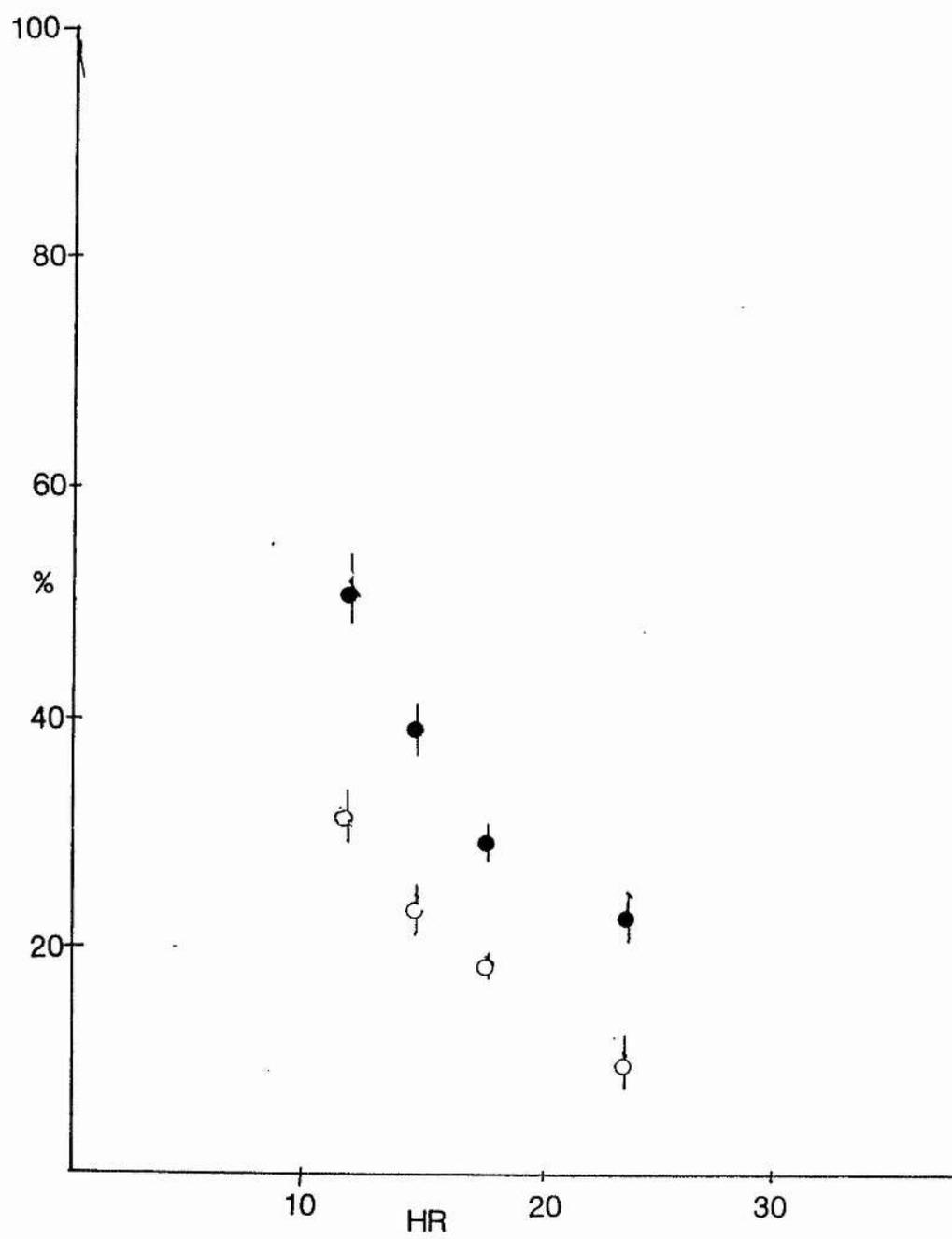
*P < 0.01 Significantly different from controls.

The results in Table 2.32 and Figure 2g show that high K medium does have a major effect on the rate of loss of ouabain from HeLa plasma membranes.

In another experiment, the effects of a range of media containing different Na and K concentrations on the internal ion concentrations were examined. The aim of this experiment was to observe whether such media conditions greatly perturbed certain parameters of cell condition - cell numbers, cell volume and internal ion concentrations. The results are shown in Table 2.33 following a 24 hr incubation of HeLa cells growing on plates in the different media conditions.

The implications of possible membrane potential effects are discussed later.

Figure 2g: Rate of loss of ouabain from HeLa plasma membranes immediately following application of a high K stress. Ordinate: % of amount of ouabain bound initially. Abscissa: time (hours). HeLa cells in a microcarrier were labelled with [³H]-ouabain, the non-specific binding washed off, the culture divided and then the cells returned to either normal medium (○) or high K medium (●). At various time intervals, the ouabain bound to the plasma membrane was determined for controls and high K incubations. Each data point is the mean ± SEM, n=4-6. Data from 2 separate experiments.



APPENDIX

Summary of results in tables 2.27-2.32

CONDITION	RATE OF LOSS OF [³ H]-OUABAIN FROM PLASMA MEMBRANE	
Control	9.8	0.7
Low K medium with 24 hrs pre-incubation in low K medium	3.9	0.5*
Control	8.8	0.5
Low K medium with no pre-incubation	4.6	0.5*
Control	9.2	0.4
Normal medium with 42 hrs pre-incubation in low K medium	9.1	0.3 ^{n.s.}
Control	9.1	0.3
Low K medium + 2% serum	4.6	0.4*
Low K medium + 10% serum	9.3	0.5 ^{n.s.}
Low K medium +20% serum	9.8	0.7 ^{n.s.}
Control	9.2	0.5*
High K medium	5.3	0.5

Results are expressed as % loss.hr⁻¹ ± SEM.

