THE INFLUENCE OF CELL POPULATION DENSITY AND VIRUS-TRANSFORMATION ON SOME MEMBRANE TRANSPORT PROPERTIES OF CULTURED MOUSE FIBROBLASTS

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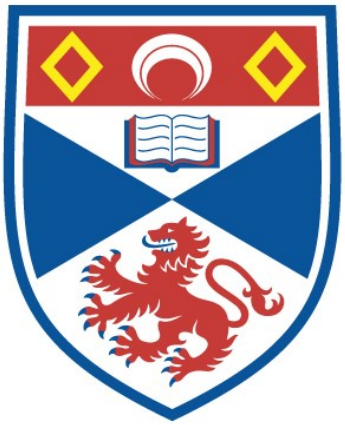

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ABSTRACT

A study has been made of the effect of cell population density and virus-transformation on the transport of K^+, inorganic phosphate (Pi) and 2-deoxyglucose in cultured mouse fibroblasts.

Normal, untransformed 3T3 cells show a marked reduction (3-5 fold) in the influx of K^+, Pi and 2-deoxyglucose with increasing cell population density. The reduction in transport begins at low cell densities and precedes the cessation of cell growth. In contrast, virus-transformed 3T3 cells do not exhibit similar density-dependent reductions in transport. In these cells the influx of K^+ and 2-deoxyglucose is independent of cell density. The influx of Pi does decrease with increasing cell density but to a lesser extent and at much higher population densities than in 3T3 cells. At low cell densities the influx of Pi and 2-deoxyglucose in 3T3 cells is only slightly lower than the influx into transformed cells. For K^+ the influx in 3T3 cells is slightly higher than in the transformed cells. At higher cell densities the transformed cells exhibit the higher rates of uptake of all three substrates due to the density-dependent transport reductions in the 3T3 cells. Virus-transformation per se does not, therefore, lead to any great increase in transport capacity.

In all cases the density-dependent transport reductions are attributable to a decreased Vmax with no change in the Km of the system. The transport sites of normal and virus-transformed cells appear to be qualitatively similar since no significant differences were found for transport Km's between the cell lines.

The density-dependent reduction of the K^+ influx in 3T3 cells is due to a decrease in "Na-pump" activity with no change in the passive permeability of the cell membrane to K^+. This reduced "Na-pump" activity is accompanied by a decrease in $[K^+]i$ and an increase in $[Na^+]i$. The $[K^+]i$ and $[Na^+]i$ of virus-transformed cells are not markedly affected by change in cell density. The $[K^+]i$ of transformed cells is slightly higher than the $[K^+]i$ of untransformed cells at low cell density and almost 2-fold greater than the $[K^+]i$ of high density untransformed cells. The cardiac glycoside, ouabain, inhibits Na-K exchange in both untransformed and transformed cells. In the transformed cells the drug also produces a secondary effect ie. a stimulation of K-K exchange. Evidence is presented which suggests that this difference is related to known changes in the lipid properties of virus-transformed cell membranes.

The inward transport of Pi occurs predominantly via a carrier-mediated, Na-dependent process. A small Na-independent influx of Pi is also present. Preliminary evidence suggests that the outward movement of Pi is also Na-dependent. A model for Na-coupled Pi transport is presented and discussed briefly. The density-dependent reduction of Pi transport in 3T3 cells is due to a decreased Na-dependent influx with no change in the Na-independent component.

The addition of fresh complete medium to quiescent 3T3 cells causes a rapid increase in the influx of Pi and 2-deoxyglucose. Fresh medium without serum does not increase transport. For both substrates the increased transport results from a higher Vmax with no alteration in Km. Under normal culture conditions this serum-stimulation of transport is independent of protein synthesis. However, an additional increase in Pi transport occurs when fresh complete medium is added to serum-starved 3T3 cells. This second increase is inhibited by cycloheximide indicating a requirement for protein synthesis.

The role of membrane transport changes in the control of cell proliferation is discussed. A model is presented which attempts to explain the effects of serum growth factors in terms of their action on transport systems.

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

by

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March 1976

CERTIFICATE

I hereby certify that Kenneth D. Brown has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1 1967), and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology, United College of St Salvator and St. Leonard, University of St. Andrews, under the direction of Professor J. F. Lamb.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October 1967 and graduated with the degree of B. Sc. Hons. (2.1) in Physiology in June 1971. I matriculated as a joint research student in the Departments of Physiology and Computational Science, University of St. Andrews, in October 1971. I matriculated as a research student in the Department of Physiology, University of St. Andrews in October 1972.

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Finally, I must thank Mary who listened to problems, wrote reference cards, filed reprints and spent many hours typing this thesis. She must now be one of the few historians with a fair knowledge of cell biology. No written acknowledgement will be enough to express my gratitude. Anyway, she knows.

If anybody shall reprove me, and shall make it apparent unto me, that in either opinion or action of mine I do err, I will most gladly retract. For it is the truth that I seek after, by which I am sure that never any man was hurt; and as sure, that he is hurt that continueth in any error, or ignorance, whatsoever.

MARCUS AURELIUS

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SUMMARY

A study has been made of the effect of cell population density and virus-transformation on the transport of K^+, inorganic phosphate (Pi) and 2-deoxyglucose in cultured mouse fibroblasts.

1) Normal, untransformed 3T3 cells show a marked reduction (3-5 fold) in the influx of K^+, Pi and 2-deoxyglucose with increasing cell population density. The reduction in transport begins at low cell densities and precedes the cessation of cell growth.

2) Polyoma or simian virus-transformed 3T3 cells (Py3T3 and SV3T3) do not exhibit similar density-dependent reductions in transport. The influx of K^+ and 2-deoxyglucose is independent of cell density. The influx of Pi decreases with increasing cell density but to a lesser extent and at much higher population densities than in 3T3 cells.

3) At low cell densities the influx of Pi and 2-deoxyglucose in 3T3 cells is only slightly lower than the influx into transformed cells. For K^+ the influx in 3T3 cells is slightly higher than in the transformed cells. At higher cell densities the transformed cells exhibit the higher rates of uptake of all three substrates due to the density-dependent transport reductions in the 3T3 cells. Virus-transformation per se does not, therefore, lead to any great increase in transport capacity.

4) In all cases the density-dependent transport reduction is attributable to a change in Vmax with no change in the Km of the system.

5) The transport sites of normal and virus-transformed cells appear to be qualitatively similar since no significant differences were found for transport Km's between the cell lines.

6) The density-dependent reduction of the K^+ influx is due to a decrease in "Na-pump" activity with no change in the passive permeability of the cell membrane.

7) The reduced "Na-pump" activity is accompanied by a decrease in $[K^+]i$ and an increase in $[Na^+]i$ with increasing 3T3 cell density.

8) The $[K^+]i$ and $[Na^+]i$ of virus-transformed cells are not markedly affected by changes in cell density. The $[K^+]i$ of transformed cells is slightly higher than the $[K^+]i$ of untransformed cells at low density and almost 2-fold greater than the $[K^+]i$ of high density untransformed cells.

9) The cardiac glycoside, ouabain, inhibits Na-K exchange in both untransformed and transformed cells. In the transformed cells the drug also produces a secondary effect ie. a stimulation of K-K exchange. Evidence is presented which suggests that this difference is related to known changes in the lipid properties of virus-transformed cell membranes.

10) The inward movement of Pi occurs predominantly via a carrier-mediated, Na-dependent process. A small Na-independent influx of Pi is also present. Preliminary evidence suggests that the outward movement of Pi is also Na-dependent. A model for Na-coupled Pi transport is presented and discussed briefly.

11) The density-dependent reduction of Pi transport in 3T3 cells is due to a decreased Na-dependent influx with no change in the Na-independent component.

12) The addition of fresh complete medium to quiescent 3T3 cells causes a rapid increase in the influx of Pi and 2-deoxyglucose. Fresh medium without serum does not increase transport. In both cases the increased transport is due to an increased Vmax with no alteration in Km.

13) Under normal culture conditions the serum-stimulation of transport is independent of protein synthesis. However, an additional increase in Pi transport occurs when fresh complete medium is added to serum-starved 3T3 cells. This second increase is inhibited by cycloheximide indicating a requirement for protein synthesis.

14) The role of membrane transport changes in the control of cell proliferation is discussed. A model is presented which attempts to explain the action of serum growth factors in terms of their action on transport systems.

INTRODUCTION

Development of model systems for the study of cell growth control

Origins of cell culture. The experiments of Harrison (1907) are generally regarded as marking the beginning of tissue culture (Paul, 1972 p.2). Harrison observed that axones grew from small pieces of nervous tissue explanted from frog embryos into clots of frog lymph. In 1912, Carrel placed pieces of chick heart in drops of horse plasma. The tissue became fixed in place when the plasma clotted and cells at the edge of the explant grew out into the plasma clot. In the absence of anti-biotics bacterial infection was a serious problem. In any case, the culture could only be kept alive for a few days which made investigation of cellular properties difficult.

Carrel (1913) quickly discovered that the life of the tissue explant could be prolonged by regularly "feeding" the culture with a few drops of whole chick embryo extract. The experimental value of an *in vitro* cell growth from tissue extracts was quickly recognised and this system produced valuable information, especially in the fields of embryology and histology. Nevertheless, the widespread application of cell culture to biological investigations only became possible after the next round of rapid development some forty years later.

Cultures from a single cell. The initial attempts to grow cultures from a single cell were not successful and this led to a widely held view that cellular connections via "protoplasmic bridges" were essential for cell proliferation (Fischer, 1946). However, in 1948 Sanford et al. demonstrated the proliferation of single cells obtained from mouse explants which had been treated with a chemical carcinogen. A single cell from the growing edge of a carcinogen-treated explant was withdrawn into a capillary tube. The end of the

tube, containing the cell, was broken off into a fresh serum clot. Sometimes, the cell divided and gave rise to a "clone" of cells. Established cell lines, known as L-cells, were obtained from these clones.

Cultures from disaggregated tissue. Moscona (1952) showed that embryonic limb-buds could be disaggregated by trypsin and that the resulting cell suspension could be cultured. Independently, Shaunon et al. (1952) and Dulbecco (1952) reported techniques for the production of cultures from trypsin digests of whole chick embryos. When these primary cultures were sub-cultured into fresh dishes it was found that the resulting secondary culture consisted mainly of one cell type. The cells were named fibroblasts (Dulbecco, 1952) since they resembled the fibroblasts obtained from the culture of connective tissue.

Establishment of fibroblast cell lines. Secondary cultures derived from normal tissue are often referred to as primary cell lines (Paul, 1972 p.25). These cell lines can be pepeatedly subcultured for several months. However, many primary cell lines then reach a crisis point, stop dividing, and die. Others develop the ability to be subcultured apparently indefinitely and are, thereafter, known as established cell lines. This designation is usually applied only after the cells have been subcultured at least 70 times (Paul, 1972 p.26).

The ability to form established fibroblast lines appears to be species-dependent. They are easily obtained from mouse and hamster cells but not from human and chick cells. An established line usually arises from dense foci of apparently altered cells which manage to survive the crisis period mentioned earlier. These cells differ in several important respects from the cells of the primary line. The established cells have an altered chromosome number whereas

primary cultures are normal in this respect. The established lines grow to higher saturation densities and, unlike most primary cell lines, will grow in suspension culture. The established cells are almost always of high tumourigenicity ie. give rise to a high incidence of tumours when injected into animals that do not immunologically reject the cells. Such cell lines are generally regarded as having undergone a "spontaneous transformation".

"Normal" fibroblast cell lines. Information on the physiological and biochemical properties of cultured cells was collected rapidly after the introduction of simplified culture procedures. However, all established lines were either derived from tumours, or, had undergone spontaneous transformation and adopted tumour cell characteristics. Observations were difficult to interpret in the absence of data from a comparable established normal cell line. In addition, a major problem remained unsolved. It was known that viruses, such as polyoma virus, could transform early-passage primary lines producing tumourigenic established lines. However, it remained "impossible to tell whether the virus was speeding up an inevitable spontaneous adaptation or introducing new virus-coded alterations in growth behaviour" (Tooze, 1973 p.88).

The solution to these problems required the establishment of permanent cell lines that exhibited the low saturation density and low tumourigenicity of normal primary lines. One such cell line was produced from kidney cultures of one-day old hamsters. The name BHK 21 was given to these cells (Stoker & MacPherson, 1961; MacPherson & Stoker, 1962). Shortly afterwards Todaro & Green (1963) reported a reproducible procedure for the production of the first established normal mouse fibroblast line. A primary line of mouse embryo fibroblasts was subcultured under carefully controlled conditions. The cells were replated every 3 days at an

innoculum of either $3x10^5$, $6x10^5$ or $12x10^5$ cells/5 cm dish. In all three conditions the population doubling time (Td) was found to increase from an original value of about 20 hours to a maximum of about 100 hours after 15-25 generations. Thereafter, the growth rate began to rise again with the Td eventually stabilising around the original values of 20 hours. All conditions gave rise to established cell lines which were named 3T3, 3T6 and 3T12. It was found that 3T6 and 3T12 cells, like previously established fibroblast lines, would continue to grow even in very crowded conditions. 3T3 cells, on the other hand, failed to divide after the formation of a confluent monolayer at about $1x10^6$ cells/dish. The 3T3 cells were also morphologically different from the other lines. In sparse culture the cells were fibroblastic but grew considerably flatter, appeared finely granular, and were difficult to trypsinise. In confluent cultures a sheet of cells with obscure individual cell borders was observed.

Todaro & Green reasoned that the 3T3 culture regime avoided cell-cell contact during establishment and resulted in a permanent line which, though abnormal in karyotype, had normal growth control properties ie. low saturation density and failure to grow in suspension. It was suggested that the cells retained the "contact inhibition" associated with primary cell lines. The authors conclude, "the malignant properties of many established cell lines may be the result of selective processes usually operating in cell culture and not related to the process of establishment per se".

The Swiss mice from which Todaro & Green obtained their cultures were not inbred and so tumourigenicity tests were not possible. Aaronson & Todaro (1968) produced similar cell lines from the embryos of Balb/c inbred mice and demonstrated that, whereas 3T6 and 3T12 cells were tumourigenic, 3T3 cells were not. Moreover, a close correlation was found between the in vitro saturation density and the

ability to produce tumours in Balb/c mice. 3T3 cells were shown to be readily transformed by two different oncogenic viruses, polyoma virus and simian virus 40 (Todaro et al., 1964). Cloned lines of Py3T3 and SV3T3 cells were produced from virus-infected 3T3 cells. The transformed cells were shown to have high saturation densities and a very high tumour-forming ability (Todaro et al., 1964; Aaronson & Todaro, 1968).

Fibroblasts of either primary cell lines (eg. chick embryo, mouse embryo) or established cell lines (3T3, BHK) have become the main model systems in the study of cell growth control. The growth characteristics of these cells may be transiently modified by various agents or permanently altered by viral or chemical transformation. It has been found that both transient and permanent changes in growth properties are accompanied by structural and functional alterations to the cell plasma membrane. For this reason it is now widely believed that the cell membrane plays a crucial role in the control of animal cell proliferation (Pardee, 1964, 1971 1975; Burger, 1971a, b, c, 1973; Rapin & Burger, 1974; Stoker, 1971; Holley, 1972; Emmelot, 1973; Hynes, 1974; Rubin, 1974). In the next section of this introduction the growth properties of the fibroblast model systems will be examined together with the associated changes in membrane structure and function. Particular emphasis will be placed on growth-related changes in membrane transport.

Growth properties of normal fibroblasts in culture

As mentioned previously normal, untransformed cells show "contact inhibition" (Todaro & Green, 1963) or "density-dependent regulation" (Stoker & Rubin, 1967) of growth. The latter term is preferable since the involvement of cell-cell contact in the regulatory process is questionable (see later). Untransformed cells grow to a certain "saturation density" and then stop growing. The resulting culture remains healthy and quiescent for some time (weeks). Transformed cells, on

the other hand, do not exhibit density-dependent regulation. These cells continue to grow until the medium is exhausted of low molecular weight nutrients. The cells do not become quiescent and will die unless transferred to fresh medium. The significance of density-dependent regulation lies in the fact that it may be an inherent regulatory mechanism in vivo, the loss of which may be a factor contributing to malignancy. Indeed tumour-forming ability in vivo has been correlated with the loss of density-dependent regulation in vitro (Aaronson & Todaro, 1968; Pollack, Green & Todaro, 1968; Abercrombie, 1970).

Normal fibroblasts in culture can thus exist in two alternative growth states; one of active proliferation or one of reversible arrest of cell division (Todaro & Green, 1963; Todaro et al., 1965).

The growing state

Progression through the cell cycle. Growing fibroblasts in culture divide every 12-24 hours depending on the cell type. The cells proceed from one division (mitosis, M) to the next by a poorly understood sequence of events. Three major phases, G1, S and G2, can be recognised during the inter-mitotic period (Howard & Pelc, 1953; Hornsey & Howard, 1956). The genetic material, DNA, replicates during the S phase. The G1 phase is the interval between mitosis and the initiation of DNA synthesis; the G2 phase is the interval between completion of DNA replication and the next mitosis. Syntheis of RNA and proteins occur throughout the intermitotic period. Which events are crucial for the progress through the cell cycle, and particularly through G1 and G2, remain major questions of cell biology.

Membrane changes during the cell cycle. Studies on the regulation of the cell cycle usually require synchronised cell populations. These can be produced by a variety of procedures which reversibly block progress through the cycle. Amino acid (Ley & Tobey, 1970) or serum (Todaro et al, 1965)

starvation stops growth in G1, hydroxyurea or excess thymidine inhibit DNA synthesis producing a block at the G1/S boundary; vinblastine, vincristine or colchicine prevent cytokinesis and thus block cells in M. When the inhibition is reversed a round of synchronised cell division can be followed.

Using these techniques several striking changes in membrane structure have been shown to take place during M and early G1. Mitotic cells, observed by electron microscopy, show numerous microvilli. After division, through G1, the cells become smoother, until, during S, few microvilli are present (Follet & Goldman, 1970; Rubin & Everhart, 1973; Hale et al., 1975). The membrane of 3T3 cells contains aggregated intramembranous particles (IMP). A disaggregation of IMP has been shown to occur during mitosis (Furcht & Scott, 1974). Normal cells are known to be less easily agglutinated by certain lectins than the corresponding transformed cells (Rapin & Burger, 1974; Nicolson, 1974). It has been reported that the agglutination of normal cells by lectins is increased during mitosis (Shoham & Sachs, 1974; Burger, 1973). An external surface protein, found on the membrane of some cells, has been shown to disappear during mitosis (Hynes, 1974). These changes in membrane structure during mitosis have led to the suggestion that they play a crucial role in determining whether the cell will proceed with the next division cycle or pass into a resting state. However, no unequivocal demonstration of this possibility has been made.

Relatively few studies have been made on variations in membrane transport through the cell cycle of mammalian cells in culture. Chinese hamster ovary (CHO) cells and L cells synchronised by isoleucine deprivation or mitotic shake-off showed reduced rates of 2-aminoisobutyric acid transport during M and early G1. As the cells progressed further into G1 the transport rate doubled and then remained constant during the rest of the cell cycle returning to the base level

the next round of cell division. These alterations in transport were due to changes in the Vmax with no change in the Km of the system (Sander & Pardee, 1972). Preliminary results suggested that uridine and thymidine transport showed similar increases during G1 (Sander & Pardee, 1972). The results for thymidine are in conflict with other observations on the same cell line (Everhart & Rubin, 1974). Using CHO cells synchronised by mitotic shake-off Everhart & Rubin demonstrated a marked increase in thymidine transport at the beginning of the S phase. The increased transport was not caused simply by increased incorporation of thymidine into DNA during S. These workers presented evidence that newly synthesised thymidine transport sites were incorporated into the membrane during late G1 and early S. Plagemann et al. (1974, 1975) obtained similar results for Novikoff cells synchronised by a double exposure to hydroxyurea. It was found that the thymidine transport capacity of these cells doubled during the S period. The increased transport was caused by an increased Vmax with no change in Km (Plagemann et al., 1974). The increase in transport was dependent on a de novo synthesis of RNA and protein (Plagemann et al., 1975). These observations provide further evidence for the proposal of Everhart & Rubin (1974) that newly synthesised thymidine sites are incorportaed into the membrane during early S phase.

Other transport systems exhibit different patterns of synthesis during the cell cycle. In Novikoff cells the transport of choline and hypoxanthine exhibited a sharp increase during early G1. The increase was abolished in the presence of either actinomycin D or protein synthesis inhibitors (Plagemann et al., 1975). In both chick embryo fibroblasts (Hale et al., 1975) and Novikoff cells (Plagemann et al., 1975) the transport of uridine and 2-deoxyglucose increased continuously through the cell cycle.

Only one report has appeared on cell cycle variations in cation content and fluxes of cultured mammalian cells. Jung & Rothstein (1967) using synchronised lymphoblasts measured a 20% loss of cell K^+ at the beginning of the S period. Over the next 5 to 6 hours the K^+ deficit was eliminated. The changes in total cell Na were more complicated showing a double fluctuation. The unidirectional K^+ influx showed a 2-fold increase at the beginning of the S period - a time at which the cells showed a net loss of K^+. It was concluded that the unidirectional K^+ efflux must also have increased by an even greater amount but this was not measured.

Taken together, these observations indicate that different transport systems are synthesised or activated at different phases of the cell cycle. The results argue against the proposal of Sander & Pardee (1972) of a general increase in membrane transport systems during early G1.

The resting state

Density-dependent regulation of cell growth. Normal, density-inhibited cells stop growing in the G1 phase of the cell cycle; that is, they have a single complement of DNA and hence have undergone cell division but not the next round of DNA replication (Nilhausen & Green, 1965). To distinguish cells resting in G1 from actively growing cells passing through G1 the terms G0 (Brown, 1968) or, less frequently, G1b (Temin, 1971) are often used. In spite of a great deal of effort over recent years the mechanism of density-dependent regulation remains incompletely understood. Two major hypotheses have developed.

One maintains that growth is restricted due to the reduced availability of essential components in the growth medium. The alternative proposal maintains that cell-cell contact directly produces a negative signal which inhibits cell growth.

Several lines of evidence support the first hypothesis. Kruse & Miedema (1965) demonstrated that continuous renewal of the culture medium by perfusion allowed the cells to reach higher saturation densities than standard culture conditions. However, the rate of cell growth was reduced at high density. High levels of serum have been shown to transiently release cells from growth inhibition. In 3T3 cells the saturation density was dependent upon the concentration of serum in the growth medium (Holley & Kiernan, 1968). In addition, in low concentrations of serum, growth was arrested before the formation of a confluent monolayer ie. before most cells are in close contact (Clarke et al., 1970). Using time-lapse cinemicography to follow individual 3T3 cells Martz & Steinberg (1972) demonstrated that cell-cell contact did not directly produce inhibition of cell division. Dulbecco & Elkington (1973) grew 3T3 cells in the same quantity of medium in dishes of different sizes. The results indicated that the saturation density was essentially independent of the dish size and depended only on the quantity of medium and the serum concentration of the medium. However, a recent modification of this procedure suggests that both medium and surface area availability are involved (Thrash & Cunningham, 1975).

The alternative hypothesis, that contact between cells in some way inhibits cell division directly, arose as an extension of earlier observations on cell movement. Abercrombie & Heaysman (1954) observed that the forward movement of cells was greatly reduced when cells collided. Later, Abercrombie et al., (1957) demonstrated that malignant cells did not show "contact inhibition" of movement. The term was expropriated to apply also to the inhibition of cell division seen in untransformed cells. The experimental evidence in favour of a direct role for cell contact rests heavily on the results of "wound-healing" experiments. This technique consists of making a fine scrape through a layer of

resting cells. It was observed that the cells at the edge of the "wound" moved out into the denuded area and commenced DNA synthesis and eventually cell division (Todaro et al., 1967; Dulbecco & Stoker, 1970). Thus it appeared that those cells which had broken intercellular contact were able to divide in the same medium that would not support the division of cells within the layer. However, serum factors have been shown to be essential for this response. In the absence of serum the cells do not migrate into the wound and do not initiate DNA synthesis (Dulbecco, 1970; Lipton et al., 1971). Also, a factor has been obtained from serum which promotes migration but not DNA synthesis and division (Lipton et al., 1971). Thus, the breaking of contacts is not sufficient to induce cell proliferation. Conversely, in the presence of serum, cells around the edge of the wound divided even when they were prevented from migrating into the cell-free area by the presence of cytochalasin B (Stoker & Piggott, 1974; Brownstein et al, 1975).

Recently, Stoker (1973; Stoker & Piggott, 1974) has explained the wound healing experiments in terms of a diffusion barrier (see Maroudas, 1974) very close to the surface of a layer of cells. The effect of a wound is to break this barrier. These experiments support the view (Holley, 1972) that a high cell density limits the availability of growth factor(s), perhaps by reducing their uptake from the medium. This possibility becomes particularly attractive if the combined effects of a diffusion barrier, medium depletion (Holley & Kiernan, 1968, 1974a, b) and reduced transport (see next section) are considered together. On the basis of this proposal, cells which do not exhibit strong density dependent regulation (ie. transformed cells), would have a reduced requirement for medium components and/or an increased ability to obtain them from depleted medium.

A great deal of research is currently directed at determining which factors in the growth medium are important in growth control. Macromolecular factors in serum and low molecular weight factors in either serum or the synthetic medium appear to be involved (Holley & Kiernan, 1968, 1974a, b). In addition, various other substances have been shown, under certain circumstances, to influence cell growth. These include: fibroblast growth factor, insulin, hydrocortisone, prostaglandins, cyclic nucleotides, sodium butyrate, lectins, calcium ions, potassium ions, phosphate ions, amino acids, and glucose. For further information and references on this topic the reader is referred to the recent reviews by Holley (1975) and Pardee (1975).

Membrane changes accompanying growth inhibition. Extensive changes appear to take place in the structure of the membrane of density-inhibited cells. Evans at al.(1974) used scanning electron microscopy to show that inhibited cells had a smooth surface with few microvilli. Growth-dependent alterations in surface glycopeptides (Buck et al., 1970; Ceccarini et al., 1975) and glycolipids (review by Hakomori, 1975) have been reported. Freeze-cleavage studies (Scott et al., 1973; Barnett et al., 1974) have demonstrated an increased aggregation of intramembranous particles with increasing population density of 3T3 cells. The activities of plasma membrane enzymes have been shown to be lower in resting cells. (Na^+-K^+)-ATPase was reduced in quiescent epithelioid MK$_2$ cells (Lelievre et al., 1971; Lelievre & Parat, 1973) and 3T3 cells (Elligsen et al., 1974). Lelievre et al. (1971) also demonstrated a reduction in 5' nucleotidase activity.

Over the past few years density- dependent changes in membrane transport have been reported. Foster & Pardee (1969) found a decreased influx of non-metabolisable amino acids in stationary 3T3 cells. The rate of uptake of metabolisable amino acids was apparently independent of cell-

density. More recently, Otsuka & Moskowitz (1975) have observed a reduced influx of both types of amino acid with increasing density of 3T3 cells. The reasons for this discrepancy are not clear, but may be related to the much higher substrate concentrations used in the first study. Griffiths (1972) found that the accumulation of a wide range of amino acids was reduced in human diploid cells at a high cell density. Unfortunately, this report provides no details of initial transport rates.

Cunningham & Pardee (1969) observed a 2 to 4-fold reduction in the rate of phosphate and uridine transport when confluent and non-confluent 3T3 cells were compared. Similar changes were later reported by Pariser & Cunningham (1971). Weber & Edlin (1971) compared phosphate transport in growing and density-inhibited cultures of 3T3 cells. They found a 5-fold reduction in the rate of phosphate transport with no change in the size of the acid-soluble phosphate pool. A common difficulty in interpreting these results is that estimations of transport rates were made using relatively long isotope labelling times (15-30 min). From the data it is impossible to determine whether initial rates of transport (ie. influx) have been obtained. The most comprehensive examination of cell-density effects on phosphate transport also suffers from this problem (Harel et al., 1975). The initial experiment showed a marked reduction in phosphate influx (measured over 10 min) with increasing population density of 3T3 cells. However, in further experiments a 40-minute labelling period was used. Values obtained in this way probably represent neither the true influx nor the true equilibrium uptake.

The effect of cell population density on transport has been most extensively investigated for glucose transport. However, glucose itself is rarely used as the substrate in these experiments. The glucose analogues 2-deoxyglucose or 3-O-methylglucose are usually employed instead. The

difficulties associated with the use of glucose and the justification for substituting an analogue are outlined in APPENDIX A.

Sefton & Rubin (1971) used a normal, primary cell line of chick embryo fibroblasts (CEF) to examine the effects of population density on 2-deoxyglucose transport. Over the population range examined (0.2 to 2.0×10^6 cells/50mm dish) a 13-fold inhibition of the influx was observed. Weber (1973) compared growing and density-inhibited CEF and found a 6-fold reduction in the rate of 2-deoxyglucose uptake. The decreased transport was associated with a 3-fold reduction in Vmax with no change in the Km of the system. Since the transport of the non-phosphorylatable glucose analogue 3-O-methylglucose showed a similar effect, Weber concluded that the reduction in transport was due to a membrane change rather than a change in intracellular phosphorylation. This conclusion is supported by the results of Kletzein & Perdue (1974a) who found similar changes in 2-deoxyglucose transport when rapidly growing and slowly growing CEF were compared. However, no differences were found for the hexokinase activities of cells with different growth rates. An analysis of the kinetics of phosphorylation in both intact cells and cellular homogenates demonstrated that transport across the membrane, and not phosphorylation inside the cell, was the rate-limiting step in the transport of 2-deoxyglucose. These authors propose that the inhibition of transport associated with increased cell density was due to a reduction in the number of functionally active transport sites. Plagemann (1973) has drawn the same conclusion from similar experiments on a primary line of mouse embryo cells.

2-deoxyglucose transport in 3T3 cells also shows density-dependent changes. This has been examined by seeding 3T3 cells at different densities and incubating for a fixed period to produce cultures with a range of cell densities. A 6 to 10-fold reduction of 2-deoxyglucose influx was found over a density range of $10^4 - 4 \times 10^4$ cells/cm^2 (Bose & Klobnick,

1973; Bannai & Sheppard, 1974). Other workers have reported similar, though less detailed, results (Schultz & Culp, 1973; Oshiro & Di Paolo, 1974). It should be noted that in these experiments, and in those of Sefton & Rubin (1971) on CEF, inhibition of transport was evident well before confluency was achieved.

The effect of cell density on 2-deoxyglucose transport in 3T3 cells has been examined in a different way. Schultz & Culp (1973) seeded cells at a single density and measured the 2-deoxyglucose influx each day for several days. In contrast to the previous results 2-deoxyglucose transport increased slightly during pre-confluent growth but then decreased rapidly when confluency was reached 5 to 6 days after seeding. A 5-fold reduction in the Vmax and a 2.5-fold increase in Km was found when growing and quiescent cells were compared. Using similar procedures Bose & Zlotnick (1973) found that the 2-deoxyglucose transport began to decline within the first 2 days after seeding. They found that the reduced transport was associated with a 7-fold reduction in Vmax with no change in Km. In view of the difficulty in accurately estimating Km, especially from Lineweaver-Burk plots (see APPENDIX B), further data is required to resolve this discrepancy.

Hassell et al. (1975) have questioned the conclusion that density-inhibited cells show reduced 2-deoxyglucose transport. These authors claim that previous investigators did not allow for density-dependent alterations in the rate of intracellular phosphorylation. This is a surprising claim in view of the particular attention paid to this point by several workers (Renner et al., 1972; Plagemann, 1973; Weber, 1973; Kletzein & Perdue, 1974a). Until further data in support of Hassell et al. is produced the combined experimental evidence strongly suggests that in cells which exhibit density-dependent inhibition of cell growth the rate

of 2-deoxyglucose transport decreases markedly with an increase in cell density.

Few studies have been made on the effects of cell density on cell cation content and fluxes. Raab & Humphreys (1974) found no significant difference for the K^+ concentration of growing and density-inhibited chick embryo fibroblasts (105 m-mole/l). However, the rate of K^+ exchange was some 30% greater in growing than in density-inhibited cells. Prigent et al. (1975) found no density-dependent effects on either K^+ influx or efflux in the ME2 epithelioid line. This is surprising in view of the large reduction in (Na^+-K^+)ATPase activity found for density-inhibited ME2 cells by the same workers (Lelievre et al., 1971). A decrease in the ouabain-sensitive K^+ "uptake" has been found in 3T3 cells at high population density (Kimelberg & Mayhew, 1975). However, since a 1 hour labelling period was used it is not clear that either true influx or true equilibrium uptake values have been obtained.

Reinitiation of cell growth in quiescent cells

Cells resting in G0 can be stimulated by a variety of agents to recommence DNA synthesis and division. Serum is the most effective and widely investigated of these stimuli (Todaro et al., 1965; 1967). Serum itself can be added to the used growth medium or a change to complete fresh medium can be made. DNA synthesis is induced after a period that is usually longer than the normal G1 period. For example, in quiescent 3T3 cells stimulated by a change of medium, DNA synthesis began some 12 hours after the change and reached a maximum at 20 hours. Dividing cells were first seen at 25 hours after the change and mitosis peaked at 30 hours (Todaro et al., 1965).

Functional membrane changes are among the earliest

events associated with the reinitiation of cell growth. The rate of transport of uridine and phosphate (Cunningham & Pardee, 1969; Pariser & Cunningham, 1971; Rozengurt & Jimenez de Asua, 1973), leucine and 2-deoxyglucose (Kram et al., 1973; Kram & Tomkins, 1973; Jimenez de Asua & Rozengurt, 1974; Oshiro & Di Paolo, 1974; Bradley & Culp, 1974) increased several fold within minutes of the serum-stimulation of resting 3T3 cells. Similar changes have been reported following serum-stimulation in mouse (Hare, 1972) and chick (Sefton & Rubin, 1971; Rubin & Fodge, 1974; Kletzein & Perdue, 1974c) primary cell lines. The degree of stimulation obtained and the precise time-course of the increased transport differs a good deal between reports from the various laboratories and even between different reports from the same laboratory. The early transport rise appears to be independent of both RNA and protein synthesis (Rubin & Koide, 1975; Jimenez de Asua & Rozengurt, 1974; Kletzein & Perdue, 1974c). However, Jimenez de Asua and Rozengurt (1974) obtained a biphasic increase for 2-deoxyglucose and phosphate transport in serum-stimulated 3T3 cells. In both cases the second phase was inhibited by cycloheximide indicating a requirement for protein synthesis. A similar, though very much smaller, cycloheximide-sensitive component was found for the serum-stimulation of 2-deoxyglucose transport in chick embryo fibroblasts (Kletzein & Perdue, 1974c).

Little evidence is available concerning changes in the kinetic characteristics of transport after serum-stimulation. The increased uridine transport appears to be associated with an increased Vmax with no change in Km (Lemkin & Hare, 1973; Jimenez de Asua et al., 1974). Kletzein & Perdue (1974c) have reported similar results for the serum-stimulated increase in 2-deoxyglucose transport in chick embryo fibroblasts. In contrast, Bradley & Culp (1974) found both an increased Vmax and a decreased Km for the 2-deoxyglucose transport of serum-stimulated 3T3 cells.

Membrane changes in virus-transformed cells

Virus-transformed cells do not show density-dependent regulation of growth. This loss of growth control is accompanied by marked changes in the structural, compositional and functional properties of the cell's plasma membrane. Transformed cells show greater agglutinability with various lectins. The glycolipid and glycoprotein compostion of the membrane surface is altered after virus-transformation. Transformed cells exhibit an enhanced proteolytic activity. Transformation appears to increase the fluidity of the lipid phase of the plasma membrane. For furtherinformation and references on these and other changes the reader is referred to the reviews by Tooze (1973) and Rapin & Burger (1974).

Changes in membrane transport properties have been shown to occur after transformation by either RNA or DNA tumour viruses. In untransformed cells the transport of sugars, some amino acids, phosphate and nucleosides decreases with increasing cell density (see earlier). In transformed cells the transport of glucose and its analogues was unaffected by cell density (Weber, 1973; Plagemann, 1973; Schultz & Culp, 1973; Böse & Zlotnick, 1973). Whether this is also true for other substrates remains to be determined since few results are available. Cunningham & Pardee (1969) found a small decrease in uridine transport and a small increase in phosphate transport with increasing Py3T3 cell density. In contrast, Harel et al.(1975) reported a small decrease in phosphate transport with increasing SV3T3 cell density. Foster & Pardee (1969) were unable to find density-dependent changes in amino acid uptake in Py3T3 cells.

Viral or chemical transformation of primary or normal established cell lines appears to increase glucose/2-deoxyglucose transport (Table 1). In general the rate of

Table 1. Summary of previous findings on the effects of cell transformation on the transport of glucose and glucose analogues.

Abbreviations used in table

cell lines	: MEF, REF, HEF, & CEF	- mouse, rat, hamster and chick embryo fibroblasts
	: NRK	- normal rat kidney cells
	: BHK	- baby hamster kidney cells
transforming agent	: MSV	- murine sarcoma virus
	: MLV	- murine leukemia virus
	: HPSV	- hamster pseudotype sarcoma virus
	: Py	- polyoma virus
	: SV40	- simian virus 40
	: RSV	- Rous sarcoma virus
	(ts indicates temperature-sensitive virus mutant)	
	: M	- N-methyl-N'-nitro-N-nitroguanidine
	: D	- 7,12-dimethylbenz(a)anthracene
substrate	: Gl	- glucose
	: 2-DOG	- 2-deoxyglucose
	: 3-MG	- 3-methylglucose
symbols	: ↑	- increase
	: ↓	- decrease
	: ND	- not determined
	: NR	- not reported

cell line	transforming agent	substrate	transport change after transformation	change attributed to alteration in Km	change attributed to alteration in Vmax	incubation period (min)	reference
MEF	MSV	Gl	20-fold ↑	↓	NR	2	Hatanaka, Huebner & Gilden, 1969
MEF	MSV	2-DOG	10-fold ↑	↓	↑	10	Hatanaka, Augl & Gilden, 1970
REF	MSV	Gl	8-fold ↑	↓	small ↑	10	Hatanaka & Gilden, 1970
REF	MSV	2-DOG	4-fold ↑	↓	no change	10	Hatanaka & Gilden, 1970
HEF	HPSV	Gl.	18-fold ↑	↓	↑	10	Hatanaka, Gilden & Kelloff, 1971
MEF	MSV or MLV	2-DOG	no change/small ↑	no change	no change/↑	5	Plagemann, 1973
NRK	MSV	Gl	variable ↑	ND	ND	20	Stephenson et al., 1973
3T3	MSV	2-DOG	no change	no change	no change	10	Bose & Zlotnick, 1973
BHK	Py	2-DOG	3-fold ↑	no change	↑	10	Isselbacher, 1972
3T3	SV40	2-DOG	2-fold ↑	no change	↑	10	Isselbacher, 1972
3T3	Py	2-DOG	2-fold ↑	ND	ND	10	Grimes & Schroeder, 1973
3T3	SV40	2-DOG	2-fold ↑	↓	no change	20	Schultz & Culp, 1973
3T3	Py(ts)	2-DOG	3-fold ↑	ND	ND	30	Eckhart & Weber, 1974
3T3	M or D	2-DOG	2-fold ↑	ND	ND	10	Oshiro & DiPaolo, 1974
3T3	D	2-DOG	2-fold ↑	no change	↑	10	Kuroki & Yamakawa, 1974
CEF	RSV	Gl	15-fold ↑	↓	NR	10	Hatanaka & Hanafusa, 1970
CEF	RSV	2-DOG	4-fold ↑	no change	↑	10	Weber, 1973
CEF	RSV	3-MG	4-fold ↑	no change	↑	1 - 10	Weber, 1973
CEF	RSV	3-MG	3-fold ↑	no change	↑	0.5	Venuta & Rubin, 1973
CEF	RSV(ts)	2-DOG	2-fold ↑	ND	ND	10	Kawai & Hanafusa, 1971
CEF	RSV(ts)	2-DOG	2-fold ↑	ND	ND	10	Martin et al., 1971
CEF	RSV(ts)	2-DOG	2-fold ↑	ND	ND	15	Bader, 1972
CEF	RSV(ts)	2-DOG	3-fold ↑	no change	↑	5	Kletzein & Perdue, 1974b
CEF	RSV(ts)	2-DOG	4-fold ↑	ND	ND	15	Hale et al., 1975

substrate uptake increased about 2-4 fold but Hatanaka and co-workers consistently found much greater increases. The results obtained with glucose itself are of doubtful value since no precautions were taken to eliminate the errors which are associated with its use (see APPENDIX A). In addition, most workers compared cells at rather higher population densities and in the untransformed cells a density-dependent reduction in transport may have been present. These results would thus be misleading since the maximum rate of transport of the untransformed cells would not have been observed.

Results on the transformation of chick embryo fibroblasts (CEF) by temperature-sensitive mutants of the Rous sarcoma virus are particularly interesting. At the non-permissive temperature (41°) cells infected with the mutant virus display normal cell morphology and growth characteristics. At the permissive temperature (37°) cells show transformed cell morphology and growth characteristics. Since virus replication occurs equally well at both temperatures, effects due to transformation and virus-replication can be separated. It has been shown that 2-deoxyglucose transport in CEF did not increase when cells were infected at the non-permissive temperature. When these cells were shifted to the permissive temperature 2-deoxyglucose transport increased 2-3 fold. The rate of transport was correspondingly reduced by the reverse shift from 37°-41° (Martin et al., 1971; Kawai & Hanafusa, 1971; Kletzein & Perdue, 1974b). The changes in either direction proceeded more or less linearly without a time lag and 50% conversion was obtained 5-8 hours after a reciprocal shift of temperature. The change from normal to transformed morphology or vice-versa followed a similar time-course (Kawai & Hanafusa, 1971). Inhibition of DNA synthesis during a shift from 41°-37° did not inhibit either the morphological transformation or the increase in 2-deoxyglucose transport. This result indicates that DNA synthesis is not required for these changes (Martin et al.,

1971; Kawai & Hanafusa, 1971). Kawai & Hanafusa (1971) found that the changes did not require RNA synthesis but were dependent on protein synthesis. In contrast, Bader (1972) has reported that either RNA or protein synthesis inhibitors prevented the increase in 2-deoxyglucose transport. This difference in results may be related to the different strains of the Rous sarcoma virus which were used.

Hatanaka and co-workers maintain that the increased hexose transport of virus-transformed cells was associated with a large reduction (20-fold) in Km with little change in Vmax. Schultz & Culp (1973) also reported a reduction in Km but the change was much smaller (2-fold). Other workers (Isselbacher, 1972; Weber, 1973; Venuta & Rubin, 1973; Plagemann, 1973; Kletzein & Perdue, 1974b) have found that the Km's for 2-deoxyglucose and 3-O-methylglucose were not altered by virus-transformation. Increased transport was, in these reports, entirely attributed to an increase in Vmax. More data is required to resolve these conflicting results.

Romano & Colby (1973; Colby & Romano, 1974) have claimed that the increased rate of 2-deoxyglucose uptake often seen in transformed cells was the result of increased intracellular phosphorylation, not of increased transport. This conclusion was based on their observation that transformed cells accumulated greater amounts of 2-deoxyglucose-6-phosphate than untransformed cells, but the amount of free 2-deoxyglucose which accumulated intracellularly was the same in both cell types. However, analysis of transport depends on initial rates of uptake not equilibrium concentrations. Examination of the data (Fig.2 , Romano & Colby, 1973) shows that the initial rates of uptake into total cell material was in fact 3-fold higher in the transformed cells.

Very little information is available on the effect of virus-transformation on the transport of substrates other

than glucose and its analogues. The rate of uptake of α-aminoisobutyric acid has been reported to be increased in BHK cells transformed by polyoma virus (Isselbacher, 1972) and in 3T3 cells transformed chemically or by polyoma or simian virus (Kuroki & Yamakawa, 1974; Foster & Pardee, 1969; Isselbacher, 1972). In all cases the increase was due to an increased Vmax with no change in Km. Cunningham & Pardee (1969) found that the rate of uptake of inorganic phosphate was slightly higher in Py3T3 than in 3T3 cells. Harel et al. (1975) found no difference for phosphate transport when 3T3 and SV3T3 cells were compared at low population densities.

Kimelberg & Mayhew (1975) have reported an increased ouabain-sensitive K^+ "uptake" in SV3T3 cells compared to 3T3 cells. However, as mentioned previously, a 1 hour labelling period was used in this study and it is not clear that either true influx or true equilibrium uptake values have been obtained. The membrane (Na^+-K^+)-ATPase activity was also found to be higher in the transformed cells. Elligsen et al. (1974) found that the (Na^+-K^+)-ATPase activity of 3T3 and SV3T3 cells were similar at low population densities but enzyme activity decreased at higher cell densities in the normal cells but not in the transformed cells. In contrast, Graham (1972) has reported a higher activity of (Na^+-K^+)-ATPase in normal BHK cells than in PyBHK cells.

Conclusions

The results which have been described provide strong evidence for the involvement of the plasma membrane in the regulation of cell division. The growth-related changes in membrane transport may be particularly important in this process. When this project began (1972) little information was available on the effects of cell population density and virus-transformation on membrane transport.

Since then more results have been reported particularly for the transport of glucose analogues. However, before any clear statement can be made concerning the role of these changes in the regulation of cell proliferation much more information on a variety of transport systems will be required. In this work the transport of K^+, inorganic phosphate and 2-deoxyglucose has been investigated in 3T3 and virus-transformed 3T3 cells.

No discussion of "in vivo" studies on cell growth regulation has been included in this INTRODUCTION. For information on this extensively investigated topic the reader is referred to the book of Bullough (1967) and the recent review by Lozzio et al. (1975).

MATERIALS & METHODS

MATERIALS

Swiss 3T3 and SV3T3 cells were originally obtained from Flow Laboratories Ltd. Py3T3 cells were kindly supplied by Dr. Michael Stoker (I.C.R.F. Laboratories, London) and by Ms. Esther Steinhardt (Department of Virology, University of Glasgow). Each cell line was ensured to be free from mycoplasma contamination (aceto-orcein staining) and kept as frozen stock in a Union Carbide LR 40 Liquid Nitrogen refrigerator. Fresh cultures were restarted from this supply every 2-3 months.

DMEM growth medium, glutamine, calf and foetal bovine serum, balanced salt solutions and amino acid and vitamin concentrates were from Flow Laboratories Ltd. Plastic Petri dishes were purchased from the Nunc Co., Denmark. Gentamicin was from Roussel Laboratories Ltd.

Glucose was obtained from Boots Co. Ltd. and Sorbitol from Laporte Industries Ltd. Ouabain glucoside was from the Laboratoire Nativelle, Paris. Cytochalasin B was purchased from Aldrich Chemicals. Other drugs and chemicals were supplied by the Sigma Chemical Co.

Radioisotopes were obtained from the Radiochemical Centre, Amersham:

^{86}RbCl (aqueous solution; 0.58mg Rb/ml; 1mCi/ml)

^{32}P-orthophosphate (in dilute HCl; ~10mCi/m-mole)

2-Deoxy-D-[1-^{3}H]glucose (aqueous solution, ~27Ci/m-mole)

Liquid scintillant was NE 250 from Nuclear Enterprises Ltd.

METHODS

Cell culture procedure

Propagation of the cell lines. All cell lines were routinely grown in Dulbecco's modification of Eagle's minimum essential medium (DMEM; Vogt & Dulbecco, 1962) supplemented with 10% calf serum. The broad spectrum antibiotic Gentamicin (0.04mg/ml) was also added to the medium whose composition is shown in Table 2. Cells were maintained at 37° in Roux bottles seeded at 2-5 x 10^4 cells/ml of complete medium. The cells were subcultured every 3-5 days. Whenever possible 3T3 cells were subcultured slightly before the formation of a confluent monolayer so avoiding extensive cell-cell contact for prolonged periods. For experimental purposes the cells were grown attached to plastic Petri dishes (plates) of 9cm or, occasionally, 5cm diameter.