* P < 0.01 Significantly different from controls.

Table 2.33. Effect of media containing different Na and K concentrations, on various cell parameters.

Growth medium	[Na] _i ^a	[K] _i ^b	Cell numbers ^c	Cell volume ^d
140 mM Na, 5 mM K	16 ± 1	164 ± 8	0.43 ± 0.03	2111 ± 34
130 mM Na, 15 mM K	14 ± 1	137 ± 8	0.39 ± 0.01	2215 ± 102
95 mM Na, 50 mM K	15 ± 1	122 ± 6	0.31 ± 0.02	2611 ± 60
40 mM Na, 100 mM K	*	133 ± 14	0.28 ± 0.02	2539 ± 192
10 mM NA, 130 mM K	*	124 ± 12	0.20 ± 0.01	2349 ± 60

All results are the mean ± SEM, n = 3. Data from a single experiment.

a, b mM

c x 10⁻⁶ cells/plate

d μl

* not detectable in assay

The results in 2.33 show that some effects on HeLa cells do occur, with incubation in the various media conditons, but such effects may not greatly affect cell 'health'.

Chronic glycoside stress

Chronic growth of HeLa cells in sub-lethal concentrations of ouabain stresses cells with respect to their normal Na, K transport as does chronic growth of HeLa cells in low K medium. The two conditions are thought to be physiologically equivalent, although this is not proved. Pollack et al. (1981a) have presented data which shown that chronic growth of HeLa cells in sub-lethal concentrations of ouabain (2×10^{-8} M), resulted in a modest 20% induction of sodium pump site numbers in the plasma membrane, compare to a 2-fold or greater induction of sodium pump numbers by chronic growth in low K medium. The induction of sodium pump numbers in the plasma membrane by chronic glycoside stress was examined. HeLa cells were grown for 4 days in a microcarrier spinner culture and then divided into 3 flasks containing equal volumes of media. The media conditions of each flask were as follows: flask A contained BME (control flask), flask B BME + 16 nM [3 H]-ouabain and flask C BME + 16 nM ouabain (non-radioactive). Each flask was incubated for a further 48 hours, flasks A and C pulsed with [3 H]-ouabain (2×10^{-7} M) for 20 minutes and the non-specific binding washed off. The ouabain bound to prepared membranes was then determined for each flask. The results are shown in Table 2.34.

Table 2.34. Effect of chronic growth in 16 nM ouabain on sodium pump site numbers of HeLa plasma membranes.

Condition	Specific activity
Control	(A) 19.883 ± 0.581
16nM [³ H]-ouabain	(B) 12.687 ± 0.665
16nM 'cold' ouabain	(C) 4.618 ± 0.364

Results are expressed as mean ± SEM, n = 6. Data from 2 separate experiments. Activities are expressed as pmoles ouabain bound per mg protein.

Pollack et al. (1981a) have shown a 20% induction of sodium pump sites in the plasma membrane following chronic growth of HeLa cells in sub-lethal concentrations of ouabain (2×10^{-8} M). If this induction occurred above then $B + C > A$ (Table 2.34) by 20%. The results in Table 2 do not show induction, (rather a slight decrease) the conclusion being that chronic growth in 16 nM ouabain does not increase sodium pump site numbers, in the HeLa plasma membrane.

APPENDIX

The Glim package

The Generalised Linear Interactive Modelling system or Glim package of the Royal Statistical Society, was developed for the fitting of generalised linear models. The package can perform a wide range of statistical operations encompassing various error structures, in addition to its wider use as a programming language for many standard functions. For normal error structures the Glim package fits lines by the least squares method.

The glycoside binding data for all experiments was treated as follows:-

Glycoside binding data was expressed as a % of the initial value, with the zero time values set at 100%. The error structure of the data was examined and showed that the variance \propto mean². Thus taking the log of the binding data gave constant variance of the errors and linearised the plot of the exponential. Log transformation is not the same as using log link with normal errors (which assumes constant variance for original binding data). The rates of loss of glycoside from HeLa plasma membranes and HeLa cells were determined by expressing the results as a single exponential and obtaining the rate loss from the slope of the fitted line (log plot). The variances of both groups of data (control and experimental/treated) were examined in order to check

that the variances of each group were not significantly different (ie a t-test is strictly only valid for comparing 2 populations with the same variance). In all cases the variances of the 2 populations (controls and experimentals) were found not to be significantly different. Hence a t-test with pooled variance estimate was used to test for possible significant difference between control line plot and experimental line plot. All of the control and experimental line plots were subjected to a test of linearity - ie a drug or condition may be altering the pattern of glycoside release from the membrane or cell. A linear and cubic model was fitted to each group of data and an f-test used to confirm that the quadratic and cubic terms were not significant. All data fitted a linear model consistent with a single exponential decay.

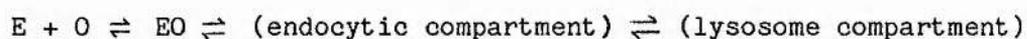
DISCUSSIONCardiac glycoside loss from HeLa plasma membranes

The results presented above have invariably used ouabain or digoxin as a label for the sodium pump in various experimental conditions. Consideration of the nature of the loss of cardiac glycoside from the HeLa cell surface is therefore of prime importance in relation to the data presented above; such considerations are discussed in depth below.