Preparation of plates of cells. The growth medium was removed from a Roux bottle and replaced by 5ml of trypsin (0.025% in Ca^{++}/Mg^{++} free Earle's Balanced Salt Solution). The cells were incubated at 37° for 10 minutes by which time the cells had detached from the substratum. The trypsin was neutralised by the addition of 45ml of complete medium to the bottle. A single cell suspension was formed by repeated aspiration into and out of a syringe fitted with a wide-bore (2mm) stainless steel needle. Medium was added to the suspension to produce the desired cell concentration and aliquots were dispensed into sterile dishes through an 'automatic' pipetting machine (BBL model 40). The dishes were stacked in plastic boxes and equilibrated with a 95% air/5% CO_2 mixture. The boxes were tightly sealed with plastic tape and incubated at 37°.

Table 2 . Dulbecco's Modification of Eagle's Medium (DMEM)

Amino acids	mg/l
L-arginine. HCl	84.00
L-cystine	48.00
L-glutamine	584.00
Glycine	30.00
L-histidine. HCl. H_2O	42.00
L-isoleucine	104.80
L-leucine	104.80
L-lysine. HCl	146.20
L-methionine	30.00
L-phenylalanine	66.00
L-serine	42.00
L-threonine	95.20
L-tryptophan	16.00
L-tyrosine	72.00
L-valine	93.60

Vitamins	mg/l
D-Ca pantothenate	4.00
Choline Chloride	4.00
Folic acid	4.00
i-inositol	7.00
Nicotinamide	4.00
Pyridoxal. HCl	4.00
Riboflavin	0.40
Thiamine. HCl	4.00

Inorganic salts and other components	mg/l
NaCl	6400.00
KCl	400.00
$MgSO_4$	98.00
$CaCl_2$	200.00
$NaH_2PO_4 \cdot H_2O$	125.00
$NaHCO_3$	3700.00
$Fe(NO_3)_3 \cdot 9H_2O$	0.10
Na pyruvate	110.00
D-Glucose	4500.00
Phenol Red Na	15.90

Measurement of the cell characteristics

Cell number and cell volume. A trypsinised cell suspension was diluted with ISOTON counting fluid and the cell number and cell volume were measured simultaneously with a Coulter Counter (model ZF) linked to a Coulter Channelyser C 1000. The instrument was calibrated with latex spheres of known volume. Fig. 1 shows a typical example of the cell volume distribution as displayed on the Channelyser for a sample of 3T3 cells. For each sample the number of cells in channels 0, 10, 20....99 were recorded and the cell number and cell volume were calculated with Simpson's rule of integration. It has been found that the accuracy thus achieved was within 1% of that obtained when the 100 channels in the full curve were employed (Boardman et al., 1974). The cell volumes were found to be log-normally distributed with a mean volume (in this case) of 3.66 $\mu l/10^9$ cells. Within a single batch of dishes little variation in cell number and cell volume was found. In a randomly chosen example the cell number S.E. was 1.9% of the mean and the cell volume S.E. was 1.4% of the mean.

Cell water. The proportion of the total cell volume occupied by water was determined by the method of Lamb & MacKinnon (1971). Cells were placed in a solution made hypertonic by the addition of KCl and the change in cell volume was measured. The intracellular water (W) was calculated from:

$$W = (V_1 - V_2)/(1 - \pi_1/\pi_2)$$

where V_1 is the cell volume at osmotic pressure π_1, and V_2 is the cell volume at osmotic pressure π_2. The results showed that there was no significant difference between cell lines for the ratio of cell water/total cell volume. A value of 0.80 was used in all calculations.

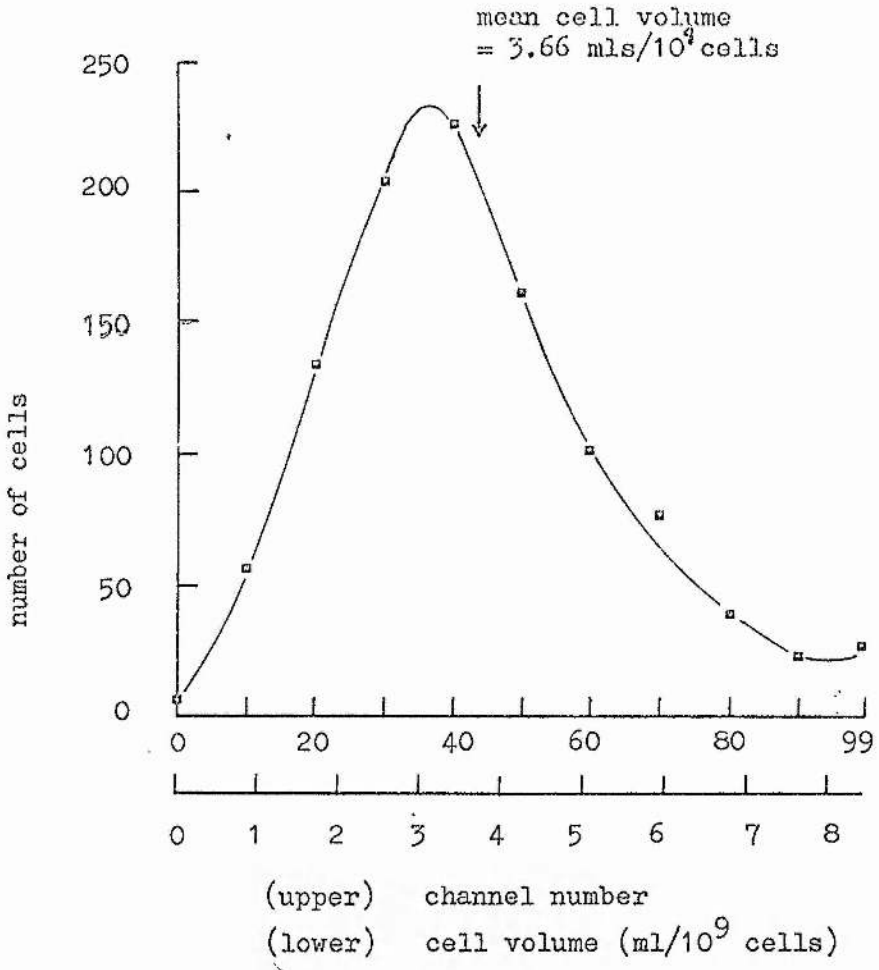


Fig. 1. Typical volume distribution of confluent 3T3 cells. Washed cells were detached from a 9cm Petri dish by incubation for 10 min at 37° with 2ml of trypsin (0.25% in Ca/Mg-free Earle's B.S.S.). Ten ml of Krebs solution were added to the dish and a single cell suspension was formed by 'syringing' through a wide-bore needle as described previously. A 1ml sample of the cell suspension was diluted with 9ml of ISOTON counting fluid and a 0.5ml sample was drawn through a Coulter Counter (model ZF) and the resulting pulse heights displayed on a C1000 pulse height analyser. The figure shows a plot of the number of cells in channels 0, 10, 20....99. The instrument was calibrated using latex spheres of known volume. The number of cells/dish and the mean cell volume/dish were calculated by integration of the curve using Simpson's rule.

Radioactive flux and uptake measurements

On influx and uptake. The terms 'influx' and 'uptake' are sometimes used interchangeably in the literature. The failure to differentiate clearly between them has led to confusion in the interpretation of results. When a radiolabelled permeant, $^{*}s$, is added to the solution bathing cells the appearance of intracellular label generally occurs exponentially as shown in Fig. 2 . The term influx must be reserved to describe the initial rate of uptake of s whilst uptake should refer to the intracellular concentration of s at equilibrium. When describing a curve such as that shown in Fig. 2 the use of the term uptake curve or simply uptake is acceptable.

The most common mistake when measuring an influx is the failure to determine rates at sufficiently early times. The unidirectional influx can only be calculated from the initial rate of uptake. Later, an apparent reduction in the forward rate occurs due to the appearance of a significant backflux.

Uptake of metabolisable substrates. When the permeant is an intracellular substrate its intracellular conversion to a non-permeant form will effectively eliminate backflux, prolong the linearity of the uptake curve and thus the period over which initial rates can be determined. In this study the uptake of phosphate and 2-deoxyglucose were affected in this way. This process of intracellular 'trapping' introduces an additional difficulty to the measurement of transport rates. In a coupled series of reactions the kinetics of the overall reaction reflect only the rate-limiting step of the whole process. How can one be sure that the measured transport rate is not limited by intracellular enzyme activities? A low level of intracellular free substrate indicates that translocation across the membrane

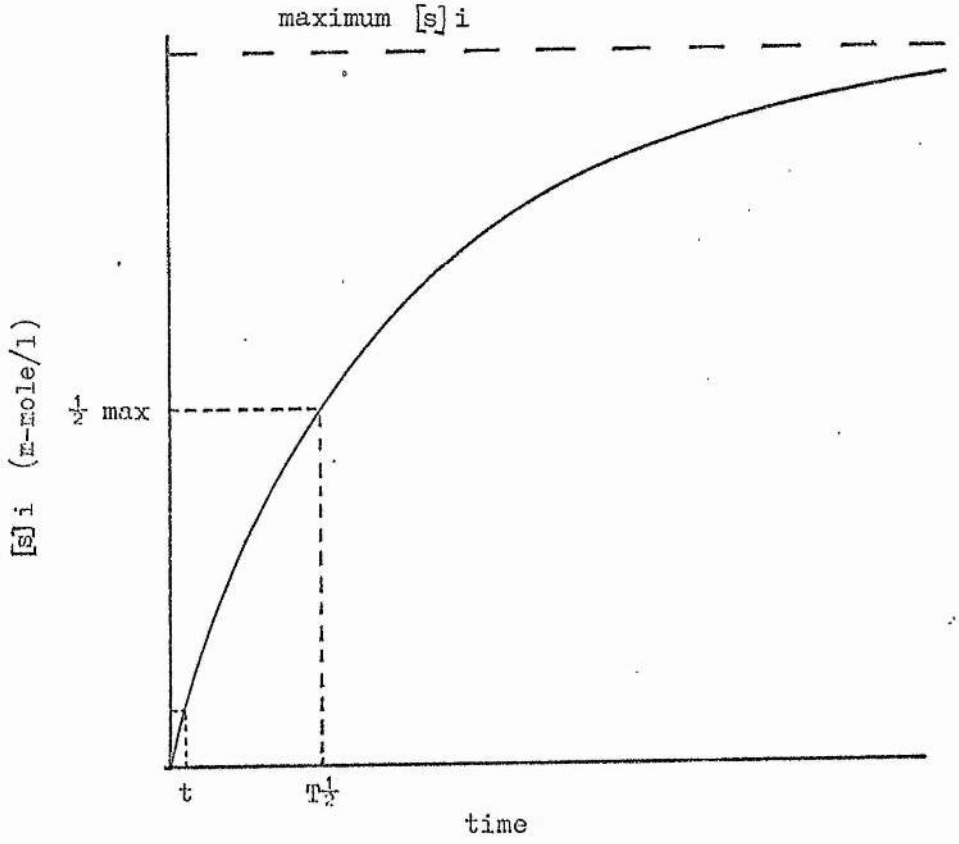


Fig. 2. Representative uptake curve for a permeant, s. Dishes of cells are incubated for increasing periods of time in an isotonic salt solution containing *s and then washed in an ice-cold salt solution to remove extracellular radioactivity. The radioactive content of the cells is measured and the intracellular molarity of s is calculated from the known specific activity of *s in the external solution. The influx is calculated from the initial rate of uptake:

$$J \text{ in} = [s]_i / t$$

where J in is the influx in m-mole/l. min and $[s]i$ the intracellular concentration of s (m-mole/l) at time t (min). For accurate influx determinations t must be small in relation to the half-time of uptake ($T_{\frac{1}{2}}$). The equilibrium concentration is approached asymptotically.

and not intracellular metabolism is limiting the rate of uptake. A demonstration that the rate of uptake of a substrate into intact cells is lower than the enzyme activity for the substrate measured in cell extracts is also evidence that metabolism is not rate-limiting.

Influx and uptake measurements. For all the substances examined the basic procedures for influx and uptake measurements were identical.

a) The growth medium was removed from dishes of cells and replaced by 10ml of radiolabelled Krebs solution (Table 3). The cells were incubated at 37° for the required period of time.

b) The incubation was terminated by aspirating off the radioactive solution and washing the cells in 4 changes of Krebs at 2°. The washing was complete within 30 seconds.

c) Washed cells were detached from the substratum by incubation for 10 minutes at 37° with 2ml trypsin (0.25% in Ca/Mg-free Earle's B.S.S.).

d) Ten ml of Krebs were added to the dish and a single cell suspension was formed by 'syringing' through a wide-bore needle.

e) A 1ml aliquot of the cell suspension was diluted with 9ml of ISOTON and the cell number and mean cell volume determined as described previously.

f) An aliquot of the cell suspension was placed in a scintillation vial for radioactive counting in a liquid scintillation spectrometer (Packard, Tri-Carb model 3320). For high energy emitters (^{86}Rb, ^{32}Pi) 10ml of the cell suspension were counted without liquid scintillant by the Cerenkov effect (Garrahan & Glynn, 1966). For ^{3}H-labelled compounds 1ml of the cell suspension was counted after the addition of 10ml of NE250 scintillant.

Table 3 Composition of experimental salt solutions

Components	Krebs		Ca-sorbitol	
NaCl	137	m-mole/l	-	
KCl	5.4	m-mole/l	-	
$CaCl_2$	2.8	m-mole/l	2.8	m-mole/l
$MgSO_4 \cdot 7H_2O$	1.2	m-mole/l	1.2	m-mole/l
NaH_2PO_4	0.26	m-mole/l	-	
KH_2PO_4	0.29	m-mole/l	-	
HCl	12	m-mole/l	12	m-mole/l
Tris base	1660	mg/l	1660	mg/l
D-glucose	1000	mg/l	-	
Sorbitol	-		49.9	g/l
Calf serum	10	ml/l	-	

Dishes without cells were run concurrently to enable correction for radioactivity adsorbed by the dish and not removed by washing. When necessary counts were quench corrected by the channel ratio method. The influx was measured using suitably short incubation periods. All calculations were made on an Olivetti P101 or P602 programme calculator. Uptake was expressed as m-mole/l of intracellular water and influx as m-mole/l. min.

Efflux measurements. A single-plate procedure was used for all efflux measurements. This involves measuring the washout of radioactivity from preloaded cells into successive changes of inactive collecting solution.

a) The growth medium was removed from dishes of cells and replaced by 10ml of a radiolabelled Krebs solution.

b) The cells were incubated with the label for a period of time sufficient to allow equilibration of intra- and extracellular radioactivity.

c) The radioactive solution was aspirated off and the loaded cells were washed (x4) with Krebs at 2° and then rinsed quickly with Krebs at 37°.

d) Ten ml of inactive Krebs were added to the dish which was then incubated at 37°.

e) After 5 minutes the effluent was quickly poured into a scintillation vial for isotope counting and a further 10ml of inactive Krebs were added to the dish. This procedure continued for a total of 30 minutes (i.e. 6 successive 5 minute intervals).

f) The cells were trypsinised and the cell number, cell volume and remaining cellular radioactivity were measured as previously described.

The fraction of radioactivity lost per 5 minute interval was calculated. For exponential efflux from a single intracellular compartment the fractional loss is constant. Any treatment affecting the efflux can be detected from a

histogram plot of the fraction lost against time.

The equilibrium intracellular concentration, $[S]_i$, the half-time of efflux ($T\frac{1}{2}$) can also be obtained from the data. The efflux (J out) can then be calculated with the equation:

$$J \text{ out} = (\ln 2 / T\tfrac{1}{2}) . [S]_i$$

This equation (Keynes & Lewis, 1951) was derived for steady-state conditions. Strictly, the equation could not be applied when the intracellular concentration was changing with time. The equation has been used when the rate of change of concentration was small compared to the control flux.

Intracellular sodium and potassium measurements

Flame photometer. Cells were washed (x4) with Ca-Sorbitol (Table 3) at 2° and extracted in 5ml of deionised water for 1 hour. Na and K were measured by comparison with an appropriate standard on an EEL (model A) flame photometer. Cell number and cell volume were determined from duplicate, unextracted dishes of cells. The Na and K were calculated and expressed as m-mole/l of intracellular water.

The Na and K concentrations of experimental solutions were checked using an EEL 450 instrument.

Isotope equilibration. Values for K were also obtained from isotope uptake measurements. The cells were incubated with ^{86}Rb labelled Krebs for 3-4 hours and the equilibrium concentration determined following the general procedure described previously.

Partition of intracellular phosphate

Isotope equilibration. The acid-soluble phosphate was extracted into perchloric acid. Molybdate, which combines with only inorganic phosphate, was added and the resulting phosphomolybdate was extracted into ethyl acetate. The acid-soluble phosphate pool can thus be separated into its organic and inorganic fractions.

Plates of cells were exposed to ^{32}Pi labelled Krebs for the required period of time. The cells were washed (x4) with Pi-free Krebs at 2°. The washed cells were kept at 2° and 3.2ml deionised water, followed by 1.0ml of PATSA [1] were added to the dish. The acid-soluble phosphate fraction was extracted for 45 minutes at 2°. Extraction for longer periods caused no significant increase in ^{32}Pi counts. A 2.1ml aliquot of the extract was placed in a test-tube and 1.5ml of molybdate reagent [2] followed by 4.0ml of ethyl acetate were added to the tube. Thorough mixing was achieved using a rotamixer (10 x 2 seconds). The tubes were left for 5 minutes to allow the phases to separate. The ethyl acetate and aqueous layers were separated and placed in scintillation counting vials and the samples counted for ^{32}Pi. Correction for chemical quenching was made by recounting samples after the addition of a small volume of a known ^{32}Pi standard to each vial. Total cellular phosphate was measured on duplicate plates with ^{32}Pi following the general uptake procedure described previously. The size of the acid-insoluble phosphate fraction was calculated by subtracting the acid-soluble fraction from the total.

1. PATSA reagent was prepared by adding 4g of tungsto-silicic acid to 5ml of 70% perchloric acid and diluting to 50ml with deionised water.
2. Molybdate reagent was prepared by dissolving 13.1g of NaCl and 1.45g of Na molybdate per 100ml of deionised water.

RESULTS

K^+ transport in 3T3 and virus-transformed 3T3 cells

Effect of the cell population density on the K^+ uptake. The short half-life of ^{42}K complicates its use as a tracer in K^+ transport studies. For this reason ^{86}Rb, which has a substantially longer half-life, is often used as an alternative tracer (Boardman et al., 1974; Cuff & Lichtman, 1975). Preliminary experiments (unreported) demonstrated that Rb^+ and K^+ were handled similarly by the cells used here. ^{86}Rb has therefore been used as a K^+ tracer throughout this work.

Initial experiments were carried out to examine the effect of cell population density on the uptake of K^+ by 3T3 (Fig. 3) and Py3T3 (Fig. 4) cells. The calculated characteristics for K^+ uptake by both cell lines are shown in Table 4 . The 2-day-old 3T3 cultures were actively growing and contained about $1x10^6$ cells/ dish. The 6-day-old cultures contained about $3x10^6$ cells/dish and had reached the stationary phase. The results show that this 3-fold increase in cell density was accompanied by a 30% reduction in cellular K^+ and a 52% decrease in the K^+ influx. The half-time of K^+ uptake was apparently unaffected by the increased cell density (Table 4). In the virus-transformed Py3T3 cell line, the cellular K^+, the half-time of K^+ uptake and the K^+ influx were all unaltered by an increase in cell density from 2 to 12 ($x10^6$) cells/dish. The results indicate that in untransformed cells K^+ transport shows a marked density-dependency. The data indicates that K^+ uptake was linear beyond 10 minutes in both cell lines and 10 minutes was therefore considered a suitable incubation period for measurement of K^+ influx. The effect of cell population density on K^+ influx was further investigated for 3T3, Py3T3 and SV3T3 cells.

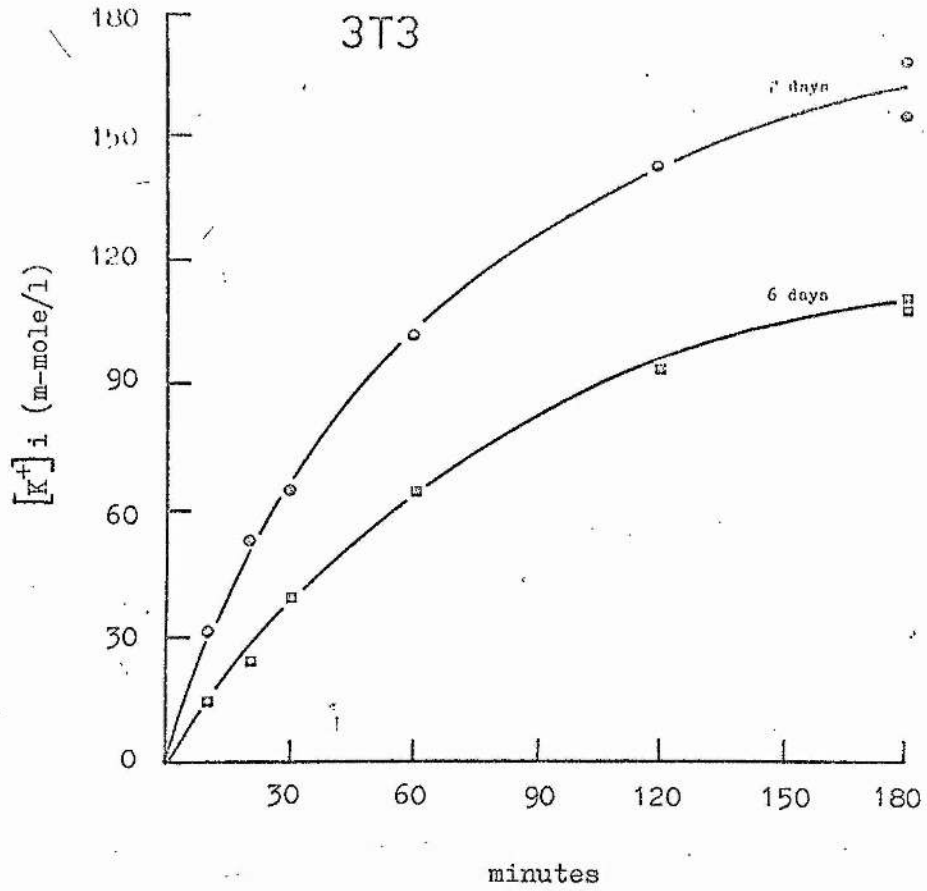


Fig. 3. Effect of the cell population density on the uptake of K^+ by 3T3 cells. Cells were seeded at 0.5×10^6 cells/9cm dish and the K^+ uptake was measured after 2 (o) and 6 (□) days growth. At zero time the growth medium was replaced with ^{86}Rb-labelled Krebs solution. After periods ranging from 10 min to 3 hours the labelling Krebs was removed, the cells washed (x4) with Krebs at 2°, detached from the dish with trypsin and analysed for ^{86}Rb activity, cell number and cell volume. The K^+ concentration of the Krebs solution was 5.40 m-mole/l and the ^{86}Rb activity was about 0.2μCi/ml. The temperature of the experimental solutions was 37° (±0.75°). Each point represents the value obtained for $[K^+]_i$ from a single plate of cells. The calculated characteristics of K^+ uptake are shown in Table 4.