Cook and Brake (1978) have presented a model which describes the release of ouabain from cells as a result of 3 processes. These processes are: (a) dissociation of ouabain from the binding site back into the extracellular medium, $t_{1/2}$ of 5 to 8 hours; (b) ouabain internalisation, $t_{1/2}$ of 5 to 8 hours; and (c) release of internalised ouabain from the cell, $t_{1/2}$ of 50 hours. Cook and Brake determined process (a) by measuring simple dissociation of bound ouabain from either isolated membranes or with cells in which internalisation has been blocked by energy inhibitors (internalisation is dependent on a supply of ATP). The consequences of such a model occurring in HeLa cells are that drug release from the plasma membrane proceeds as a result of 2 processes - dissociation and internalisation and so cannot be treated as a single exponential process. Griffiths, Lamb and Ogden (1983) have computed the observed data for glycoside accumulation by HeLa cells, on 2 models: (a) model 1 assumed that the cardiac glycosides bind to the sodium pump on the cell surface and dissociate, i.e. no internalisation occurs, (b) model 2 assumed that cardiac glycosides bind to the sodium pump and the pump:glycoside

complex is internalised, i.e. no dissociation occurs. The glycoside accumulation data was found to closely correlate with model 2. One of the consequences of this internalisation model is that no dissociation of glycoside from the sodium pump occurs; yet in isolated membranes and in cells where internalisation is stopped (see above), cardiac glycosides have been shown to dissociate with a $t_{1/2}$ of 5 to 8 hours.

However in the intact cell, glycoside uptake by HeLa cells is a series of reactions which may be described by the following scheme:



E represents the sodium pump, O ouabain or other cardiac glycoside.

This model is probably too simplistic (i.e. more steps may exist) but what it suggests is that glycosides may be driven along this route into the cell. In other words, the effective dissociation constant (K_d) of ouabain binding to the sodium pump in the physiological (dynamic) state, is much smaller than that measured in studies with isolated membranes; thus when glycoside binds to the sodium pump of HeLa cells in the physiological state, the reaction may be effectively regarded as near irreversible.

The direct measure of the turnover (or degradation) rate by Pollack, Tate and Cook (1981b) gave a value of $12\% \text{ hr}^{-1}$, which is very similar to the value obtained for the rate of loss of glycoside from HeLa plasma membranes of $10\% \text{ hr}^{-1}$ (Table 2.2).

The consequences of this observation is that it suggests that loss of glycoside from the plasma membrane of HeLa cells is achieved solely by normal pump turnover. Furthermore, it also suggests that glycosides do not induce sodium pump internalisation; sodium pump

internalisation occurs at the same rate in the presence or absence of ligand. All of the above observations taken together (rate of loss of glycoside from the plasma membrane, the measured turnover rate of the sodium pump and the model calculations on glycoside accumulation by HeLa cells), suggest that a measure of ouabain loss from the HeLa plasma membrane essentially reflects both ouabain internalisation and sodium pump turnover; with the ligand (glycoside) and sodium pump intrinsically associated throughout the internalisation process.

An important principle arises out of the above scheme and that is internalisation is not turnover. Turnover reflects degradation of receptors with corresponding de novo synthesis of new receptors, while internalisation is the rate with which receptors leave the plasma membrane. The turnover of the asialoglycoprotein receptor has been calculated to be $t_{1/2}$ 20 hours in primary cultures of rat hepatocytes and is unaltered in the presence or absence of ligand; yet this receptor has been shown to be internalised and recycled rapidly back to the cell surface (Tanabe, Pricer and Ashwell, 1979).

Similarly, the LDL receptor is rapidly internalised ($t_{1/2}$ of several minutes) and recycled back to the plasma membrane, yet the LDL receptor turnover rate is thought to be about $t_{1/2}$ 25 hours (Goldstein, Basu, Brunschede and Brown, 1976). The internalisation rate of receptor can therefore be much more rapid than receptor turnover rate due to recycling; but the converse cannot be true, i.e. internalisation rate can never be slower than turnover rate. The fact that for the sodium pump internalisation occurs at almost the same rate as turnover, is both convenient and informative; i.e. this observation argues against receptor recycling. The above observation also simplifies

somewhat the model of glycoside uptake by HeLa cells (supporting the ideas mentioned previously), that a measure of ouabain loss from the plasma membrane is achieved solely by internalisation and that this happens as part of the turnover of the native sodium pump.

The nature of cardiac glycoside uptake in HeLa cells has been investigated by the use of protein synthesis inhibitors. Aiton and Lamb (1984) have shown that cycloheximide causes a 2% decline in sodium pump numbers in HeLa cells after a 3 hour lag. This value ($2\% \text{ hr}^{-1}$) should therefore be an indirect measure of the sodium pump turnover rate, however is not in agreement with the measured turnover rate of sodium pumps by Podlack et al. (1981b). A possible explanation of this could be that sodium pumps are turning over at this reduced rate in response to the cycloheximide condition. However, the rate of loss of glycoside from the plasma membrane (Tables 2.10 and 2.11) or internalisation rate, is unchanged from that found in controls ($10\% \text{ hr}^{-1}$). This argues against any such reduction in turnover rate because such a reduction in turnover rate would be correspondingly reflected in a reduced internalisation rate. Another idea to explain this $2\% \text{ hr}^{-1}$ reduction in sodium pump sites in the presence of cycloheximide, is that HeLa cells do not recycle receptors, rather they possess preformed stores of sodium pumps within the cell which are utilized in the presence of cycloheximide. Lamb and Ogden (1982) have shown that in recovery experiments (cells blocked with a pulse of glycoside then allowed to recover) in the presence of cycloheximide, recovery of pump function reaches 60 - 70% of controls. These results are consistent with the idea that HeLa cells possess a store of preformed sodium pumps within the cell. Stores of pumps may be verified

in an experiment which may be more difficult to carry out in practice and that is to block all the cell surface sodium pump sites, lyse the cell and attempt to specifically label any internal store of sodium pump sites.

In the present study, the measurements obtained are for the rate of loss of glycoside from HeLa plasma membranes, which has been shown (above) to reflect glycoside internalisation. Other studies have used biochemical stripping techniques to obtain a measure of the internalisation rate of ligand from the plasma membrane, (e.g. Karin and Mintz (1981) investigated binding and internalisation of transferrin using such methods). Since the internalisation of many ligands by receptor-mediated endocytosis is very rapid ($t_{1/2}$ of several minutes), binding of ligand is studied separately by inhibiting internalisation; receptor-mediated endocytosis is inhibited by either low temperature (4°C) or by the use of energy inhibitors (receptor-mediated endocytosis is ATP dependent). Internalisation rate is then measured at 37°C by separating the surface bound and cellular (internalised) pools of labelled ligand at various times. This biochemical stripping was used to measure the internalisation rate of ouabain in HeLa cells. The results in Tables 2.4 and 2.5 show that at time 0, acetic acid was effective at removing most of the surface bound ouabain while pronase did not. After 5 hours when roughly half of the ouabain should have been internalised however, the results obtained with acetic acid stripping did not reflect this - 85 - 95% of the ouabain initially bound was still releasable after 5 hours. This result does not agree with the calculated ouabain internalisation rate, but such treatment of HeLa cells with acetic acid was found

to greatly perturb ion gradients suggesting that the plasma membrane structure was being severely disrupted. Measurement of ouabain internalisation by such biochemical stripping experiments was therefore thought to be a rather severe procedure and give spurious results. A less severe treatment however may resolve the question of non-specific cellular disruption and provide a true measure of ouabain internalisation in HeLa cells; attempts to achieve this however proved elusive.

As discussed above, a measure of glycoside loss from the membrane reflects internalisation and that this occurs as part of normal sodium pump turnover. These separate measurements (glycoside internalisation and sodium pump turnover) are quantitatively very similar, which strongly suggests, but does not prove, that glycoside internalisation is equivalent to internalisation of the sodium pump itself. In order to obtain definitive proof a direct measure of the internalisation rate of the native sodium pump is required. A body of evidence has been presented in the last few years showing that in many different cell types, receptors and ligands are cointernalised in receptor-mediated uptake systems (see Introduction, Chapter 2). Furthermore, it is now thought likely that the pathway of receptor-mediated endocytosis which has been shown to exist for several well studied ligands, is also the pathway by which all 'dynamic' membrane components turnover, e.g. Hopkins and Trowbridge (1983) using monoclonal antibodies to the transferrin receptor, have shown that the receptor is internalised (at the same rate) in the absence of ligand and that it is found in endocytic vesicles. The work of Geuze, Slot, Straus, Lodish and Schwarz (1983) and Evans (1985) has provided direct biochemical

evidence for the cointernalisation of ligand and receptor. For cardiac glycosides binding to HeLa cells, the fact that the measured rates of glycoside internalisation and turnover of the sodium pump are the same is good evidence, but nevertheless indirect evidence, that sodium pumps and cardiac glycosides are cointernalised along the same pathway.

In conclusion, the binding and uptake of cardiac glycosides by HeLa cells, has been treated in the present study as a receptor-mediated endocytic process - certainly glycoside uptake by HeLa cells shares many of the features of receptor-mediated endocytosis in that: (a) only receptor-bound glycoside enters the cell, (b) glycosides are internalised along with receptors, (c) glycosides are separated from the receptors inside the cell and are found in the lysosome fraction and (d) the glycosides are released (probably by exocytosis) unmetabolised from the cell. Furthermore, as has been discussed above, it is likely that this pathway for uptake of cardiac glycosides in HeLa cells is also the route for turnover of the native sodium pump. These receptor-mediated endocytic and exocytic events were investigated, by the use of specific inhibitors or conditions which have been reported to perturb receptor-mediated endocytosis of other uptake systems - the results are discussed below.

Chloroquine

The results in Tables 2.6 and 2.7 show that chloroquine does not alter the rate of loss of either ouabain or digoxin from HeLa plasma membranes. Griffiths et al. (1983) has previously shown that chloroquine however slows the excretion rate (or rate of exocytosis) of digoxin but not that of ouabain and this is confirmed here in Tables 2.6 and 2.7. Taken together the results suggest that chloroquine slows the excretion of digoxin from the cell, not by affecting the internalisation process, but by affecting the excretion rate of digoxin from the cell. Chloroquine is a weak base and is thought to act by raising the pH of lysosomes thus inhibiting lysosomal processing of internalised ligand. Many studies have shown that internalised ligand is accumulated in cells in the presence of chloroquine (for review see Goldstein, Anderson and Brown, 1979), unable to be degraded due to inhibition of lysosomal enzymes. Al Gharably (1985) has shown that in the presence of chloroquine, the amount of digoxin in the lysosomal fraction of HeLa cells is increased from that found in controls. Griffiths et al. (1983) have proposed a model to explain why chloroquine affects digoxin (and digitoxin) handling but not ouabain handling in HeLa cells. The model supposes that the rate limiting step for the more lipid soluble digoxin is the lysosomal processing step, while the rate limiting step for the handling of the less lipid soluble ouabain is diffusion from the cell; inhibiting the lysosomal processing step will therefore only affect the excretion rate of digoxin from the cell and not that of ouabain. This model is in agreement with the results in Tables 2.6 and 2.7, and with the observation by Al Gharably. Al Gharably (1985) has also

suggested that ouabain is found in the lysosome interior, while digoxin is found in the lysosomal membrane; presumably this may be the consequence of different lipid solubilities.