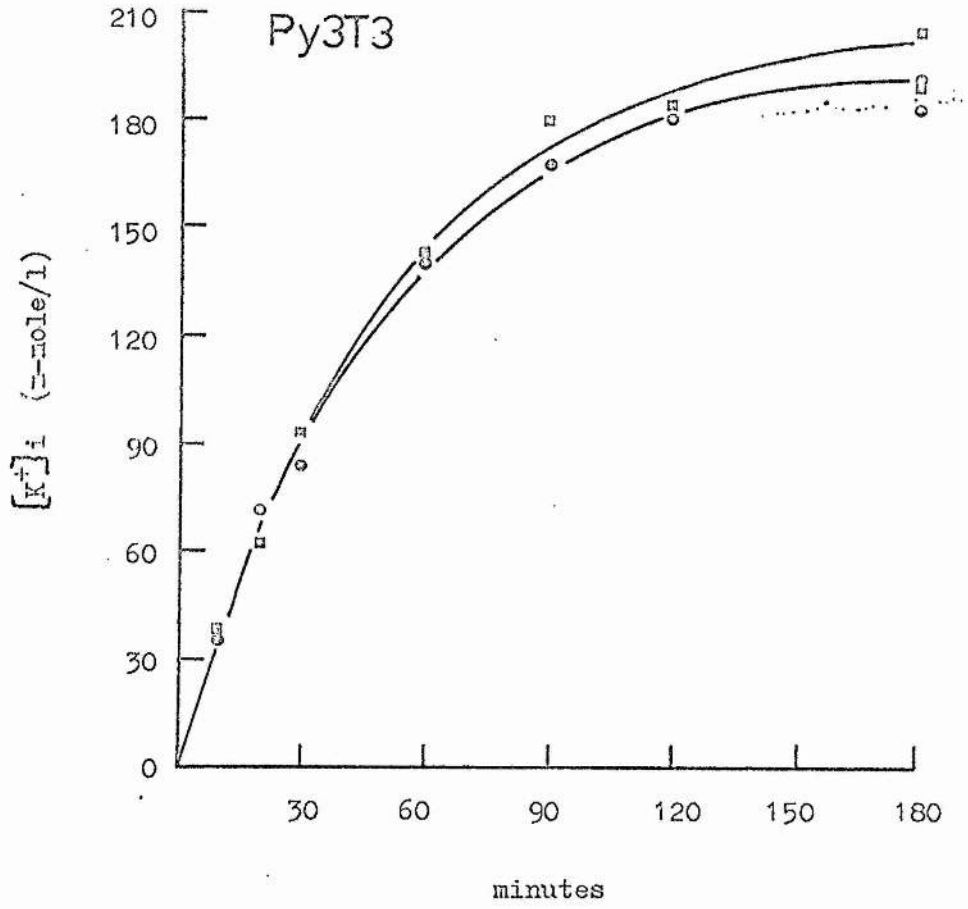


Fig. 4. Effect of the cell population density on the uptake of K^+ by Py3T3 cells. Cells were seeded at 0.8×10^6 cells/9cm dish. After 2 (o) and 6 (□) days growth the K^+ uptake was measured according to the procedure described in the legend to Fig. 3. Each point represents the value obtained for $[K^+]i$ from a single plate of cells. The calculated characteristics of K^+ uptake are shown in Table 4.

Table 4. Effect of the cell population density on the K^+ uptake characteristics of 3T3 and Py3T3 cells

cell line	days of growth	mean cell no. /dish ($\times 10^{-6}$)	$[K^+]_i$ m-mole/l	$T_{\frac{1}{2}}$ uptake (min)	K^+ influx m-mole/l.min
3T3	2	1.04 ($\pm$0.04)	166.3	39.6	3.13
	6	2.98 ($\pm$0.11)	116.0	40.7	1.49
Py3T3	2	1.94 ($\pm$0.09)	218.5	25.9	5.56
	6	11.81 ($\pm$0.21)	215.2	27.1	3.82

The values shown here were calculated after logarithmic transformation of the data presented in Figs. 3 & 4.

Effect of the cell population density on the K^+ influx.
Fig. 5 shows the results of an experiment in which the K^+ influx of 3T3 cells was measured each day, for several days, after cell seeding. The K^+ influx showed a small increase over the first 3 days reaching a maximum value of 3.85 m-mole /l. min. The influx then steadily decreased to a value of 1.03 m-mole/l. min. after 7 days of growth. The onset of the decrease in the K^+ influx appears to precede the cessation of cell growth since the influx fell markedly between days 3 and 5, a period of rapid cell growth.

It was possible that this reduced K^+ influx was not directly related to cell density but was a reflection of the culture age. In order to eliminate this possibility the effect of the cell poulation density on the K^+ influx into 3T3 cells was more extensively investigated using an alternative procedure. Cells were seeded at various densities in the range 0.2 to 1.0 ($x10^6$) cells/9cm dish and allowed 3 or 4 days of growth. In this way the K^+ influx of cultures of the same age but with a range of cell densities could be measured. The combined results of several experiments are shown in Fig. 6 . A 4-fold reduction in the K^+ influx was found over the cell density range examined. The values for 3- and 4-day-old cultures were found to lie on the same curve. The cultures seeded at high initial densities ($1.0x10^6$ cells/dish) had reached saturation within 4 days since there was no further increase in cell numbers during the following 48 hours. These results indicate that the reduction in K^+ transport was directly related to an increased cell density. The data shown previously in Fig. 5 has been included in Fig. 6 to facilitate a direct comparison of the two sets of results. In general the results are in close agreement. However, the small rise in K^+ influx seen at low cell densities in Fig. 5 was not apparent in the data shown in Fig. 6 . Possible reasons for this difference will be considered in the DISCUSSION.

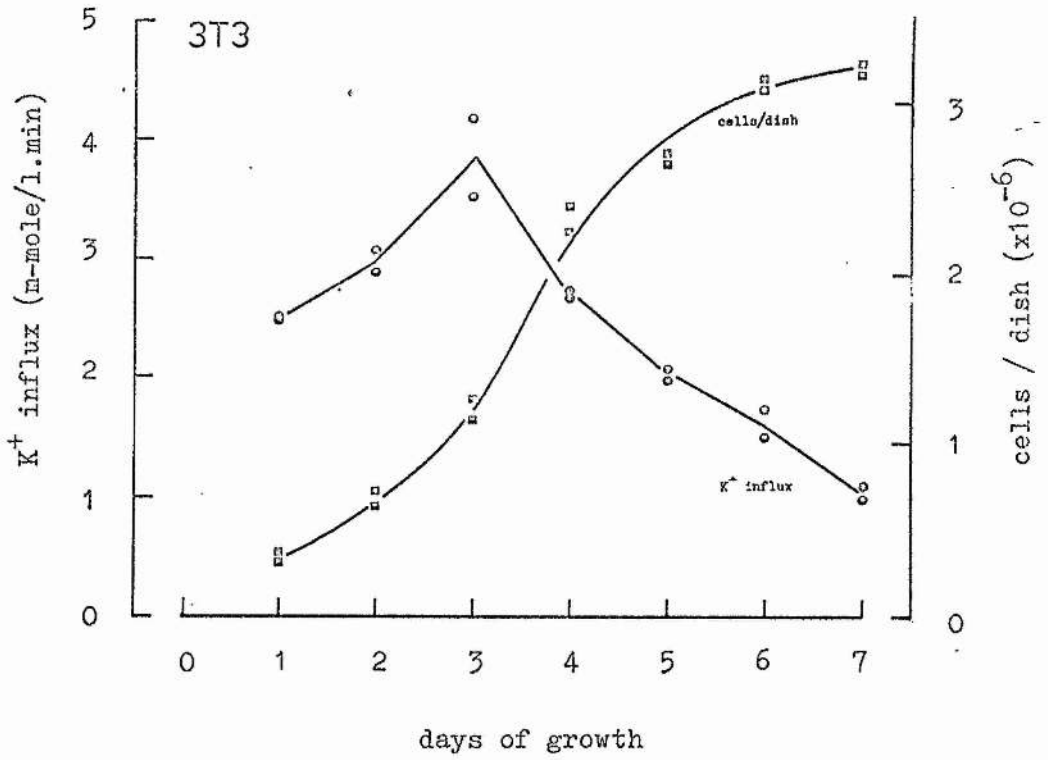


Fig. 5. Effect of the cell population density on the K^+ influx into 3T3 cells. Cells were seeded at $0.5x10^6$ cells/ 9cm dish. The K^+ influx (○) was measured at the same time each day from 1 to 7 days after seeding. Incubations with ^{86}Rb were carried out for 10 min at 37° according to the procedure described in the legend to Fig. 3. The number of cells/dish (□) at the time of influx measurement has also been included in the figure. Each point represents the value for K^+ influx or cell number obtained from a single plate of cells. The lines were drawn by eye.

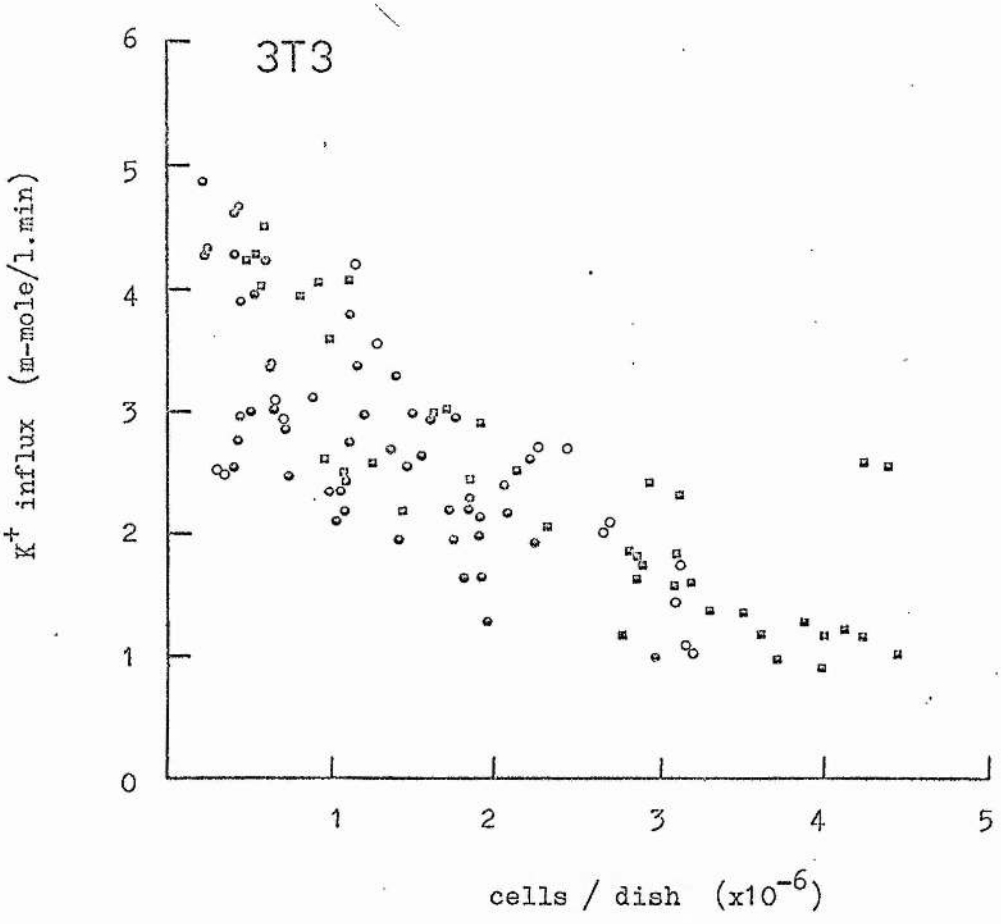


Fig. 6. Effect of the cell population density on the K^+ influx into 3T3 cells. Cells were seeded at various densities in the range 0.?-1.0 ($x10^6$) cells/9cm dish and the K^+ influx measured after 3 (○) or 4 (□) days growth. Incubations with ^{86}Rb were carried out for 10 min at 37° according to the procedure described in the legend to Fig. 5. Each point represents the value for K^+ influx obtained from a single plate of cells. The results show that the K^+ influx decreased with increasing cell density and that the values obtained after 3 and 4 days growth lie on the same curve. The data previously presented for 3T3 cells in Fig. 5 (○) has been included in this graph to facilitate a direct comparison of the two sets of results.

The effect of cell population density on the influx of K^+ into Py3T3 and SV3T3 cells was followed using a similar procedure to that described for 3T3 cells in Fig. 6 . The combined results of several experiments are shown in Fig. 7 . At low cell densities the Py3T3 cells showed a wide range of values for the K^+ influx. At higher cell densities the K^+ influx appeared to be somewhat less variable. There was no evidence of a reduction in the K^+ influx with increasing cell density. Indeed, when individual experiments were analysed a small, but significant, increase in the K^+ influx accompanied increased cell density. Fewer observations were made for the other transformed cell line, SV3T3. The values for K^+ influx were generally slightly lower than in Py3T3 cells but, once again, there was no tendency for the influx to decrease with increasing cell density.

These results suggest that the K^+ transport capacity of 3T3 cells is not markedly altered by virus-transformation. At low cell densities all three cell lines showed similar values for the K^+ influx. However, an important change after virus-transformation was the abolition of the density-dependent decrease in K^+ influx observed in the 3T3 cells. This reduced K^+ transport could be caused by a reduction in either the 'active' or 'passive' entry of K^+ or a combination of both components. This problem was investigated by measuring the K^+ influx into 3T3 cells at different population densities in the presence or absence of either ouabain or metabolic inhibitors. The results (Fig.8) show that after treatment for 1 hour with DNP+IAA the K^+ influx was reduced to a low level which was independent of cell density (mean value = $\cdot 156 \pm \cdot 025$). If this low level represents the 'passive' entry of K^+ into the cell, this result indicates that the permeability of the 3T3 cell membrane is unaffected by an increase in cell density. The reduced K^+ influx with increased cell density must thus be very largely caused by a reduction in 'active' K^+ transport. The results

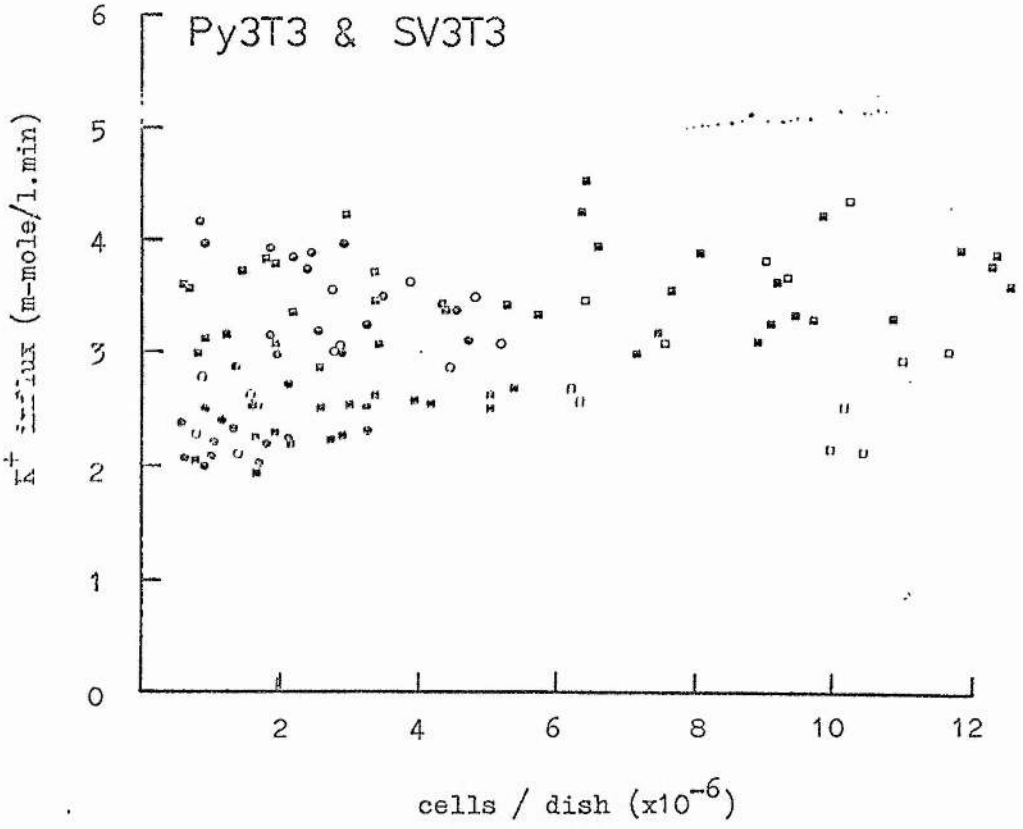


Fig. 7. Effect of the cell population density on the K^+ influx into Py3T3 (closed symbols) and SV3T3 (open symbols) cells. Cells were seeded at various densities in the range 0.2-1.5 ($x10^6$) cells/9cm dish and the K^+ influx measured after 3 (●) or 4 (■) days of growth. Incubations with ^{86}Rb were carried out for 10 min at 37° according to the procedure described in the legend to Fig. 3. Each point represents the value for K^+ influx obtained from a single plate of cells.

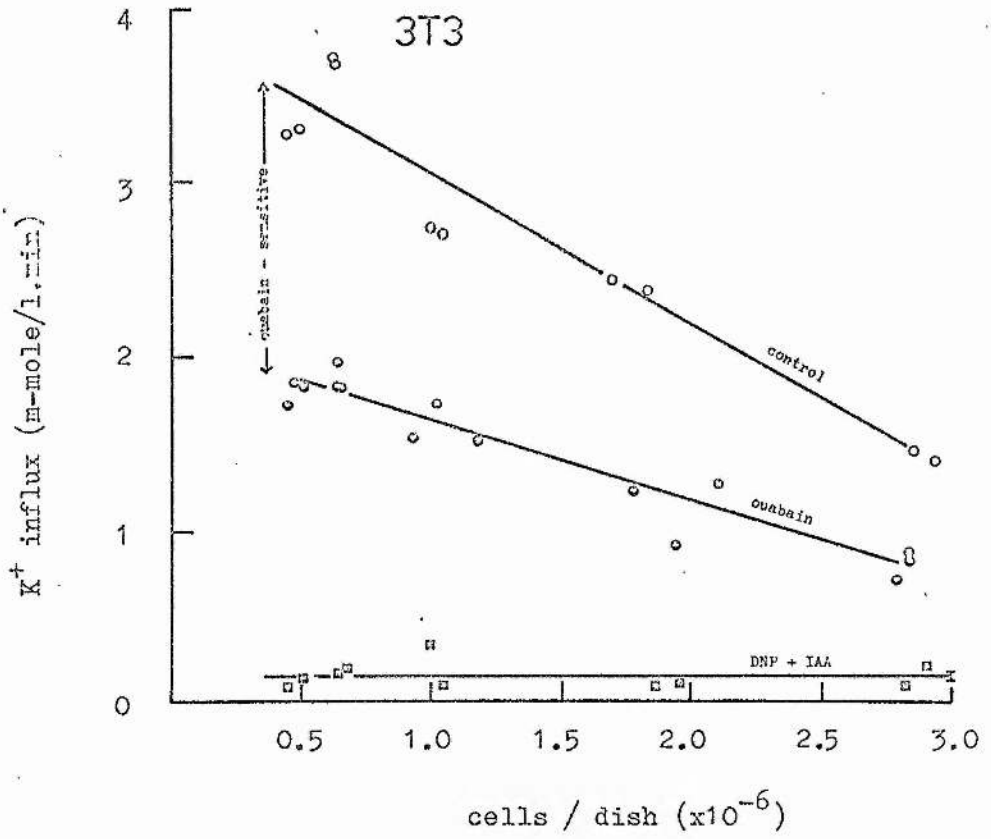


Fig. 8. K^+ influx into 3T3 cells as a function of cell population density in the presence and absence of transport inhibitors. Cells were seeded at 0.2, 0.3, 0.4, 0.7 or 1.0 ($x10^6$) cells/9cm dish and used after 4 days of growth. The K^+ influx, in the presence or absence of inhibitors, was measured for each initial seeding density. The cells were pretreated for 1 hour with control Krebs (O), Krebs containing 10^{-3} M ouabain (●) or 10^{-3} M DNP+ 10^{-4} M IAA (□). Incubations with ^{86}Rb were carried out for 10 min at 37° according to the procedure described in the legend to Fig. 3. The inhibitors were included in the influx solution. Each point represents the value for K^+ influx obtained from a single dish of cells.

in the presence of ouabain (Fig. 8) support this conclusion. As the total K^+ influx decreased the ouabain-sensitive K^+ influx decreased. Since ouabain is considered to specifically inhibit active Na-K exchange across the cell membrane this result indicates that the reduction in the K^+ influx was due to a decrease in "Na-pump" activity at the higher cell densities. The apparent reduction in the size of the ouabain-insensitive K^+ influx seen in this experiment is a consequence of the incomplete inhibition of Na-K exchange produced by ouabain in these cells which, being mouse cells, are relatively insensitive to the drug. At all cell densities ouabain produced an inhibition of 40 to 50% of the control K^+ influx.

Effect of cell population density on cellular K^+ and Na^+. Preliminary experiments (Table 4) suggested that the reduction in the K^+ influx in 3T3 cells at higher cell densities was accompanied by a decrease in the cellular K^+ concentrations. The effect of the cell population density on the K^+ and Na^+ concentrations of the 3 cell lines was therefore examined in more detail using flame photometry.

Cells were seeded at a single population density and the cell K^+ and Na^+ concentrations were measured after 2 and 6 days of growth. The results (Table 5) show that in 3T3 cells the $[K^+]_i$ decreased markedly with the increase in cell density between days 2 and 6. The $[Na^+]_i$ increased over the same period. In both the transformed lines the $[K^+]_i$ rose slightly between days 2 and 6 with little change in $[Na^+]_i$ (Table 5). The values obtained for 3T3 and Py3T3 cells in this experiment were in reasonable agreement with those obtained in the isotopic equilibration study reported in Table 4 .

Table 5 . Effect of the cell population density on cellular K^+ and Na^+ concentrations

		3T3	Py3T3	SV3T3
2 days	$[K^+]$ i m-mole / l	162 ± 3	170 ± 4	188 ± 4
	$[Na^+]$i	6.8 ± 0.9	14.8 ± 1.5	19.2 ± 0.9
6 days	$[K^+]$ i m-mole / l	117 ± 4	192 ± 2	211 ± 8
	$[Na^+]$i	12.3 ± 2.4	19.5 ± 0.3	21 ± 4.0

3T3 cells were seeded at 0.5×10^6 cells/dish, Py3T3 and SV3T3 cells at 0.7×10^6 cells/dish. After 2 and 6 days of growth cellular K and Na were determined by flame photometry as described in METHODS. Duplicate plates of cells were used to measure cell number/dish and mean cell volume/dish. The values show the mean (± S.E.) for 6 determinations of [K]i and [Na]i. Cell densities (cells/dish) at the time of measurement were:

3T3 0.88 ± 0.02 and 3.14 ± 0.05

Py3T3 1.87 ± 0.04 and 8.93 ± 0.16

SV3T3 2.08 ± 0.04 and 10.16 ± 0.15

Effect of the cell population density on the K^+ efflux. 3T3 cells were seeded at three different densities and allowed to grow for 3 days. After this period dishes of cells at each density were loaded with ^{86}Rb for 3 hours. The washout of isotope from the cells into successive changes of collecting solution was then followed during 5 minute intervals for 30 minutes. The results (Fig. 9) show that the "fractional loss" of K^+ was not significantly ($P>0.25$) different for the cells at different population densities. With the exception of the values for the first 5 minutes the "fractional loss" was effectively constant indicating a single exponential loss of K^+ from these cells. In these experiments the values at 5 minutes are often erroneous due to the technical problems of washing the cells, raising the temperature from 2° to 37° and maintaining accurate timing. From the data shown in Fig. 9 the half-time of K^+ exit and the K^+ efflux can be calculated. The values at the different cell densities are shown in Table 6 . Since the "fractional loss" was independent of cell density, $T\frac{1}{2}$ was also independent of cell density. The $[K^+]_i$, which was calculated from the total cellular ^{86}Rb counts, decreased with increasing cell density. The efflux thus decreased with increasing cell density. The values for the efflux at the various cell densities examined were in close correspondence with the influx values at similar cell densities (Fig. 6). The half-time of exit was in close agreement with the half-time of uptake in 3T3 cells (Table 4).