Other reports have appeared which demonstrate that chloroquine can inhibit the internalisation of various ligands (Fitzgerald, Morris and Saelinger, 1980; Sando, Titus-Dillon, Hall and Neufield, 1979). The proposed mechanism of action on the internalisation step is that chloroquine raises the pH of endocytic vesicles thus inhibiting internalisation. This raises an interesting question as to why chloroquine does not inhibit the internalisation step of ouabain or digoxin if these ligands are internalised within endocytic vesicles (this question is also directed at other ligand uptake systems in which chloroquine is reported to inhibit the lysosomal step but not internalisation). The answer to this question is not known, but a possible reason may be that endocytic vesicles rapidly turnover and therefore do not limit the transfer of ligand from the plasma membrane into lysosomes.

Cycloheximide

The effects of cycloheximide have largely been discussed above. Table 2.11 shows that cycloheximide slightly slows the loss of digoxin from HeLa cells. This finding is in agreement with that of Griffiths *et al.* (1983) that cycloheximide may reduce the supply of lysosomal enzymes and therefore slow the rate of release of digoxin from the cell. This observation was supported by the demonstration by these authors that chloroquine (which inhibits lysosomal processing in HeLa cells) nullifies any effect by cycloheximide.

Monodansylcadaverine

The results in Tables 2.12 and 2.13 show that monodansylcadaverine has no effect on the rate of loss of glycoside from HeLa plasma membrane or HeLa cells. Higher concentrations of monodansylcadaverine also had no effect on the above rate losses. The lack of effect of monodansylcadaverine is surprising due to the numerous reports showing it to inhibit the internalisation of various ligands. Dickson, Schlegel, Willingham and Pastan (1982) have presented morphological evidence which shows that monodansylcadaverine prevents the clustering of α 2M:receptor complexes into coated pits. It may be that cardiac glycosides in HeLa cells do not cluster in coated pits and so uptake of these ligands is unaffected by monodansylcadaverine (preliminary autoradiographic evidence by Lamb and Ogden, suggests that ouabain does not cluster, but may be located in coated pits which are evenly distributed over the surface). Another possibility is that inhibition of the enzyme transglutaminase by monodansylcadaverine does not occur in HeLa cells or that this enzyme is not present in HeLa cells.

Calcium

The results in Tables 2.14 to 2.19 show that altering $[Ca]_o$, $[Ca]_i$ or inhibition of the calcium regulating protein calmodulin by TFP, did not affect the rates of loss of glycoside from HeLa plasma membranes or HeLa cells. Calcium has many cellular effects, and so such experiments above are likely to be of a non-specific nature, with an inherent difficulty in identifying a specific reaction that may be calcium sensitive. The results however show that any

non-specific calcium effects do not affect glycoside loss from HeLa plasma membranes or HeLa cells. Calcium has also been implicated as an essential cofactor for the enzyme transglutaminase (above) (Levitzki, Willingham and Pastan, 1980). The results in Tables 2.14 to 2.19 are in general agreement with the results in Tables 2.12 and 2.13 - that transglutaminase may not be necessary for cardiac glycoside uptake in HeLa Cells.

Monensin

The results in Tables 2.20 and 2.21 show that monensin affects the rate of loss of digoxin from HeLa cells but does not affect the other measured rates. These results are quantitatively very similar to those obtained for chloroquine (Tables 2.6 and 2.7). This may be expected as both monensin and chloroquine are thought to produce the same effects - both raise the pH of acidic vesicles such as lysosomes. However their mechanisms of action are different; weak bases may accumulate in the lysosomes as non-ionised molecules thus raising the pH due to increased buffering of the lysosome interior, while monensin (an ionophore) equilibrates protons across the lysosome. Monensin has been used in many studies to inhibit internalisation and is thought to act by inhibiting the function of endocytic vesicles, however such effects were not found in this present study. Dickson et al. (1982) have presented morphological evidence which shows that monensin allows clustering of ligand in coated pits but prevents transfer of ligand into 'receptosomes' or endocytic vesicles. Evans (1985) working with isolated endocytic vesicles from hepatocytes, has identified a monensin-activated Mg^{2+} -ATPase as a marker for

endocytic vesicles, but whether this is a universal marker for such vesicles is not known.

Nocodazole and cytochalasin B

The results in Tables 2.22 to 2.25 show that these cytoskeletal disrupting agents do not affect the rates of loss of glycoside from HeLa plasma membranes and HeLa cells. Willingham and Pastan (1984) have suggested that receptosomes move by saltatory motion and that this motion is controlled by the cytoskeleton, but such a role for the cytoskeleton has not been implicated in the above studies.

Conclusions

Most of the inhibitors/conditions used in the above studies did not have an effect on the receptor-mediated internalisation or the excretion (exocytosis) of cardiac glycosides (notable exceptions were chloroquine and monensin). Almost all of the systems in which these inhibitors/conditions have been used, are systems in which the internalisation rate of ligands by receptor-mediated endocytosis is very rapid often with a $t_{1/2}$ of internalisation of a few minutes (e.g. transferrin has a $t_{1/2}$ of 3 minutes in mouse tetradecanoyl phorbol acetate (TPA) induced rat hepatoma cells). Internalisation of cardiac glycosides proceeds at a rate of hours rather than minutes ($10\% \text{ hr}^{-1}$) and the possibility remains that such slow rate internalisation events may occur by a different pathway to the rapid rate internalisation events. However the pathway of internalisation of glycoside by HeLa cells seems indeed similar to the pathway of receptor-mediated endocytosis originally elucidated for LDL and its receptor; i.e. glycoside (ouabain) uptake in HeLa

cells can only occur by binding to the sodium pump, and the envisaged release of glycoside from the sodium pump is within an endocytic-like compartment.

Cook and co-authors have attempted to identify some of the intermediate steps of glycoside internalisation (such as identification or isolation of endocytic vesicles) but with no success at present; certainly more detailed biochemical knowledge is required of this process. Other information which should be sought is a measure of the internalisation rate of the pump itself (e.g. by means of a monoclonal antibody to the pump); the results presented above follow the sodium pump by the use of the ligand ouabain and by measuring the turnover (not the same as internalisation) of the native sodium pump. A useful experiment would be to follow ouabain internalisation and sodium pump internalisation at the same time - this may be achieved if the binding site for a monoclonal antibody was distinct from the binding site for ouabain. Morphological data obtained from such an experiment may prove conclusively that glycoside and sodium pump are cointernalised, as well as yielding other information such as: intracellular location of pump and glycoside, separation of pump:glycoside complex, and if sodium pumps are also located in the lysosome? etc.

Morphological investigations may also yield information on whether endocytic or exocytic vesicles exist and how they may traffic internalised ligands or receptors inside the cell.

Study of the receptor-mediated internalisation of cardiac glycosides along with sodium pump turnover can also give information about the possible role of receptor-mediated endocytic events in cellular regulation. Such aspects are discussed below.

MODULATION OF SODIUM PUMP NUMBERS IN HeLa PLASMA MEMBRANES.

In the various conditions examined above, sodium pump numbers are modulated over a period of hours - days; this represented long term regulation of pump numbers as opposed to short term modulation of activity. The level of Na/K ATPase or any other enzyme, can be described by the expression $[E] = \frac{k_{syn}}{k_{tur}}$, where $[E]$ is the concentration of enzyme, k_{syn} the enzyme synthetic rate and k_{tur} the turnover rate of the enzyme. In the steady state condition either $k_{syn} = k_{tur}$ or $k_{ins} = k_{int}$, where k_{ins} is the insertion rate and k_{int} the internalisation rate. It has already been discussed above that these expressions are not necessarily equivalent, i.e. turnover is not the same as internalisation and increased synthesis may not lead to increased insertion; however for cardiac glycoside uptake in HeLa cells it has been suggested that the above expressions are equivalent (discussed previously). The results presented in Tables 2.26 to 2.35 are discussed in the context of the considerations presented above.

Serum effects

The results in Table 2.26 show that the rate of loss of ouabain from the HeLa plasma membrane (or internalisation rate of ouabain - i.e. it has been previously discussed above that ouabain loss from the membrane is due totally to internalisation and not dissociation into the medium) is the same in low serum (1%) and high serum (20%).

Aiton and Lamb (1984) have shown however that such high serum conditions applied chronically (for 24 hours) to HeLa cells causes an increase in the sodium pump numbers (after a 6 hour lag) until a new, elevated steady state is reached after 16 - 24 hours with up

to a x2 increase of sodium pump numbers compared to that found in the low serum treated cells. Aiton and Lamb (1984) have shown that the sodium pump numbers increase linearly as a function of the log of the serum concentration. Taken together, the above results suggest that the increase of sodium pump sites due to the high serum condition, is not a result of a change in the internalisation of the sodium pump (or turnover of the sodium pump) but is due to an increase in the synthetic rate of the sodium pump. Cycloheximide has been shown to completely abolish (as expected) the serum stimulated increase in pump site numbers demonstrating the requirement for de novo protein synthesis. A potential criticism of the above scheme may be that cycloheximide alters the normal drug release pattern such that loss of ouabain from the plasma membrane occurs by both internalisation and dissociation into the external medium, so giving a false measure of the rate of ouabain internalisation. However cycloheximide was found not to alter the drug release pattern (as was true for all other conditions/inhibitors), strongly suggesting that the ouabain loss from the membrane occurs only by internalisation. Assuming that the internalisation rate of ouabain reflects sodium pump turnover in the high serum condition, then the above scheme to explain the up-regulation of sodium pump numbers - that high serum causes an increase in the synthetic rate but does not affect the turnover rate of sodium pumps, is a valid model.

Lo and Edelman (1976) have shown that the increase in V_{max} activity of the Na/K ATPase in kidney cortex as a result of thyroxine (T_3) treatment in rats, is due also to an increase in the synthetic rate of the Na/K ATPase with no change in the turnover rate of this enzyme occurring.

Low K effects

It was previously shown by Boardman, Huett, Lamb, Newton and Polson (1974) and Pollack, Tate and Cook (1981a) that if cells are incubated chronically in low K medium, then this results in up to a x2 increase of sodium pump numbers in the plasma membrane. These results were confirmed in this present study, with a 144% increase in the density of sodium pump numbers observed during chronic low K stress. Pollack, Tate and Cook (1981b) have shown that the turnover of the sodium pump is decreased from 3.1/generation to 1.3/generation during such low K stress conditions. Pollack et al. (1981b) have suggested that this decrease in turnover alone, can account for all the observed increase in sodium pump numbers in the plasma membrane; i.e. in the scheme $[E] = \frac{k_{syn}}{k_{tur}}$ a reduced turnover rate, assuming that the synthetic rate remains constant, will cause an increase of [E] (sodium pumps in this case) until a new steady state is achieved. The results in Tables 2.27 and 2.28 show that the rate of loss of ouabain from HeLa plasma membranes (or ouabain internalisation rate) is reduced by over 100% from that found in controls, and essentially reflect the results of Pollack et al. (1981b) above. Taken together, these results obtained for ouabain internalisation and pump turnover strongly suggest that ouabain internalisation is achieved solely by

sodium pump turnover, even when turnover is dramatically reduced in response to low K stress conditions. Furthermore, the low K media conditions were shown not to alter the pattern of drug release from the plasma membrane, the inference being that the loss of ouabain from the plasma membrane is achieved solely by internalisation in low K media conditions (as is the situation in normal medium). What is assumed both by Pollack et al. (1981b) and in the present study, is that the synthetic rate of the sodium pump does not change during low K stress conditions. Certainly the assumption is valid in terms of the model presented above, however no direct evidence for this exists (it is also worth pointing out that no direct evidence exists for the increased rate of synthesis of sodium pumps in response to high concentrations of serum). Lamb and Ogden (1982) have suggested that a direct measure of the synthetic rate (or more correctly the insertion rate) can be obtained by a measure of the rate of recovery of sodium pump sites from a glycoside block; the rate obtained is about $10\% \text{ hr}^{-1}$. By a repeat of such an experiment with low K stressed cells, this indirect measure of the insertion rate may be obtained.