The effect of cell population density on the washout of ^{86}Rb from preloaded SV3T3 cells is shown in Fig. 10 . This was examined by seeding cells at a fixed density and measuring the efflux after 1, 2 and 6 days of growth. The results show that the "fractional loss" at 1 day (a) was not significantly ($P>0.50$) different from the "fractional loss" after 6 days growth (c). However, at 2 days (b) the "fractional loss" was significantly larger ($P<0.01$),

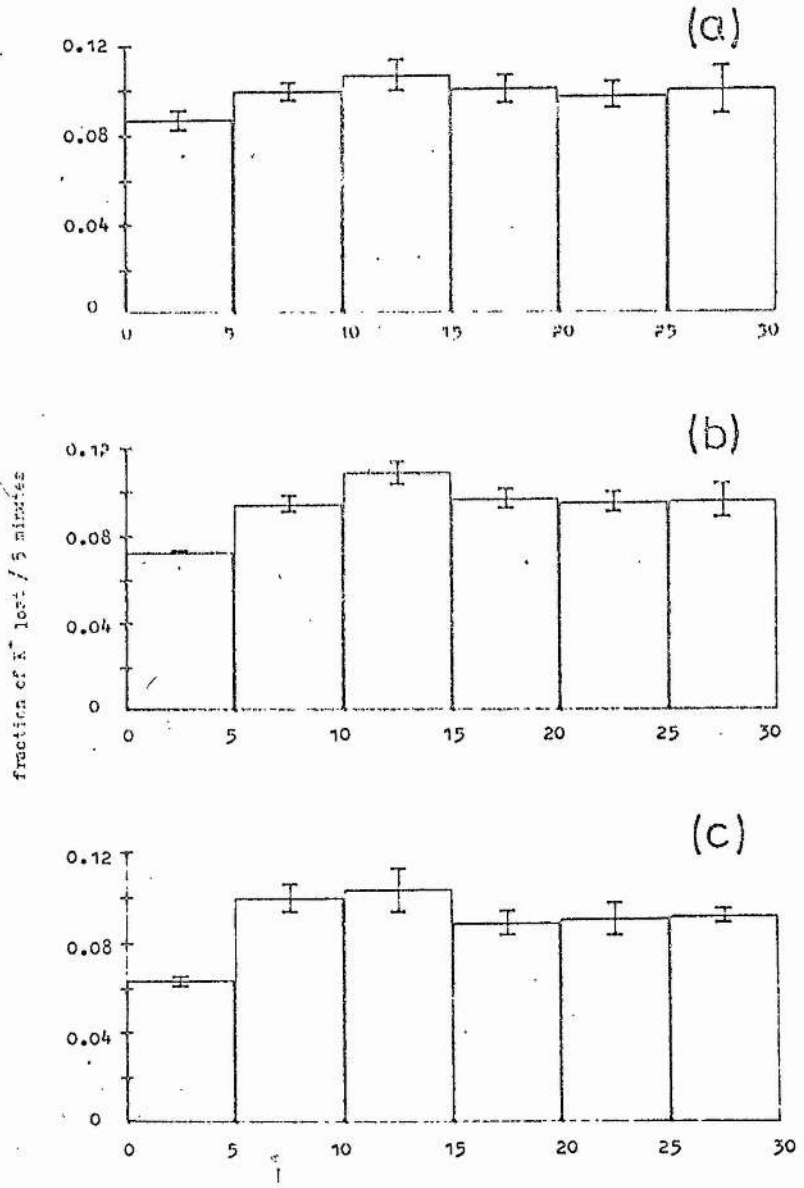


Fig. 9. Effect of the cell population density on the K^+ efflux from 3T3 cells. Cells were seeded at 0.3 (a), 0.5 (b) or 1.0 (c) ($x10^6$) cells/9cm dish and used after 3 days of growth. The cells were "loaded" with ^{86}Rb for 3 hours and then washed (x4) with Krebs at 2° and rinsed rapidly with Krebs at 37°. After 5 min the solution was quickly poured from the dish into a scintillation vial for ^{86}Rb counting. A further 10ml of Krebs were added to the dish and the procedure pepeated for a total of 30 min (6x10ml samples). The cells were then trypsinised and the cell number, cell volume and residual cellular radioactivity were determined as described in METHODS. The column heights represent the mean value (n=2) for the fractional loss of K^+ from the cells into the extracellular solution during successive 5 min collecting periods. The calculated characteristics for K^+ exit from 3T3 cells are shown in Table 6.

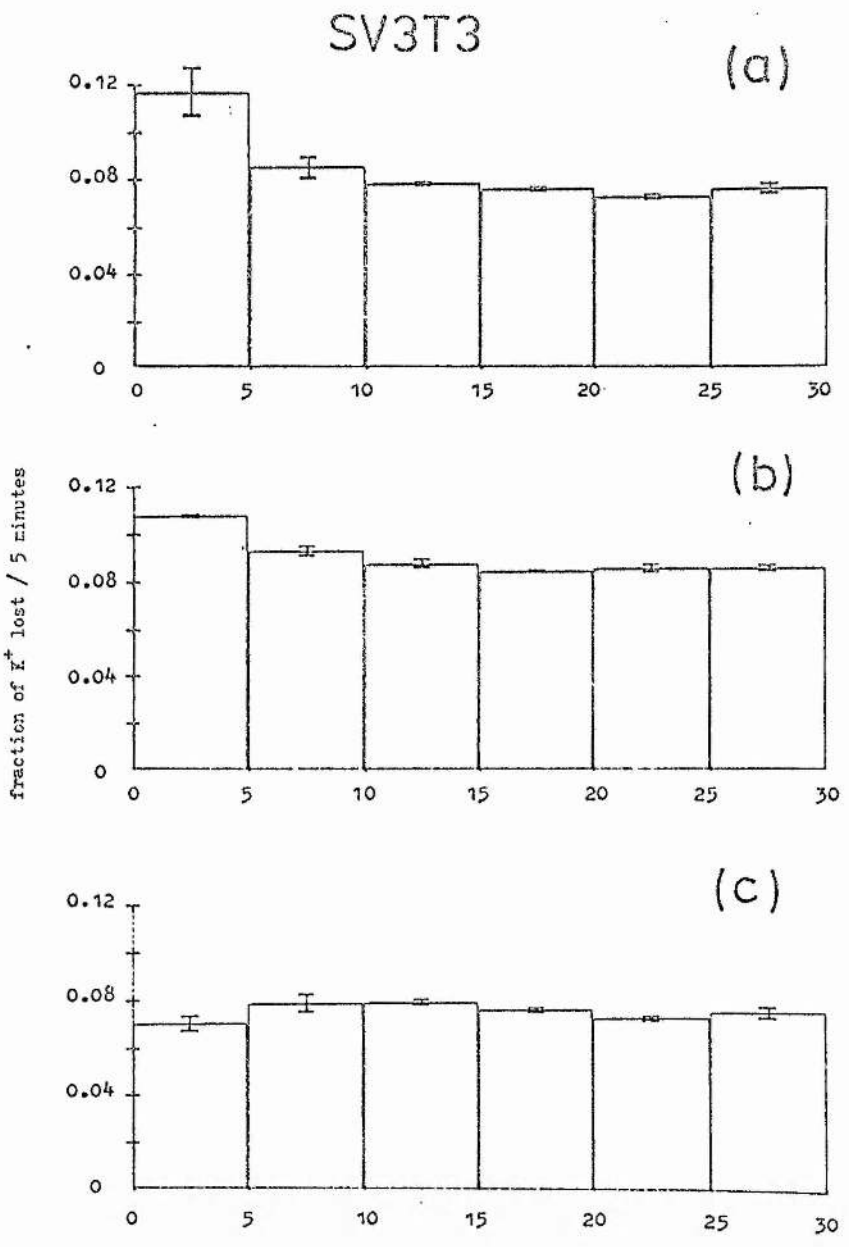


Fig. 10. Effect of the cell population density on the K^+ efflux from SV3T3 cells. Cells were seeded at 1.0×10^6 cells/9cm dish and the K^+ efflux was followed after 1 (a), 2 (b) and 6 (c) days of growth. Experimental details were the same as those described in the legend to Fig. 9. The column heights represent the mean value (n=3) for the fractional loss of K^+ from the cells into the extracellular solution during successive 5 min collecting periods. The calculated characteristics for K^+ exit from SV3T3 cells are shown in Table 6.

Table 6. Effect of cell population density on the characteristics of K^+ exit from 3T3 and SV3T3 cells

		mean cell no./ dish ($\times 10^{-6}$)	mean "fractional loss" /min	$T_{\frac{1}{2}}$ exit (min)	mean $[K^+]_i$	efflux (J out) n-mole/l. min
3T3	(a)	0.65 ± 0.01	0.0202	34.3	185 ± 8	3.74
	(b)	1.01 ± 0.03	0.0197	35.2	163 ± 2	3.21
	(c)	2.02 ± 0.09	0.0190	36.5	136 ± 4	2.58
SV3T3	(a)	1.15 ± 0.03	0.0155	44.7	197 ± 5	3.05
	(b)	2.46 ± 0.07	0.0175	39.6	211 ± 9	3.69
	(c)	10.08 ± 0.40	0.0152	45.6	191 ± 5	2.90

The values shown here were calculated from the ^{86}Rb washout data shown in Figs. 9 and 10 .

but the difference was very small (9%) and could easily have been caused by small differences in technique on the different days when the experiments were carried out. The values for $T\frac{1}{2}$, $[K^+]_i$ and K^+ efflux (Table 6) show no consistent dependence on cell density in these cells. The efflux values are slightly larger than the influx values for SV3T3 cells shown in Fig. 7 , though still within reasonable agreement.

Effect of the cell population density on the kinetic constants for K^+ transport. Cells were seeded at different densities and the K^+ influx from a range of extracellular concentrations (0.58-10.16 m-mole/l) was measured 3 days later. A linear transformation (v against v/s) of the data obtained is shown in Fig. 11 together with a summary of the calculated kinetic constants. The results show that the decreased K^+ transport associated with increased cell density can be attributed to a reduction in Vmax with little change in the Km of the system. This conclusion is strongly supported by the combined results of several experiments which show (Fig. 12a) a close inverse correlation between the cell density and the Vmax for K^+ influx in 3T3 cells. On the other hand, Km (Fig. 12 b) appears to be independent of cell density with a mean value of 1.50 ± 0.10 m-mole/l.

The kinetics of K^+ influx into Py3T3 cells are shown in Fig. 13 . In the transformed cells the Vmax appears to increase with increasing cell population density. The values for Km were similar to those found for 3T3 cells and showed no dependency on cell density.

An altered response of virus-transformed cells to ouabain

It is well known that ouabain causes an inhibition of Na^+ and K^+ movement across cell membranes (Glynn, 1964).

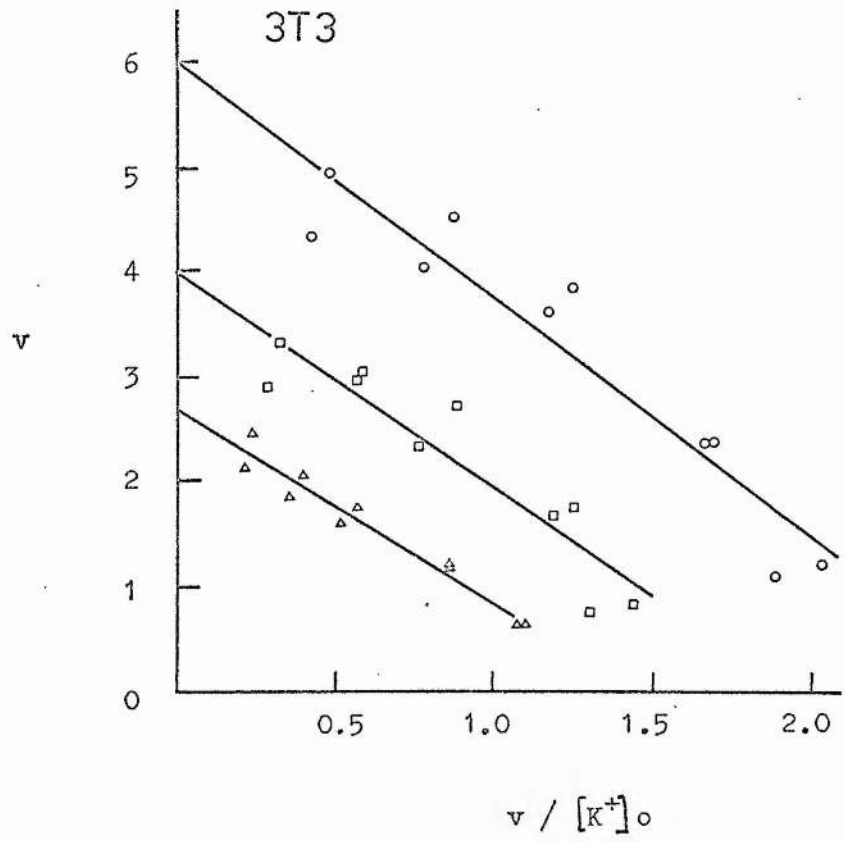


Fig. 11. Effect of the cell population density on the kinetic constants for K^+ transport by 3T3 cells. Cells were seeded at 0.25 (○), 0.50 (□) or 1.0 (△) ($\times 10^6$) cells/9cm dish and used after 3 days of growth. The K^+ influx was determined from Krebs with $[K^+]$ in the range 0.58-10.16 m-mole/l. Incubations with ^{86}Rb were carried out for 10 min at 37°, according to the procedure described in the legend to Fig.3. The data is shown after linear transformation (v against v/S; APPENDIX B). each point represents a single value for the influx (v) which is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km and Vmax, are shown in the attached table.

seeding density	mean cell no./ dish ($\times 10^{-6}$)	Km m-mole/l	Vmax m-mole/l. min
○ 0.25	0.63 ($\pm$0.02)	2.17	6.01
□ 0.50	1.60 ($\pm$0.04)	2.04	3.98
△ 1.00	2.51 ($\pm$0.06)	1.82	2.67

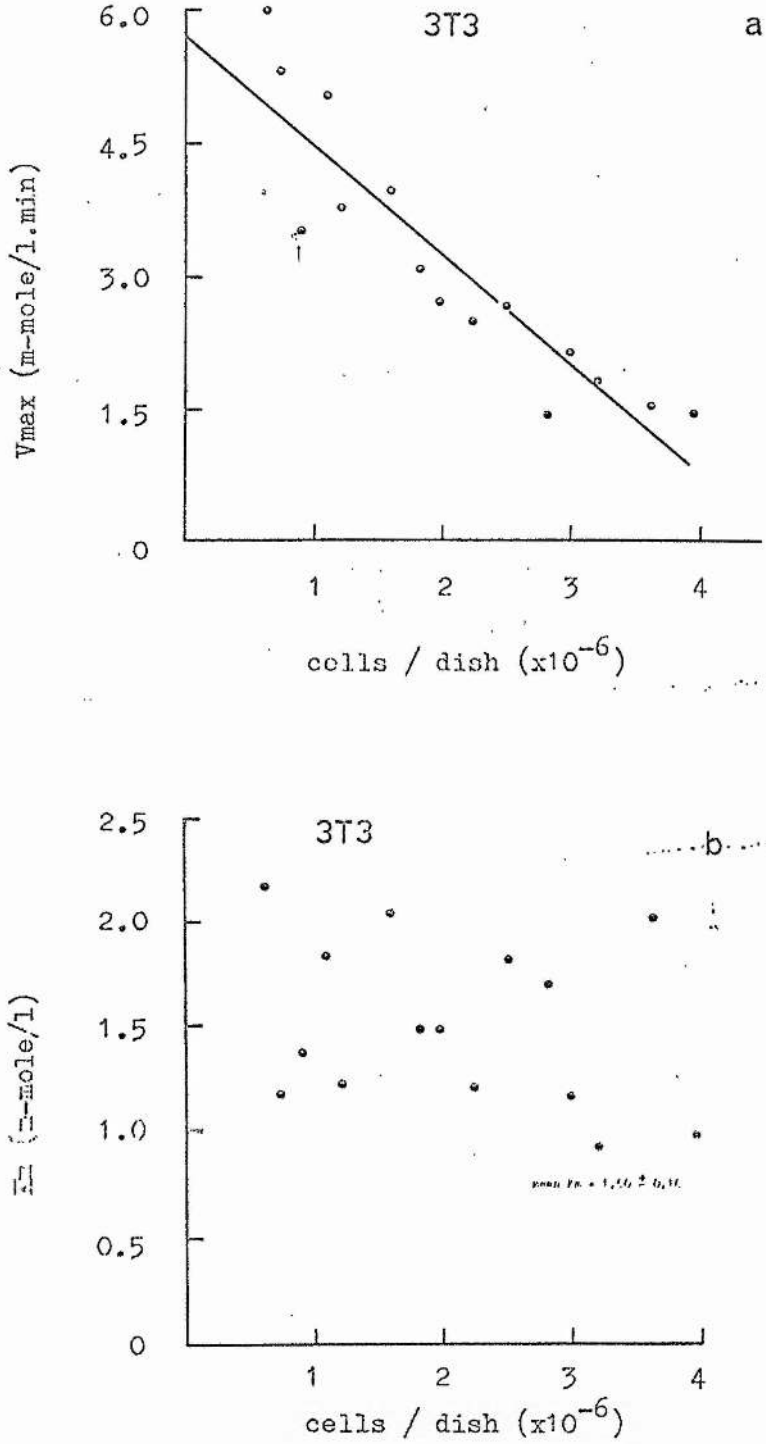


Fig. 12. Relationship between 3T3 cell population density and the Vmax (a) and Km (b) for K^+ transport. The combined results from several experiments similar to that described in the legend to Fig. 11 are shown. The line (a) was fitted by a linear regression programme without weighted factors. The results show a close correlation between cell density and Vmax ($r=0.911$; $P<0.01$) with no effect of cell density on Km ($r=0.276$; $P>0.05$).

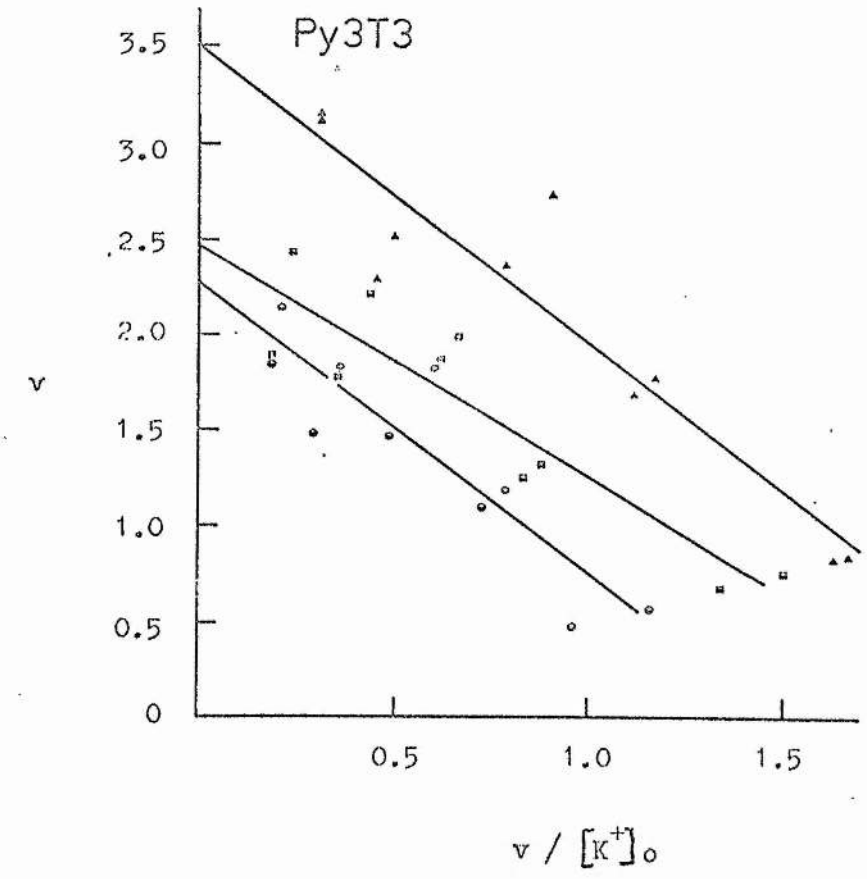


Fig. 13. Effect of the cell population density on the kinetic constants for K^+ transport by Py3T3 cells. Cells were seeded at 0.4 (⊙), 0.7 (⊡) and 1.0 (△) (x10) cells/ 9cm dish and used after 4 days growth. Experimental details were identical to those described for 3T3 cells in the legend to Fig. 11.

seeding density	mean cell no./ dish ($x10^{-6}$)	Km m-mole/l	Vmax m-mole/l. min
⊙ 0.4	1.75 ($\pm$0.05)	1.52	2.28
⊡ 0.7	3.98 ($\pm$0.11)	1.21	2.47
△ 1.0	6.49 ($\pm$0.10)	1.55	3.52

In mouse 3T3 cells a concentration of 10^{-3}M is required to produce a 40% reduction of the K^+ influx. The response was not significantly altered by up to 60 minutes pretreatment with the drug (Fig. 14). This result demonstrates, in agreement with others (Bonting et al., 1962; Mayhew & Levinson, 1968; Lamb & Mackinnon, 1971) that mice cells retain the low sensitivity of the species to this drug. When the experiment was repeated using Py and SV40 transformed 3T3 cells it was found that the initial inhibition (40%) of the K^+ influx was followed by an increase in the influx (Fig. 14). The chloride influx was not increased ($P>0.50$) suggesting that the effect on K^+ was not caused by non-specific membrane damage (unreported observation).

The effect of ouabain on the efflux of K^+ from 3T3 and virus-transformed 3T3 cells was investigated. Fig. 15 shows the effect of various bathing solutions on the rate of loss of ^{86}Rb from preloaded Py3T3 cells. Extracellular ouabain caused an increase in the rate of ^{86}Rb loss from these cells. The time-course of the increase in influx and efflux was similar and maximal after about 30 minutes exposure to the drug (Figs. 14 & 15). The stimulation of the K^+ efflux by ouabain was abolished by the removal of extracellular K^+ (Fig. 15). The efflux of K^+ from SV3T3 cells showed a similar response to ouabain whereas the drug was without effect on the efflux of K^+ from 3T3 cells (unreported observations). These results indicate that the effect of ouabain on the K^+ fluxes of transformed cells was due to the stimulation of K-K exchange across the cell membrane.

It appears, then, that the K^+ influx of the transformed cells was initially reduced by the inhibition of Na-K exchange and later increased after the onset of K-K exchange. To test whether inhibition of the Na-K exchange continued after the initiation of K-K exchange Py3T3 cells were treated with ouabain and the intracellular Na^+ and K^+ concentrations

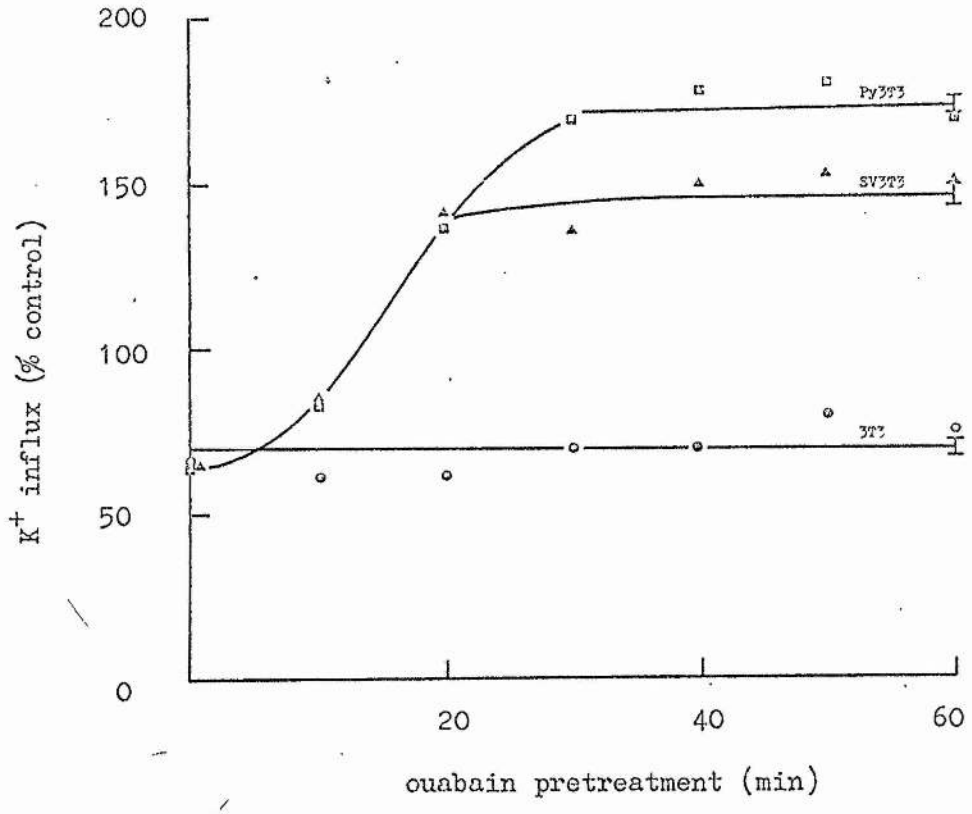


Fig. 14. Effect of ouabain pretreatment on the K^+ influx into 3T3 (○), Py3T3 (□) and SV3T3 (△) cells. The growth medium was removed and the cells incubated at 37° in 10ml of a Krebs solution with or without ouabain (10^{-3}M) for periods up to 60 min and the K^+ influx measured in the presence or absence of ouabain. Incubations with ^{86}Rb were carried out for 10 min according to the procedure described in the legend to Fig. 3. Each point represents the value for the K^+ influx obtained from a single plate of cells, expressed as a % of the control value. The lines were drawn by eye; the error bars ($\pm$ S.E.) refer to the horizontal lines.

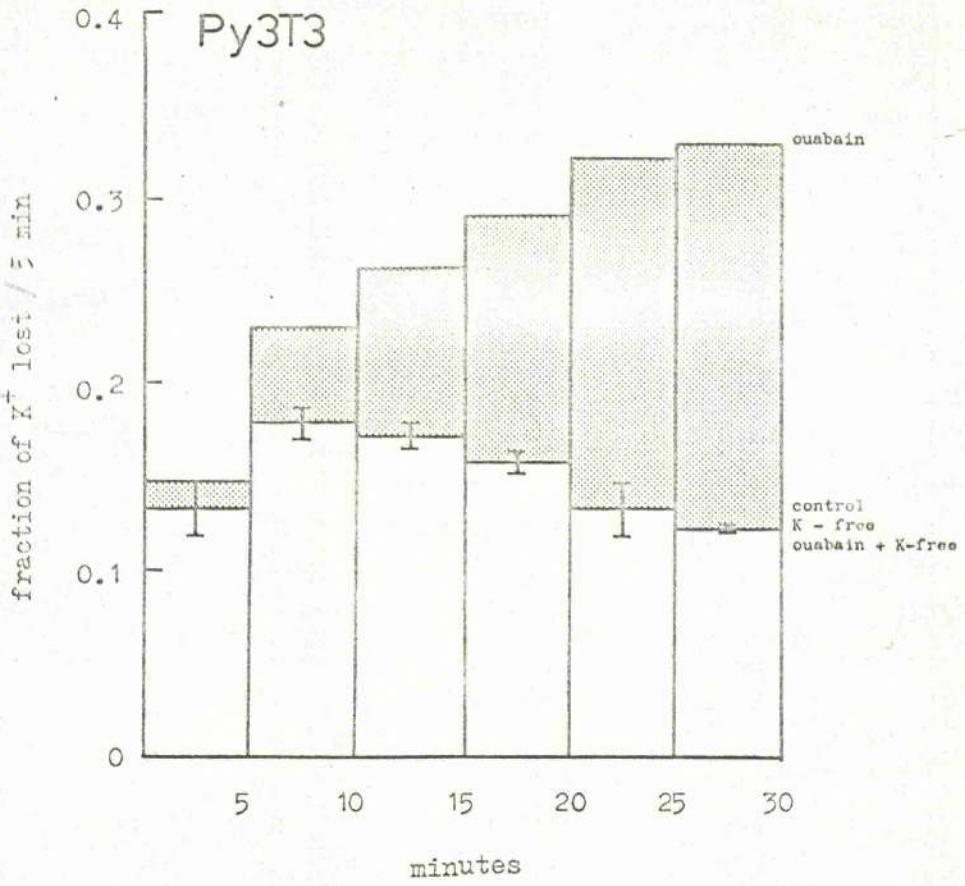


Fig. 15. Effect of ouabain on the rate of loss of K^+ from Py3T3 cells. Cells were "loaded" for 3 hours in an ^{86}Rb-labelled Krebs solution. The radioactive solution was removed and the cells were washed rapidly with 3 changes of cold (2°) inactive Krebs and rinsed once with Krebs at 37°. Tem ml of one of the following solutions were then added to a dish of cells : 1) control Krebs 2) Krebs + 10^{-3} M ouabain 3) K^+-free Krebs 4) K^+-free Krebs + 10^{-3} M ouabain. After 5 min the solution was quickly poured into a vial for ^{86}Rb counting, and another 10ml of the same solution was added to the dish. The washout of ^{86}Rb during 6 successive 5 min periods is expressed as the fraction of ^{86}Rb lost/5 min. The total column height represents the fractional loss from cells into Krebs + 10^{-3} M ouabain. Since the fractional loss into solution 1, 3 and 4 were not significantly different ($P > 0.10$) the mean ($\pm$ S.E,) is shown. The shaded column height thus represents the additional loss of ^{86}Rb caused by ouabain. The increased efflux was blocked by the removal of extracellular K^+.

were measured by flame photometry (Fig. 16). Over a 60 minute period the $[Na^+]_i$ rose exponentially from 20 to 70 m-mole/l and the $[K^+]_i$ fell exponentially from 200 to 140 m-mole/l indicating a continuing inhibition of the Na-K exchange over 60 minutes. Therefore, in the transformed cells, ouabain caused a simultaneous reduction of Na-K exchange and a stimulation of K-K exchange.

The dose-response of the two effects produced by ouabain has been examined. Fig. 17 shows the results of an experiment in which the effect of ouabain on the K^+ influx into Py3T3 cells was measured with or without 30 minutes pretreatment with the drug (10^{-8} to 10^{-3}M). The results show that the inhibition of Na-K exchange and stimulation of K-K exchange exhibited a similar dependence on ouabain concentration. There was neither inhibition (no pretreatment) nor stimulation (pretreatment) of the K^+ influx at ouabain concentrations below 10^{-4}M. There was a small degree of inhibition and stimulation at 10^{-4}M ouabain with larger effects at the maximum drug concentration of 10^{-3}M.

The reversibility of these effects has been investigated. SV3T3 cells were treated with ouabain for 60 minutes after which the cells were transferred to a control Krebs solution. The K^+ influx was measured at 15 minute intervals for 120 minutes after the removal of ouabain (Fig. 18). The K^+ influx decreased during the first 60 minutes, thereafter, the influx remained constant at a value some 40 to 50% below that of the untreated control. The ouabain-induced K-K exchange was thus more readily reversible than ouabain inhibition of Na-K exchange.

The effect of a reduction in experimental temperature to 20° on the response of the three cell lines to ouabain was examined. The results (Fig. 19) show that at

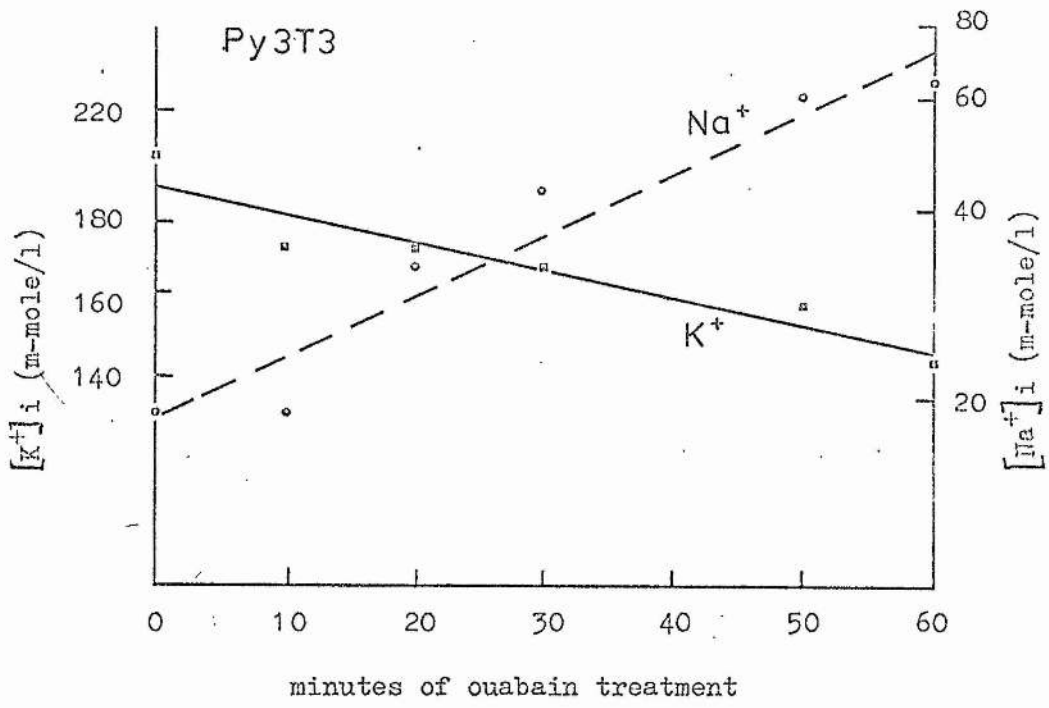


Fig. 16. Effect of ouabain treatment time on the $[K^+]i$ and $[Na^+]i$ of Py3T3 cells.[1] The K^+ and Na^+ of Py3T3 cells was measured by flame photometry after periods (0-60 min) of treatment with 10^{-3} M ouabain. Duplicate plates were used to measure mean cell number and cell volume/plate. The intracellular K^+ (▫) and Na^+ (○) concentrations were calculated and are expressed as m-mole/l. Each point represents the value for $[K^+]i$ and $[Na^+]i$ obtained from a single plate of cells. The lines were fitted by linear regression after logarithmic transformation

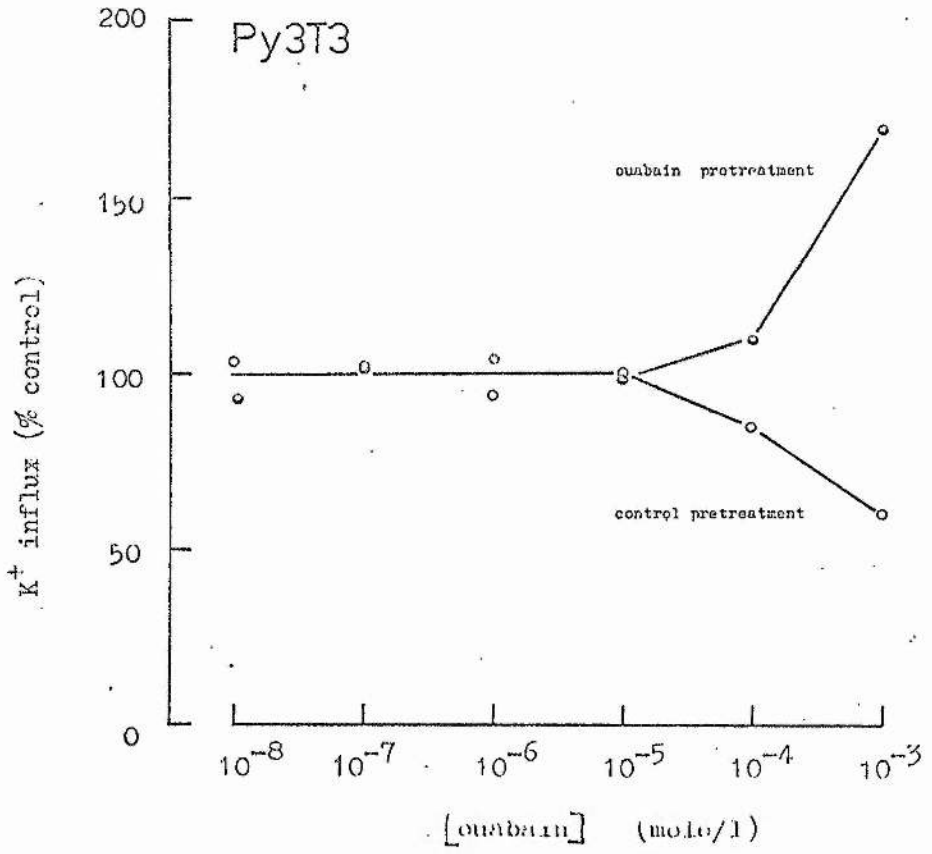


Fig. 17. Dose-response of the K^+ influx in Py3T3 cells to ouabain with and without drug pretreatment. The K^+ influx was measured in the presence of various concentrations of ouabain in the range 10^{-8} to 10^{-3} M. The cells had previously been treated for 30 min with either control Krebs (O) or Krebs containing ouabain (⊖) at the indicated concentration. Each point represents the value for K^+ influx obtained from a single plate of cells expressed as a % of the control K^+ influx. The results show that ouabain inhibition of Na-K exchange (control pretreatment) and ouabain stimulation of K-K exchange (drug pretreatment) exhibits a similar sensitivity to ouabain concentration.

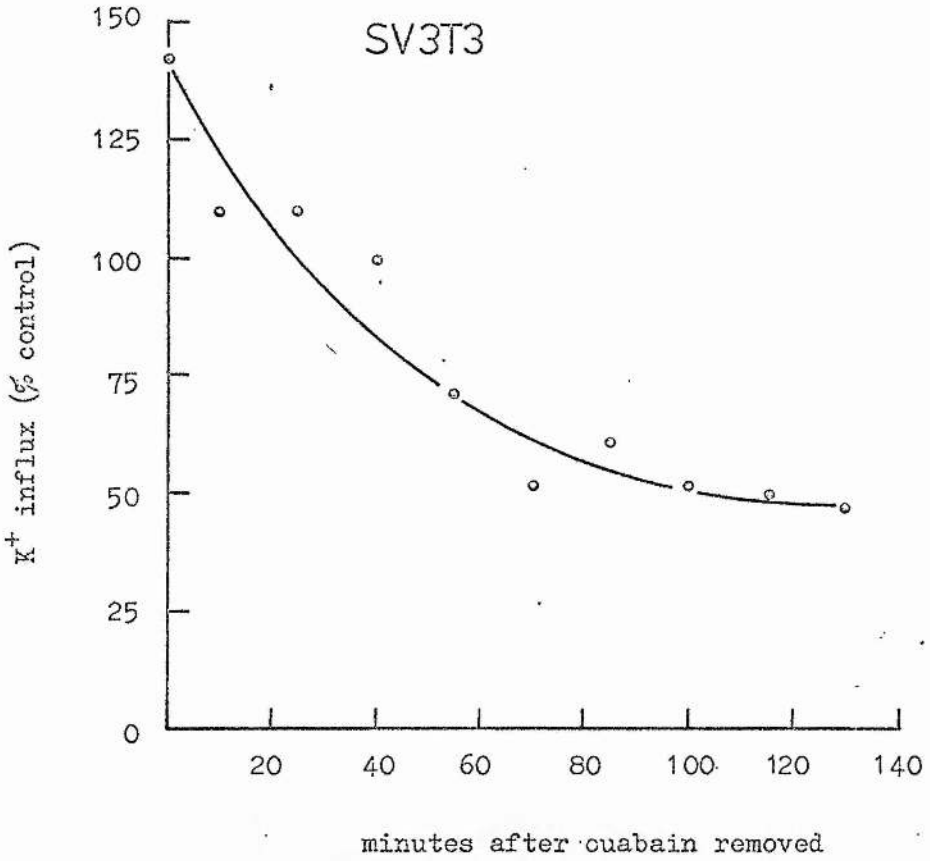


Fig. 18. Reversibility of the response of virus-transformed cells to ouabain. SV3T3 cells were treated with Krebs $\pm$ 10^{-3}M ouabain for 60 min and then transferred to a control Krebs solution for periods ranging from 0-120 min. The K^+ influx was measured by incubation with ^{86}Rb-Krebs for 10 min according to the procedure described in the legend to Fig. 3. Each point represents a single value for the K^+ influx after ouabain treatment expressed as a % of the values obtained for the untreated controls. The 10 min influx measurement period has been added to the reversal period to give the total time after ouabain reversal. The value at 0 min was measured in the continued presence of ouabain.

the reduced temperature K-K exchange was not induced by ouabain in either transformed line. The possible significance of these observations will be considered in the DISCUSSION.

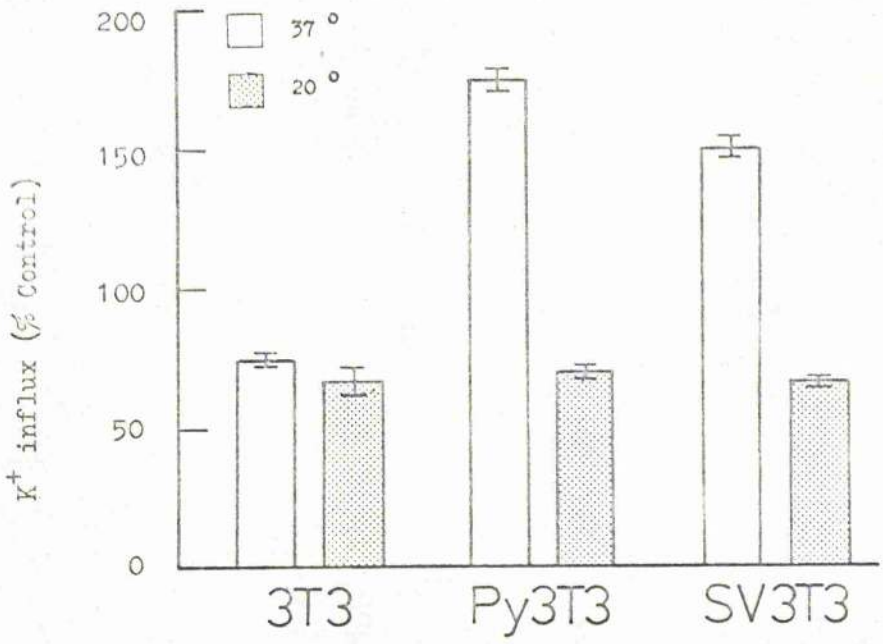


Fig. 19. Effect of a decreased experimental temperature on the response to ouabain. 3T3, Py3T3 and SV3T3 cells were treated with Krebs $\pm$ 10^{-3}M ouabain for 60 min at either 37° or 20°. The K^+ influx was then measured at the same temperature in the presence or absence of ouabain. Incubations with ^{86}Rb were carried out for 10 min according to the procedure described in the legend to Fig. 3. The column heights represent the mean value ($\pm$ S.E.) for 3 determinations of the K^+ influx in the ouabain-treated cells, expressed as a % of the value obtained for the K^+ influx in the absence of the drug. The results show that the increased K^+ influx produced by ouabain in transformed cells at 37° did not occur at 20°.

Phosphate transport in 3T3 and virus-transformed 3T3 cells

Effect of the cell population density on the phosphate

The uptake of phosphate (Pi)[1] into 3T3 cells at different population densities is shown in Fig.20. The parameters of uptake, calculated after logarithmic transformation of the data shown in Fig.20, are presented in Table 7. The initial rate of Pi uptake (influx) decreased with increasing cell density. The half-time of uptake increased with increasing cell density. However, the intracellular phosphate concentration at equilibrium was apparently independent of the cell population density. Weber & Edlin (1971) found no significant difference in the size of the acid-soluble phosphate pool in growing and density-inhibited cultures of 3T3 cells. They obtained an equilibrium value of 80 n-mole/10^6 cells. The total intracellular phosphate concentration of 20.75 m-mole/l (Table 7) was equivalent to 78 n-mole/10^6 cells. Since the amount of ^{32}Pi incorporated into the acid-insoluble fraction during the labelling period is small (~15%; Table 8) the value presented here is in close agreement with that of Weber & Edlin.

The uptake of Pi by Py3T3 and SV3T3 cells at a single cell population density is shown in Fig.21 and the calculated parameters of uptake are presented in Table 7. The intracellular phosphate concentration at equilibrium was similar in all three cell lines. However, the transformed cells, after 6 days growth, transported Pi more rapidly than 3T3 cells after a similar growth period. Under these

1. The symbols Pi, ^{32}Pi represent inorganic phosphate ions, with no specific valence being assigned.

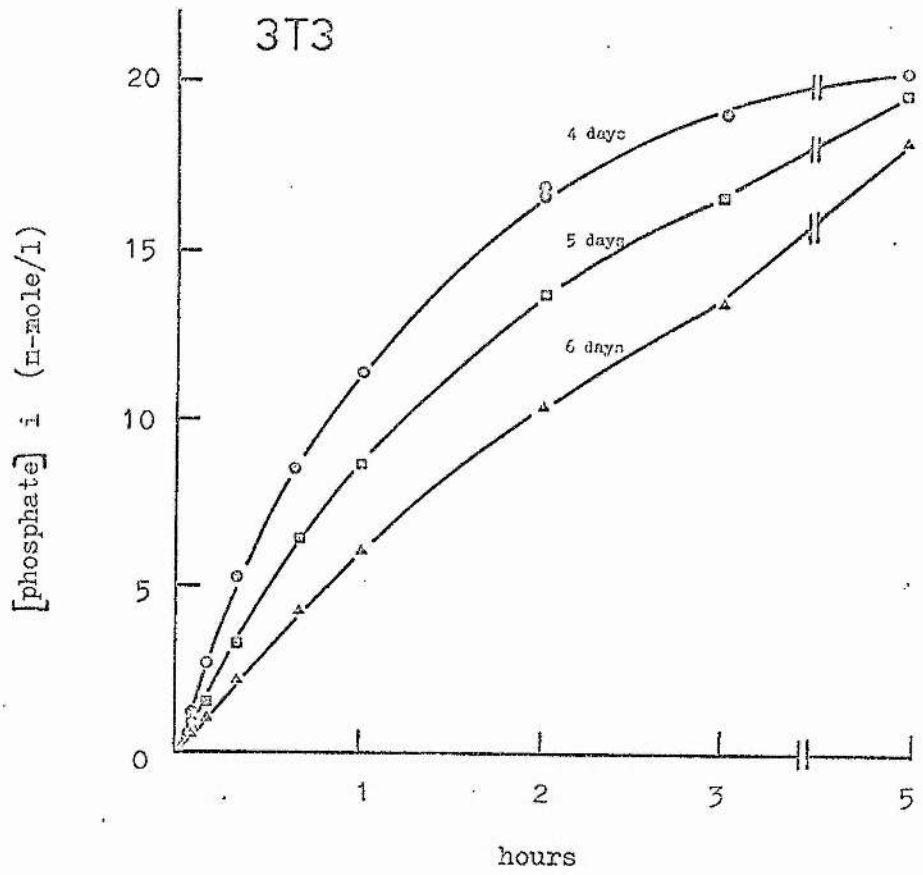


Fig. 20. Effect of the cell population density on the uptake of Pi by 3T3 cells. Cells were seeded at 0.4×10^6 cells/9cm dish and the Pi uptake measured after 4 (○), 5 (□) and 6 (△) days of growth. At zero time the growth medium was replaced with ^{32}P-labelled Krebs solution. After periods ranging from 5 min to 5 hours the ^{32}P Krebs was removed, the cells washed (x4) with Krebs at 2°, detached from the dishes with trypsin and analysed for ^{32}P activity, cell number and cell volume. Each point represents the value of [phosphate] i obtained from a single plate of cells. The lines were fitted by eye. The Pi concentration of the Krebs solution was 0.55 m-mole/l and ^{32}Pi activity was about 0.1 µCi/ml. The temperature of the growth and experimental solutions was 37° (±0.75°). The calculated characteristics of Pi uptake are shown in Table. 7.