Boardman et al. (1974) has shown that the increase in pump site numbers during low K stress conditions is caused by an increase in $[\text{Na}]_i$ rather than the lowered $[\text{K}]_o$. This was demonstrated in experiments in which the $[\text{K}]_o$ was lowered without raising $[\text{Na}]_i$ (achieved by substitution of external Na with sorbitol), which resulted in no increase in sodium pump numbers after 24 hours. An interesting question follows from this and that is, does a lowered $[\text{Na}]_i$ reduce sodium pump numbers when applied chronically to HeLa cells? Kim and Smith (1985) have recently shown a decrease in sodium pump site numbers in chick ventricular cells growing in low Na medium.

The results in Table 2.28 and Figure 2d suggest that on the application of low K medium, HeLa cells respond by immediately altering the internalisation rate of ouabain. This contrasts with the situation on the immediate application of high serum medium - in this situation a lag period of about 6 hours is apparent before any increase in sodium pump numbers can be detected. Devreotes and Fambrough (1976) have called this lag period the transit time, which is the time taken for transport of newly synthesised sodium pumps from the endoplasmic reticulum through the Golgi apparatus to eventual insertion in the plasma membrane. As discussed above, serum stimulation of sodium pump numbers likely occurs by increased synthesis hence the observed lag period. However the increase of pump sites by low K medium occurs by a reduction in the turnover (or internalisation rate) of the sodium pump and so can begin immediately; this scheme fits the observed data in Table 2.28 and Figure 2d.

Pollack et al. (1981b) have shown that when HeLa cells growing in low K medium are returned to normal medium, sodium pump site numbers return promptly to control levels. These authors claimed that this lack of any lag period or transit time was further evidence that the elevated sodium pump numbers observed during low K stress is due to modulation of the sodium pump turnover rate rather than the synthetic rate. If cells were modulating the sodium pump site numbers by changes in the synthetic rate, then a lag period would have been found in the above experiment, due to sodium pumps synthesised at an increased rate still in transit within the cell on return of cells to normal medium. The results in Table 2.29 and Figure 2e show that the ouabain internalisation rate (previously shown to be about

4% hr⁻¹ during low K stress) promptly returns to the control rate (about 9% hr⁻¹). Although the experiment carried out (data shown in Table 2.29 and Figure 2e) measures ouabain internalisation rate in cells returned to normal medium, while Pollack et al. (1981b) measured ouabain binding sites, the data is in agreement with the model proposed by Pollack et al. (1981b); there is a major change in the internalisation rate or turnover rate of the sodium pump (as measured with ouabain) when HeLa cells in low K medium are returned to normal medium and importantly, the change is immediate. The faster internalisation rate of ouabain on return to normal medium, can account for the observed return of sodium pump site numbers to control levels. Furthermore, as mentioned previously, low K medium was not found to alter the ouabain release pattern from the plasma membrane, inferring that loss of ouabain from the plasma membrane is achieved solely by internalisation as part of sodium pump turnover during such low K stress conditions.

In conclusion the measurements of ouabain internalisation are in full agreement with both the direct measure of sodium pump turnover during low K stress and also the model proposed by Pollack et al. (1981b) to account for the up-regulation of sodium pump numbers in response to low K stress conditions.

Interactive effects

The model presented above showing that the increase in sodium pump numbers during low K stress is caused by a reduction in the sodium pump turnover rate, should mean that this pathway is independent of protein synthesis. However in HeLa cells in normal medium for which the sodium pump turnover rate is $12\% \text{ hr}^{-1}$, cycloheximide was found to cause a 2% reduction in sodium pump numbers following a 3 hour lag period. Boardman et al. (1974) observed that with HeLa cells in low K medium in which the sodium pump turnover rate is only $4\% \text{ hr}^{-1}$, cycloheximide caused the same 2% reduction in sodium pump numbers found in controls (above). With a much reduced pump turnover rate (which in turn causes sodium pump numbers to increase) low K stressed cells should be expected to behave differently to control cells when all protein synthesis is stopped; a much reduced increase in sodium pump numbers or at least no decrease may have been expected with low K stressed cells. The conclusion from the observations above is that de novo protein synthesis is essential for any effect on sodium pump numbers caused by low K stress. It is apparent that this cycloheximide result conflicts with the model proposed by Pollack et al., (1981b) above. However a possible explanation compatible with the Pollack model, is that de novo protein synthesis is required for a protein essential to the mechanism of up-regulation which occurs during low K stress. Such a protein may be a rapidly turning over kinase necessary for intracellular signal transduction or a general transport protein essential for any translocation of pumps within the cell. In short, such a consideration above would mean that the

Pollack model for low K stress conditions would still be correct but lack of synthesis of an essential protein would cause cessation of such a mechanism.