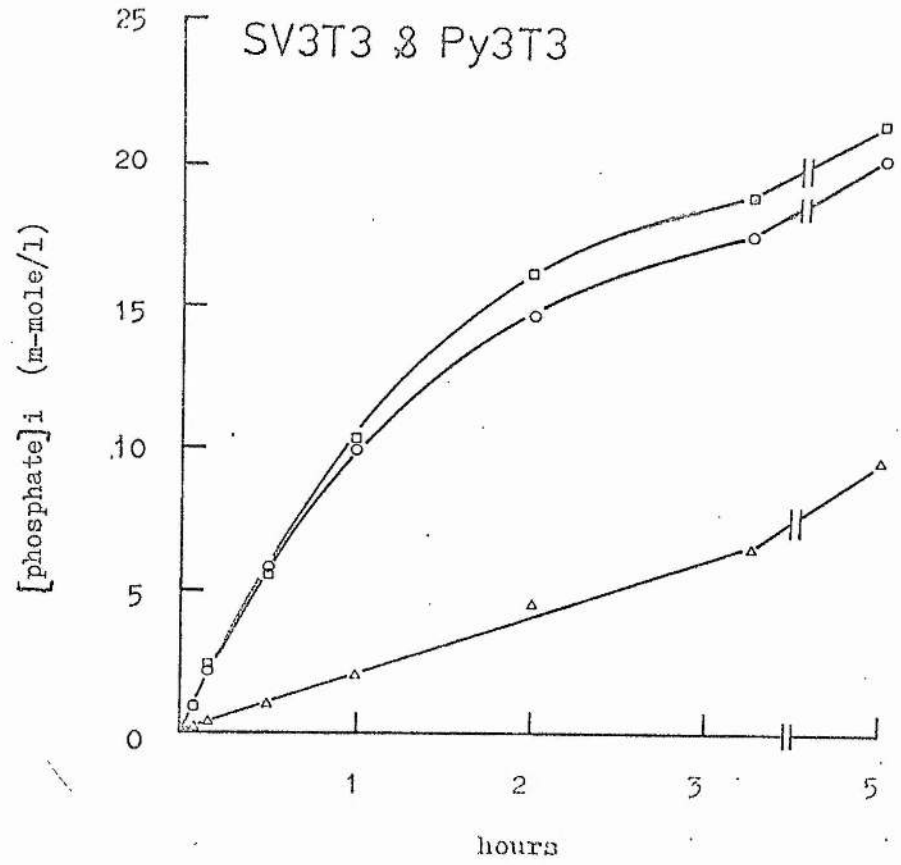


Fig. 21. The uptake of Pi by virus-transformed 3T3 cells. SV3T3 (○) and Py3T3 (□) cells were plated at 0.6×10^6 cells/ 9cm dish. After 6 days growth the Pi uptake was measured as described in the legend to Fig. 20. The effect of a reduction in the incubation temperature from 37° to 20° on the Pi uptake of SV3T3 cells has been included (△). Each point represents the value for [phosphate]i obtained from a single plate of cells. The lines were fitted by eye. The calculated characteristics of Pi uptake at 37° are shown in Table. 7.

Table 7. The characteristics of phosphate uptake by 3T3 and virus-transformed 3T3 cells

cell line	days of growth	mean cell no. /dish ($\times 10^{-6}$)	[phosphate] i m-mole/l	$T_{\frac{1}{2}}$ uptake (h)	Pi influx m-mole/l. min
3T3	4	1.31 ($\pm$0.05)	20.75	0.76	0.24
	5	1.93 ($\pm$0.09)	20.05	1.11	0.15
	6	2.11 ($\pm$0.06)	18.41	1.62	0.10
SV3T3	6	12.04 ($\pm$0.36)	19.50	1.16	0.19
Py3T3	6	10.71 ($\pm$0.28)	21.40	1.03	0.20

The values shown here were calculated after logarithmic transformation of the data shown in Figs.20&21.

conditions, the population density of the transformed cells was 5-7 times greater than that of the 3T3 cells. This result indicates that the rate of phosphate transport in transformed cells is less dependent on cell density than in untransformed cells. For all cell lines under all conditions examined the phosphate uptake was linear out to at least 10 minutes. Five minutes was therefore chosen as a suitable incubation period for determination of Pi influx.

Fig.21 also shows the effect of a reduction in experimental temperature on the uptake of phosphate by SV3T3 cells. When the temperature was reduced from 37° to 20° the Pi influx decreased from 0.194 m-mole/l. min to 0.044 m-mole/l. min. This result emphasises the need for careful temperature control when measuring phosphate transport. In the experiments reported here the incubation temperature during labelling and the temperature of the experimental solutions was maintained at 37° (±0.75°).

Partition of intracellular phosphate. The total cell phosphate is divided between the acid-soluble and the acid-insoluble pools. The former can be further subdivided into an organic and inorganic fraction. The effect of cell population density on the equilibrium distribution of ^{32}Pi between these three compartments in 3T3 and SV3T3 cells is shown in Table 8. Most of the total phosphate was consistently found in the acid-soluble organic fraction. Virus-transformation caused no major change in the partition of phosphate between the various compartments. The results in Table 8 indicate that, especially in 3T3 cells, the size of the cellular Pi fraction decreased with increasing cell density. A reduction of 49% for 3T3 cells and 23% for SV3T3 cells was found when 3-day and 6-day old cultures were compared. Previous results (Fig.20) indicated that the Pi influx decreased with increasing cell density. It is possible that this reduced Pi influx is the cause of the

Table 8. Effect of the cell population density on the partition of cellular phosphate

		[phosphate]i m-mole/l (% total)			
		total	acid-sol. inorg.	acid-sol. org.	acid-insol.
3T3	3-days growth	20.68 (100)	2.40 (11.6)	14.91 (72.1)	3.37 (16.3)
	6-days growth	18.16 (100)	1.23 (6.7)	14.17 (78.0)	2.76 (15.3)
SV3T3	3-days growth	19.13 (100)	2.20 (11.5)	13.66 (71.4)	3.26 (17.1)
	6-days growth	18.87 (100)	1.69 (9.0)	13.81 (73.2)	3.37 (17.8)

3T3 and SV3T3 cells were preloaded with ^{32}Pi (0.2 μCi/ml) for 4 h. At the end of incubation the cells were washed (x4) with inactive Krebs at 2°. The perchloric acid soluble phosphate was extracted and separated into the organic and inorganic fractions using the phosphomolybdate/ethyl acetate procedure described in the METHODS. The neutralised samples were then counted for ^{32}P. Duplicate plates were used for the determination of cell numbers, cell volume and total [phosphate]i. Subtraction of the acid-soluble [phosphate]i from the total gave the values for the acid-insoluble organic fraction.

reduction in the size of the cellular Pi pool. The effect of cell population density on the Pi influx has been examined in more detail.

Effect of cell population density on the phosphate influx. Fig.22 shows the results of an experiment in which the Pi influx of 3T3 cells was measured each day, for several days, after plating. The number of cells/dish at the time of measurement has also been included in the figure. The Pi influx increased for the first 3 days after plating reaching a maximum of 0.325 m-mole/l. min. The influx then steadily decreased to 0.060 m-mole/l. min after 7-8 days growth. It can be seen that the onset of the decrease in the Pi influx precedes the cessation of cell growth. The Pi influx fell between days 3 and 5, a period during which significant cell growth occurred. The influx continued to decrease rapidly between days 5 and 8 after the cessation of cell growth. The effect of cell population density on the Pi influx of SV3T3 cells is shown in Fig.23. In these cells the Pi influx also increased to a maximum and then declined. However, the maximum Pi influx (0.431 m-mole/l. min) was slightly higher than in 3T3 cells and was reached one day later; that is, after 4 days of growth. The minimum influx (0.187 m-mole/l. min) measured after 7 days growth was some 3 times larger than the minimum Pi influx into 3T3 cells. In this experiment no measurement was possible at 8 days. Even very gentle changes of solution caused the thick cell layer to detach from the dish.

The different effect of cell population density on the Pi influx of 3T3 and SV3T3 cells is seen more clearly in Fig.24. Here, the data in Figs.22 and 23 have been used to plot the Pi influx against the number of cells/dish (log scale). In 3T3 cells the Pi influx began to decrease at about 1×10^6 cells/dish (approx. 40-50% confluent). In SV3T3 cells the Pi influx continued to increase until the dish

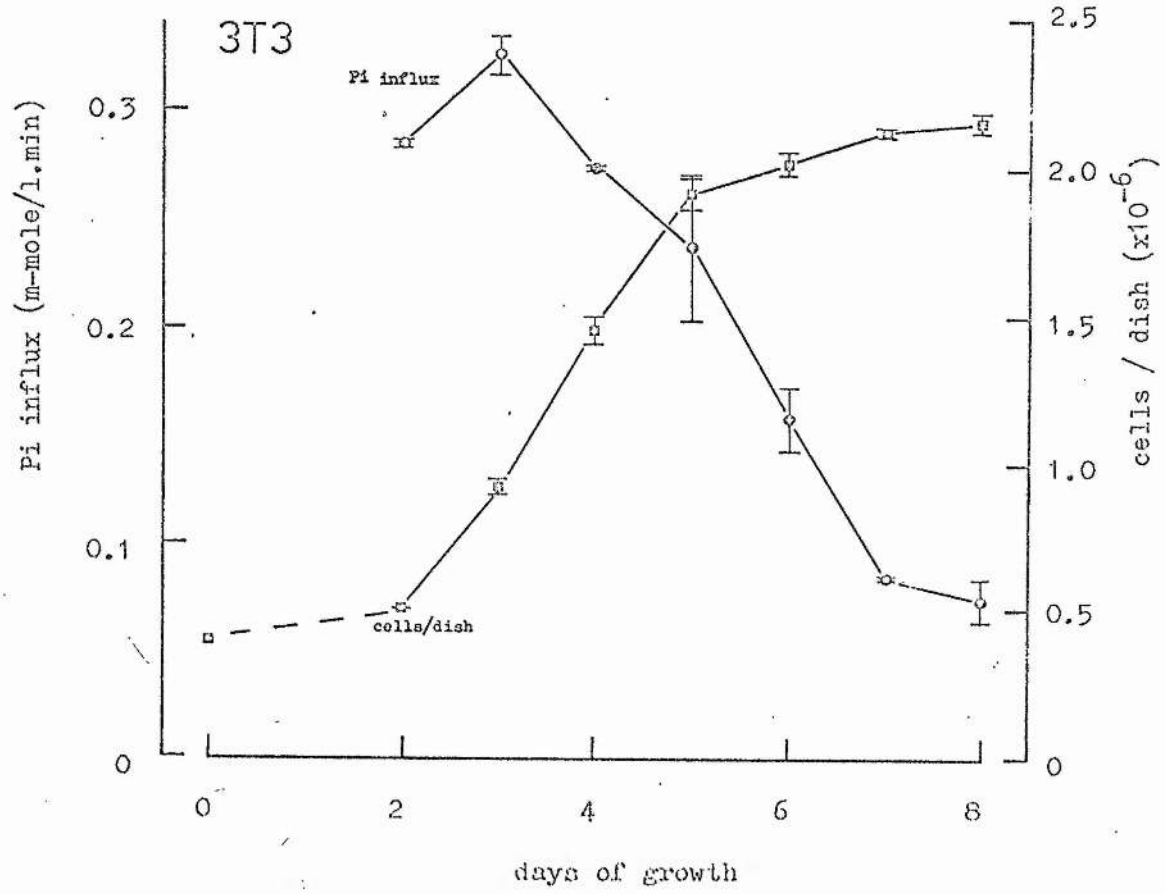


Fig. 22. Effect of the cell population density on the Pi influx into 3T3 cells. Cells were seeded at 0.4×10^6 cells/9cm dish. The Pi influx (O) was measured at the same time each day from 2 to 8 days after seeding. Incubations with ^{32}Pi were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 20. The number of cells/dish (□) at the time of the influx measurement has also been incuded in the figure. Each point represents the mean value for 3 determinations of the influx or cell number. The error bars indicate ± S.E.

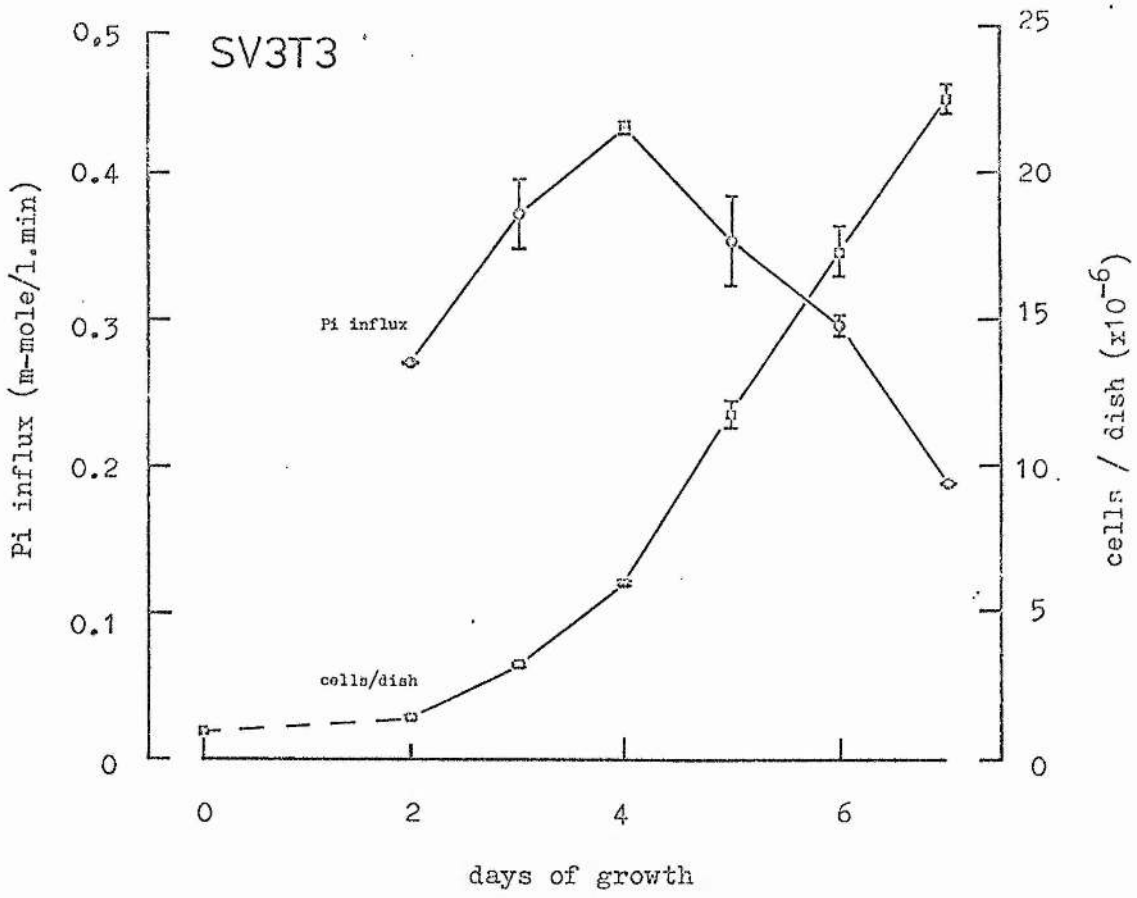


Fig. 23. Effect of the cell population density on the Pi influx into SV3T3 cells. Cells were plated at 1.0×10^6 cells/9cm dish. The Pi influx (○) was measured at the same time each day from 2 to 7 days after plating. Incubations with ^{32}Pi were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 20. The number of cells/dish (□) at the time of the influx measurement has also been included in the figure. Each point represents the mean value for 2 determinations of the Pi influx and cell number. The error bars indicate ± S.E.

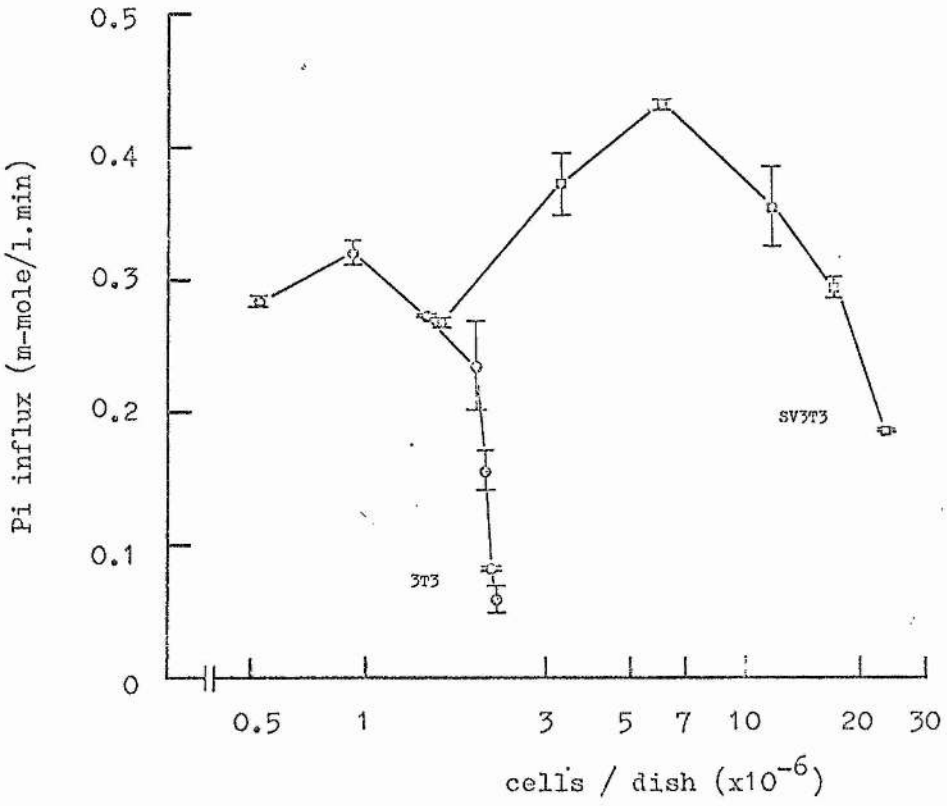


Fig. 24. Effect of cell population density on the influx of Pi into 3T3 (⊙) and SV3T3 (⊡) cells. Plot of Pi influx against the number of cells/dish (log scale) using the data shown in Figs. 22 and 23.

contained about $6x10^6$ cells (approx. 2-3x confluent). Thus, in untransformed cells, the Pi influx decreases before most cells are in close contact with one another; in the transformed cells the Pi influx does not decrease until after a multilayer of cells has formed.

Effect of cell population density on the kinetic constants for phosphate transport. The effect of cell density on the kinetics for Pi transport by 3T3 cells is shown in Fig.25. The Pi influx from a range of extracellular Pi concentrations (0.1-4.0 m-mole/l) was measured 3, 5 or 6 days after plating. As expected the kinetics of Pi transport were dependent on cell density. A linear transformation (v against v/s) of this data is shown in Fig.26 together with a summary of the calculated kinetic constants. These results show that the decreased Pi transport associated with increased cell density can be attributed to a reduction in Vmax with no change in the Km of the system. Similar findings have been reported for the decreased transport of 2-deoxyglucose with increasing population density in several cell lines (Plagemann, 1973; Bose & Zlotnick, 1973; Kletzein & Perdue, 1974). Levinson (1972) found values of $0.33x10^{-3}$M and 0.73 m-moles/kg.min for the Km and Vmax of Pi transport by Erlich ascites tumour cells. The values reported here (Fig.26, TABLE) are in reasonable agreement with Levinson's results.

The results of a similar experiment using SV3T3 cells are shown in Fig.27. Here again, increasing cell density led to a reduction in the Vmax with little change in the Km for Pi transport. The maximum Vmax measured in the transformed cells was slightly greater (10%) than the maximum Vmax of the untransformed cells. It should be emphasised that in SV3T3 cells the maximum Vmax occurs at a much higher cell density than in 3T3 cells. The differences observed in Km between the cell types were small and, though

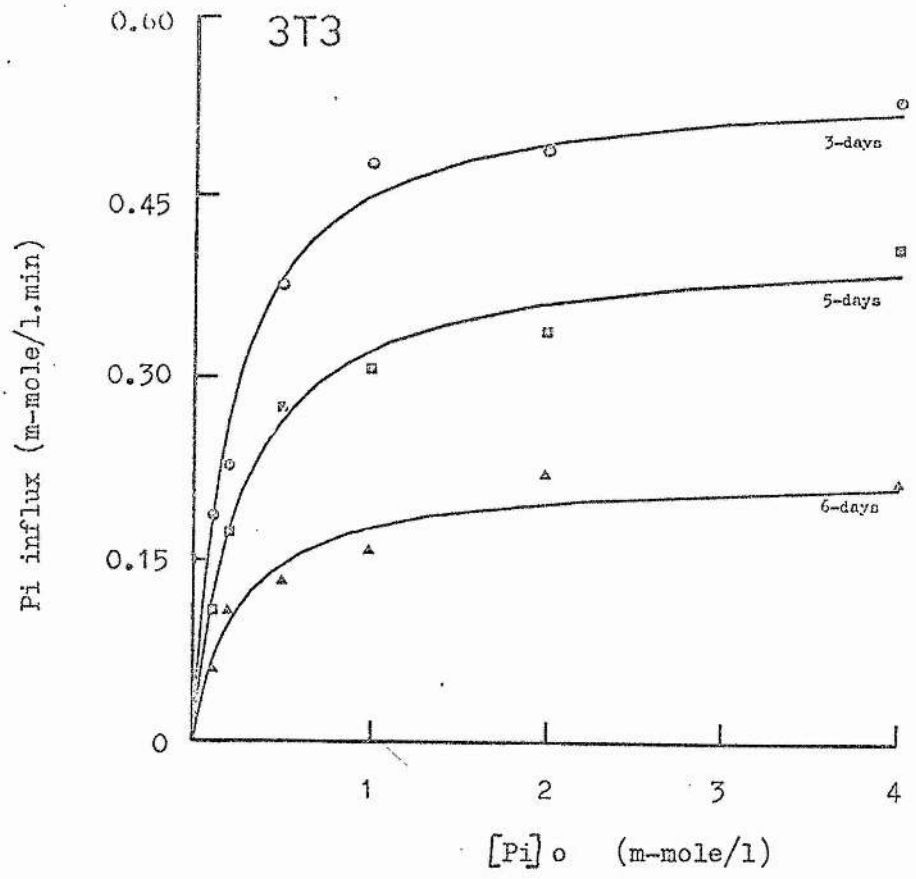


Fig. 25. Effect of the cell population density on the influx of Pi into 3T3 cells as a function of the extracellular Pi concentration. The influx of Pi from Krebs with [Pi] in the range 0.1 to 4.0 m-mole/l was determined for 3 (○), 5 (▣) and 6 (△)-day-old cultures of 3T3 cells. Incubations with ^{32}Pi were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 20. Each point represents the value for Pi influx obtained from a single plate of cells. The curves were fitted by linear regression after linear transformation of the data.