Aiton and Lamb (1984) have shown that the low K and serum effects interact; an increased serum concentration diminishes the ability of low K medium to increase the sodium pump numbers in the plasma membrane. This is not expected from consideration of the mode of action of either condition, i.e. low K causes an increase in sodium pump sites by reducing the turnover rate of the sodium pump while high serum causes increased synthesis of sodium pumps. It should have been expected that such an interaction of the 2 conditions would be additive with an even greater stimulation of sodium pump numbers, instead of the much reduced effects observed. The results in Table 2.30 show that increased concentrations of serum abolishes the low K induced reduction of ouabain internalisation rate. This result can provide a mechanism for the findings of Aiton and Lamb (1984) above, i.e. increased serum concentration gradually turns off the reduction in internalisation rate (or the turnover rate) of the sodium pump resulting in no observed increase in sodium pump numbers. The results of Aiton and Lamb (1984) and those in Table 2.30 not only suggest that the low K effect is turned off (i.e. normal sodium pump turnover rate is observed) but also suggest that the serum stimulated increase in synthesis of sodium pumps is switched off by low K medium. It is clear that the pathways for modulation of sodium pumps by low K (really $[Na]_i$) or high serum are not independent, with each input able to exert an inhibitory effect on the other. Hume and Lamb (1976)

have shown that a 10 minute exposure of HeLa cells to 1 mM ATP causes a delayed increase in sodium pump numbers and that this increase is abolished by an increased serum concentration. This ATP induced increase may represent another input pathway for modulation of sodium pump numbers in the plasma membrane, which like the low K effect is dependent on the serum concentration of the medium. Boardman, Hume, Lamb and Polson (1975) have shown that growth of HeLa cells in Li (as a substitute for Na) medium also causes an increase in sodium pump site numbers, but that this effect is independent of serum concentration. This observation suggests that Li may modulate sodium pump numbers by a pathway independent of the serum control pathway and perhaps the low K pathway.

The intracellular signalling for each pathway and how they interact is not known. It would be interesting to establish whether the levels of any known second messengers (e.g. cAMP, cGMP, phosphatidylinositol) change in response to any of the conditions described above.

High K effects

Larking, Brown, Goldstein and Anderson (1983) showed that incubation of fibroblasts in hypotonic medium for 5 minutes followed by transfer to K-free medium, led to a rapid depletion of $[K]_i$. When the $[K]_i$ level fell below a threshold level (40% of controls), coated pit formation and the rate of receptor-mediated endocytosis were arrested, despite normal ligand binding. It was thought possible that these effects were perhaps due to changes in the membrane potential rather than changes in the level of $[K]_i$; the possible effects

of membrane potential on the receptor-mediated internalisation of ouabain in HeLa cells were investigated. The results of a preliminary experiment in which the membrane potential was drastically altered, shows a marked change in the ouabain internalisation rate (Table 2.32). The data is qualitatively similar to that obtained for the ouabain internalisation rate for HeLa cells in low K medium. Table 2.33 shows that the high K medium used (135 mM K, 12 mM Na, 2% dialysed serum) causes a reduction in both $[Na]_i$ and $[K]_i$ after 24 hours. If it is then assumed that the reduced ouabain internalisation rate during high K stress results in a corresponding increase in sodium pump numbers (as is the case for HeLa cells in low K medium), then this increase cannot be accounted for by a response to raised $[Na]_i$ as has been shown to operate for HeLa cells during low K stress; in short 2 independent mechanisms would operate during low and high K stress. It has still to be established however that the noted reduction in the ouabain internalisation rate during high K stress does in fact correlate with an increase in sodium pump numbers in the plasma membrane as has been shown for HeLa cells during low K stress. It would also be interesting to discover whether the high K effect is diminished by increased serum (as is the case for low K incubations). The initial observations (above) showing the reduced ouabain internalisation rate in HeLa cells during high K stress may lead to the establishment of another mechanism by which HeLa cells modulate sodium pump numbers in the plasma membrane in response to changes in the membrane potential.

Excretion rate

The results in Table 2.31 show that during low K stress, the excretion rate of ouabain does not change despite an approximate 100% reduction in the internalisation rate of ouabain during such low K stress. Lamb and Ogden (1982) have shown that the excretion rate of ouabain is independent of sodium pump site recovery under various conditions (e.g. cycloheximide, vinblastine, serum). The results in Table 2.31 are in agreement with the observation of Lamb and Ogden and suggest that the excretion of ouabain from the cell is independent of ouabain internalisation during low K stress conditions. If this is so then an interesting question is what would happen in such low K experiments if digoxin was used instead of ouabain? - presumably both digoxin and ouabain would be internalised by HeLa cells during a low K stress at the reduced rate of about $4\% \text{ hr}^{-1}$. However the rate of digoxin excretion is $10\% \text{ hr}^{-1}$ (for ouabain it is about $4\% \text{ hr}^{-1}$) and such a rate could not be faster than the rate of internalisation during low K stress of $4\% \text{ hr}^{-1}$; i.e. the rate limiting step would be internalisation of digoxin, thus slowing the rate of excretion of digoxin out of the cell.

Conclusions

An increased understanding of the process of receptor-mediated internalisation of cardiac glycosides in HeLa cells (as discussed earlier) would also lead to an increased understanding of the mechanisms involved in modulation of sodium pump numbers. The probable increased rate of synthesis of sodium pump sites in response to an increased serum concentration can be further investigated by establishing techniques to directly measure any increase of sodium pump synthesis. A possible technique could be the isolation of HeLa cell mRNA, followed by determination of the level of mRNA specific for the sodium pump by hybridisation with Na/K ATPase cloned DNA. It is envisaged that the level of sodium pump mRNA would be higher in HeLa cells growing in high serum compared to HeLa cells growing in low serum. The specific signals which mediate the modulation of sodium pump numbers in the conditions described (high serum, low K, Li medium, ATP), are not known. Preliminary experiments may be carried out to determine whether any change in the levels of cellular second messengers occurs during conditions in which sodium pump numbers are modulated; such experiments it is envisaged, may yield information as to the nature of signalling during modulation of sodium pumps.

It is concluded that a greater understanding of the biochemical mechanisms of receptor-mediated internalisation of cardiac glycosides in HeLa cells would also contribute to increased understanding of the specific signals involved in long term regulation of sodium pump numbers.

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