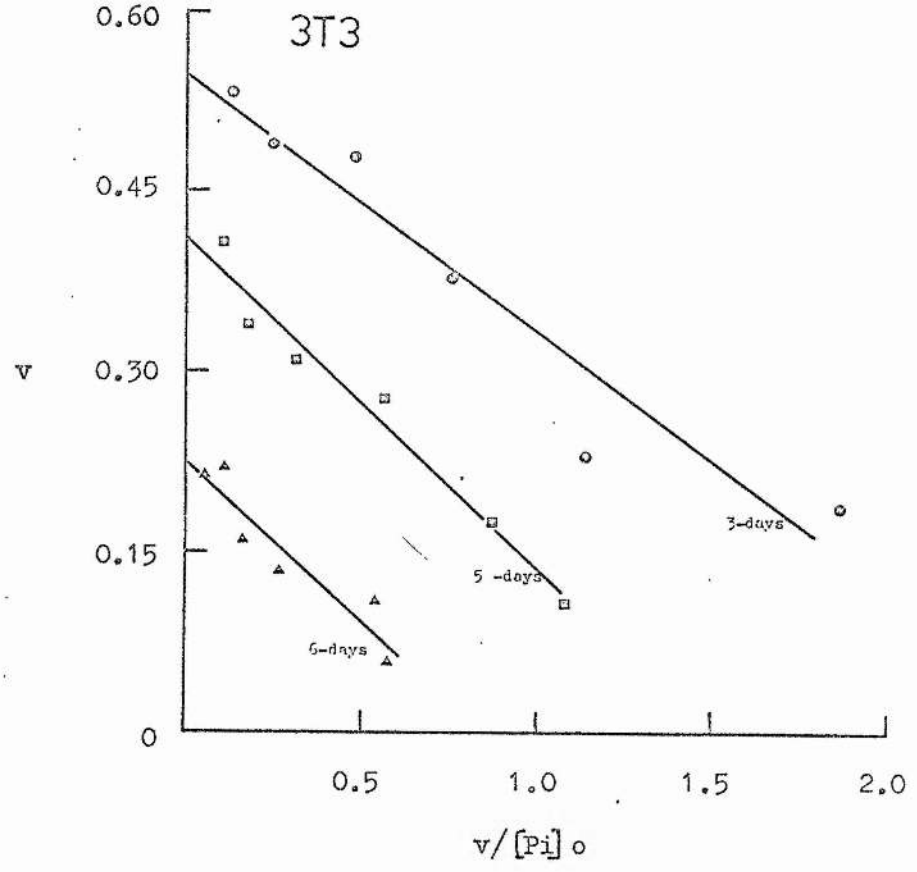


Fig. 26. Effect of the cell population density on the kinetic constants for Pi transport by 3T3 cells. Linear transformation (v against v/S; see APPENDIX B) of the data shown in Fig. 25. The influx (v) is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km and Vmax, for the 3 (○), 5 (□) and 6 (△)-day-old cultures are shown in the attached table.

Days of growth	mean cell no./ dish ($\times 10^{-6}$)	Km m-mole/l	Vmax m-mole/l. min
○ 3	0.87 ($\pm$0.06)	0.21	0.55
□ 5	2.31 ($\pm$0.05)	0.28	0.41
△ 6	2.54 ($\pm$0.09)	0.26	0.22

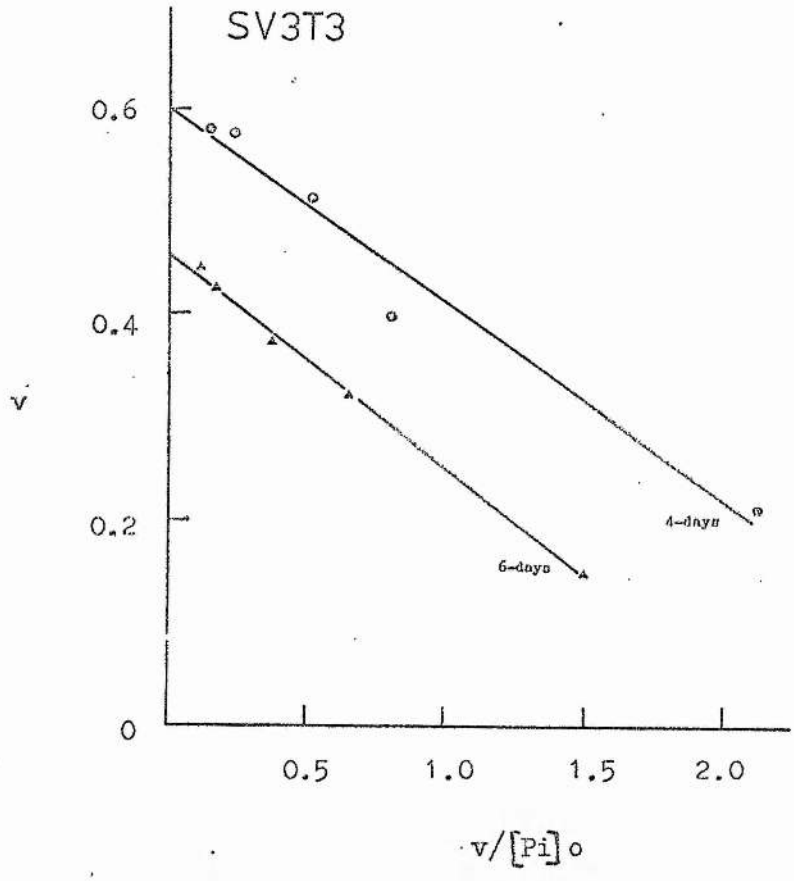


Fig. 27. Effect of the cell population density on the kinetic constants for Pi transport by SV3T3 cells. v against v/S plots of the influx of Pi into 4(⊙) and 6 (▲) day-old cultures of SV3T3 cells. Each point represents a single value for Pi influx determined as described in the legend to Fig. 25. The influx (v) is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km and Vmax, are shown in the attached table.

Days of growth	mean cell no./ dish ($x10^{-6}$)	Km m-mole/l	Vmax m-mole/l. min
⊙ 4	4.61 ($\pm$0.14)	0.19	0.60
▲ 6	13.08 ($\pm$0.20)	0.21	0.46

difficult to test, are probably not significant.

For both cell lines, under all conditions, the curves of influx against substrate concentration showed clear evidence of saturation. The good fit obtained for the straight line transformations demonstrates that Pi transport "follows Michaelis-Menten kinetics". This is consistent with the view that Pi transport in these cells is carrier-mediated.

Sodium-dependent phosphate transport in 3T3 and SV3T3 cells

Effect of the extracellular Na^+ concentration on the influx and uptake of phosphate. In previous studies I observed an effect of the $[Na^+]_o$ on Pi transport in L cells (Brown, 1971). Scholnick et al (1973) have also reported an effect of the $[Na^+]_o$ on the Pi influx in Erlich ascites tumour cells. These observations suggest that Pi transport may exhibit a Na-dependency similar to that demonstrated for the transport of several organic solutes (review; Schultz & Curran, 1970). The effect of varying the $[Na^+]_o$ on the influx and uptake of Pi by 3T3 and SV3T3 cells has been examined. The results for 3T3 cells are shown in Fig.28 and for SV3T3 cells in Fig.29 . As the $[Na^+]_o$ was reduced from the control level of 136 m-mole/l there was little effect of the Pi influx until below about 70 m-mole/l after which the Pi influx decreased markedly. Both cell types exhibited a similar Na-dependency indicating that virus-transformation did not significantly affect this property of Pi transport. The 3T3 cells appeared to have a slightly larger Na-independent component as shown by the higher Pi influx when the $[Na^+]_o$ approached zero (1.6 m-mole/l)($P<0.01$). The equilibrium phosphate concentration was also Na-dependent. However, this value did not begin to decrease until after the $[Na^+]_o$ was reduced below about 40 m-mole/l. In all the experiments described choline was substituted for Na^+ to maintain isotonicity.

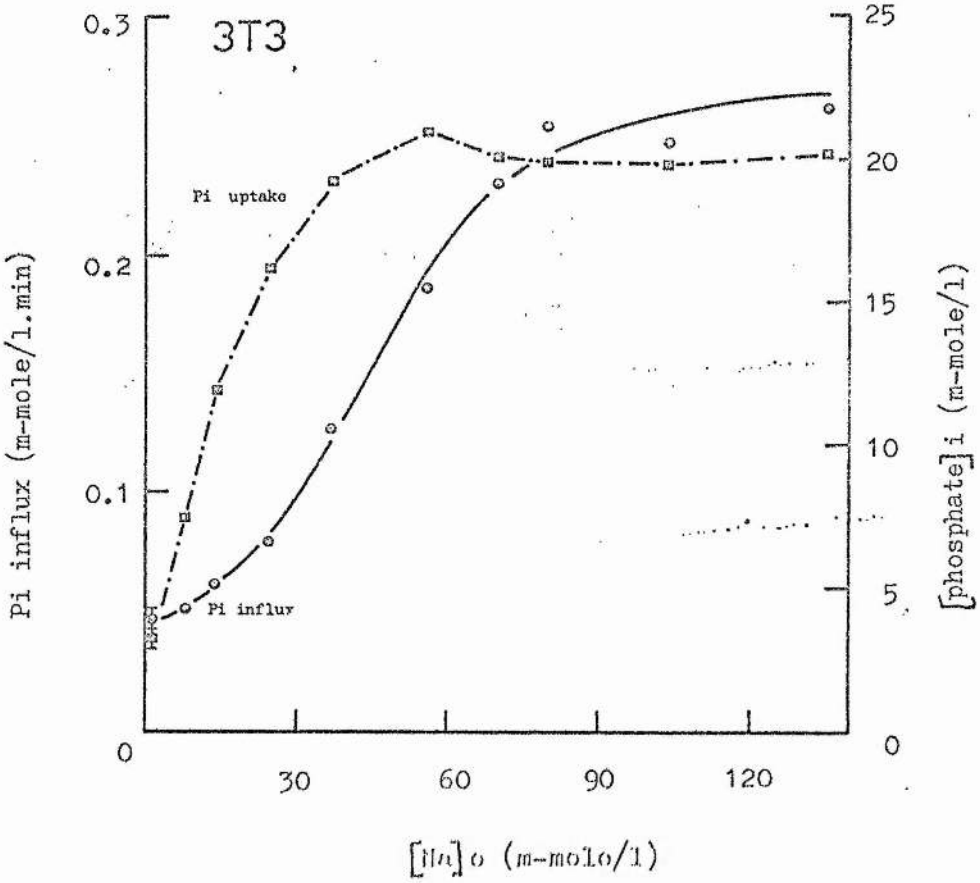


Fig. 28. Effect of the extracellular Na^+ concentration on the influx and uptake of Pi by 3T3 cells. The Pi influx and uptake were measured from Krebs solutions in which the $[Na^+]$ was varied between 1.6 and 136 m-mole/l. Choline was substituted for Na^+ to maintain isotonicity. Incubations with ^{32}Pi were carried out for 5 min (influx, ⊙) or 4h (uptake, ⊡) according to the procedure described in the legend to Fig. 20. Each point represents a single value for Pi influx or [phosphate]i. The lines were drawn by eye.

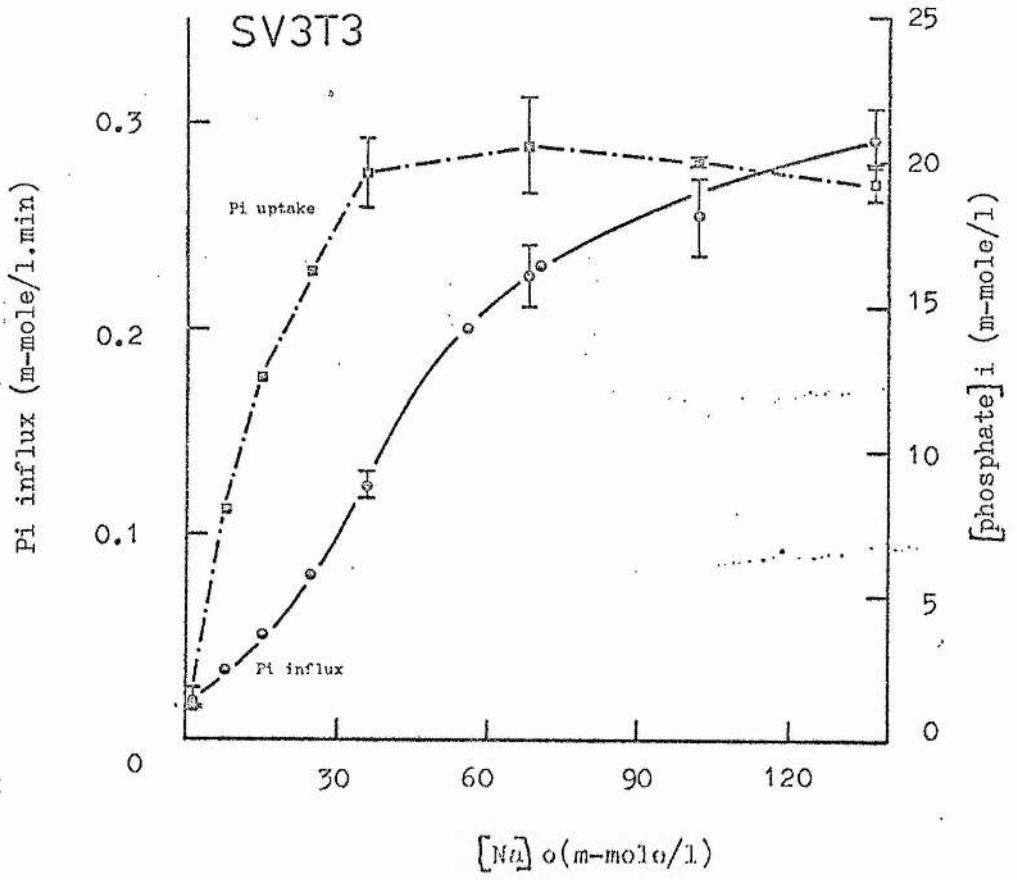


Fig. 29. Effect of the extracellular Na^+ concentration on the influx and uptake of Pi by SV3T3 cells. The Pi influx and uptake were measured from Krebs solutions in which the $[Na^+]$ was varied between 1.6 and 136 m-mole/l. Choline was substituted for Na^+ to maintain isotonicity. Incubations ^{32}Pi were carried out for 5 min (influx, ⊙) or 4h (uptake, ■) according to the procedure described in the legend to Fig.20. The points without error bars represent a single value for Pi influx or [phosphate]i. Where error bars ($\pm$S.E.) are included the points represent the mean value of 2 to 4 observations. The lines were drawn by eye.

Similar results (unreported) were obtained when Li^+ was used as the Na^+ substitute.

These results indicate that Na^+ facilitates Pi transport in both 3T3 and SV3T3 cells. A possible explanation for this effect is similar to that suggested for the Na-coupled transport of organic solutes (Schultz & Curran, 1970 p. 672). A simplified statement of this proposal would be that the binding of Na^+ to the phosphate transport carrier increases its affinity for Pi and thus facilitates Pi transport.

Effect of the extracellular Na^+ concentration on the kinetic constants for phosphate transport. If the above interpretation is correct, a reduction of the $[Na^+]_o$ should lead to an increase in the Km for Pi transport. Fig. 30 shows the results of an experiment in which the kinetic characteristics for the Pi influx were measured at different levels of $[Na^+]_o$. The values calculated for the kinetic constants are shown in Table 9. Reduction of $[Na^+]_o$ from the control level of 136 m-mole/l to 36 m-mole/l led to an increased Km and a decreased Vmax. Km was most affected, increasing by 280% whilst the Vmax fell by 22%. When the $[Na^+]_o$ was further reduced to 1.6 m-mole/l the Km increased by 375% and the Vmax fell by 59% compared to the control values. The kinetics for Pi transport by SV3T3 cells showed a similar response to $[Na^+]_o$ (Fig. 31 ; Table 9).

The observation that reducing $[Na^+]_o$ increased the Km of the system supports the proposal that Na^+ increases the affinity of the transport carrier for Pi. However, the fact that Vmax also appears to be dependent on $[Na^+]_o$ suggests that the affect of Na^+ is not only on the carrier affinity. This possibility will be considered in more detail in the DISCUSSION.

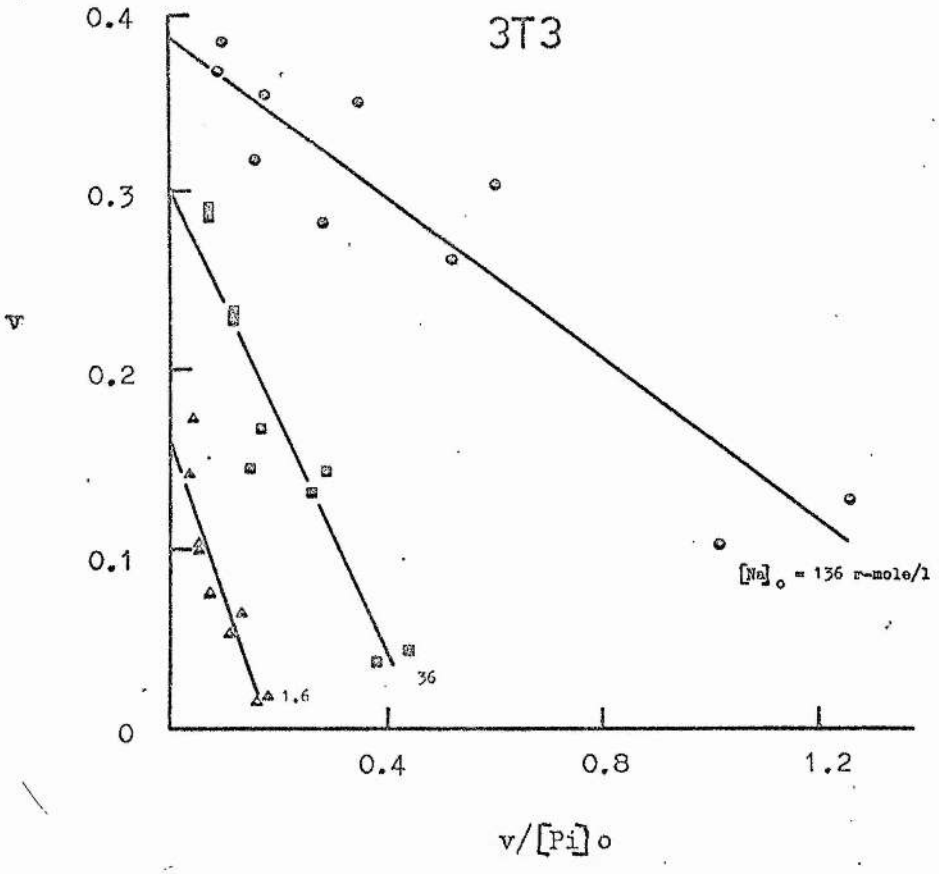


Fig. 30. Effect of the extracellular Na^+ concentration on the kinetic constants for Pi transport by 3T3 cells. The Pi influx from Krebs with [Pi] in the range 0.1 to 4.0 m-mole/l was determined at 3 levels of $[Na^+]o$ (○, 136; □, 36; △, 1.6 m-mole/l). Incubations with ^{32}Pi were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 20. Choline was substituted for Na^+ to maintain isotonicity. Each point represents the value for Pi influx from a single plate of cells. The data is shown after linear transformation (v against v/S). The influx (v) is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km and Vmax, are shown in Table 9.

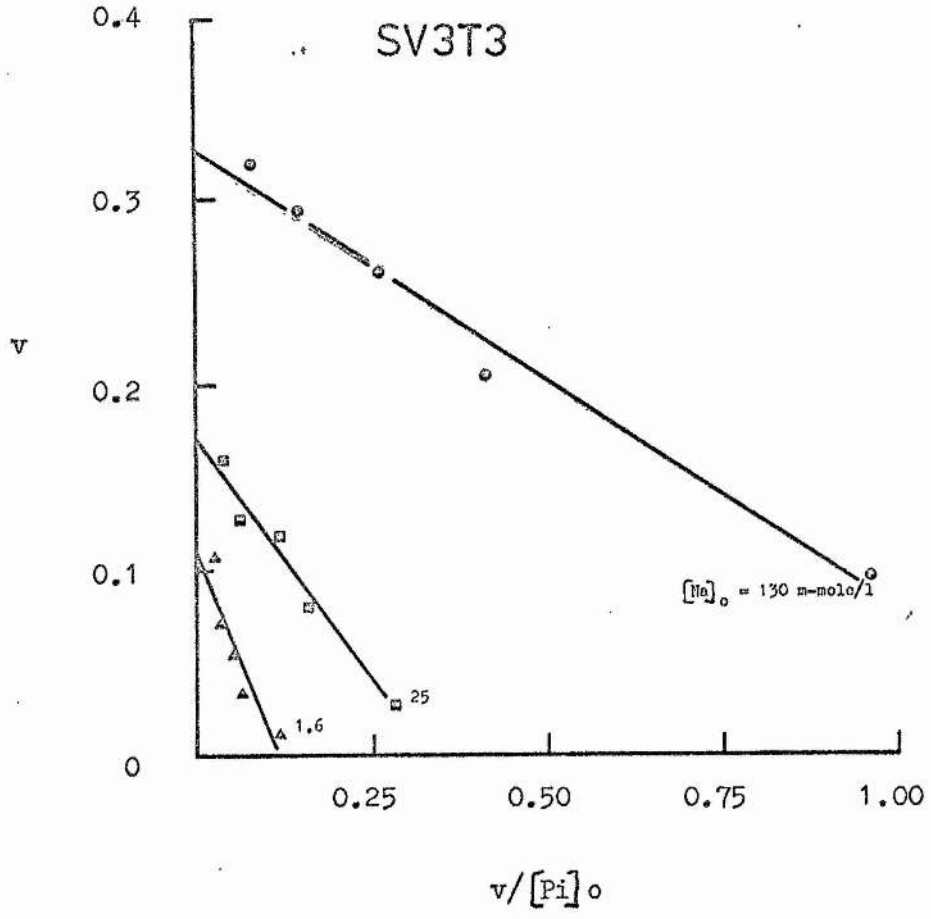


Fig. 31. Effect of the extracellular Na^+ concentration on the kinetic constants for Pi transport by SV3T3 cells. The experimental details were as described in the legend to Fig. 30, except that the $[Na^+]o$ were slightly different: (⊙, 130; ▣, 25; ▲, 1.6 m-mole/l). The calculated kinetic constants, Km and Vmax, are shown in Table 9.

Table 9. Effect of the extracellular Na^+ concentration on the kinetic constants for Pi transport.

	$[Na^+]_o$ (m-mole/l)	Km (m-mole/l)	Vmax (m-mole/l. min)
3T3	136	0.226	0.388
	36	0.642	0.302
	1.6	0.850	0.158
SV3T3	130	0.250	0.328
	25	0.535	0.172
	1.6	0.922	0.114

The values shown here were calculated from the data presented in Figs. 30 and 31.

Effect of the intracellular Na^+ concentration on the phosphate efflux. True efflux measurements can be conducted only with substrates that are not metabolised by the cells. This is clearly not the case for Pi. As shown previously (Table 8) most of the Pi which enters the cell is incorporated into organic compounds. Since the cell membrane is relatively impermeable to these compounds the phosphate is effectively 'trapped' inside the cell. Nevertheless, when cells are pre-loaded to equilibrium with ^{32}Pi a fraction of the total label is present in the intracellular Pi pool (Table 8). Measurements of the rate of loss of ^{32}Pi from this pool should provide some indication of changes in the outward transport of Pi under various conditions. Since no major differences in the Na-dependency of Pi transport were found between 3T3 and SV3T3 cells the investigation of the effects of Na^+ on Pi transport was continued using only SV3T3 cells. These cells were chosen because the higher cell numbers obtained simplifies the measurement of the transport parameters.

The effect of $[Na^+]_i$ on the outward movement of Pi from SV3T3 cells has been investigated. This was achieved by pre-loading cells with ^{32}Pi for several hours and measuring the loss of ^{32}Pi into successive changes of collecting solution. In some cells the $[Na^+]_i$ was raised by the addition of ouabain (10^{-3}M) during the final 90 minutes of loading. This was shown to raise the $[Na^+]_i$ from 19±4 m-mole/l to 61±6 m-mole/l. At the end of incubation the total cellular ^{32}Pi in the control condition was 537×10^3 c.p.m. and in ouabain-treated cells, 480×10^3 c.p.m. The washout of ^{32}Pi from control and ouabain-treated cells is shown in Fig.32 . The ^{32}Pi counts in successive 10 ml batches of collecting solution are shown after normalisation for the small difference in total cellular ^{32}Pi. Approximately equal amounts of ^{32}Pi were lost from the cells during each 5 minute interval. In a simple one-compartment system the washout of label from

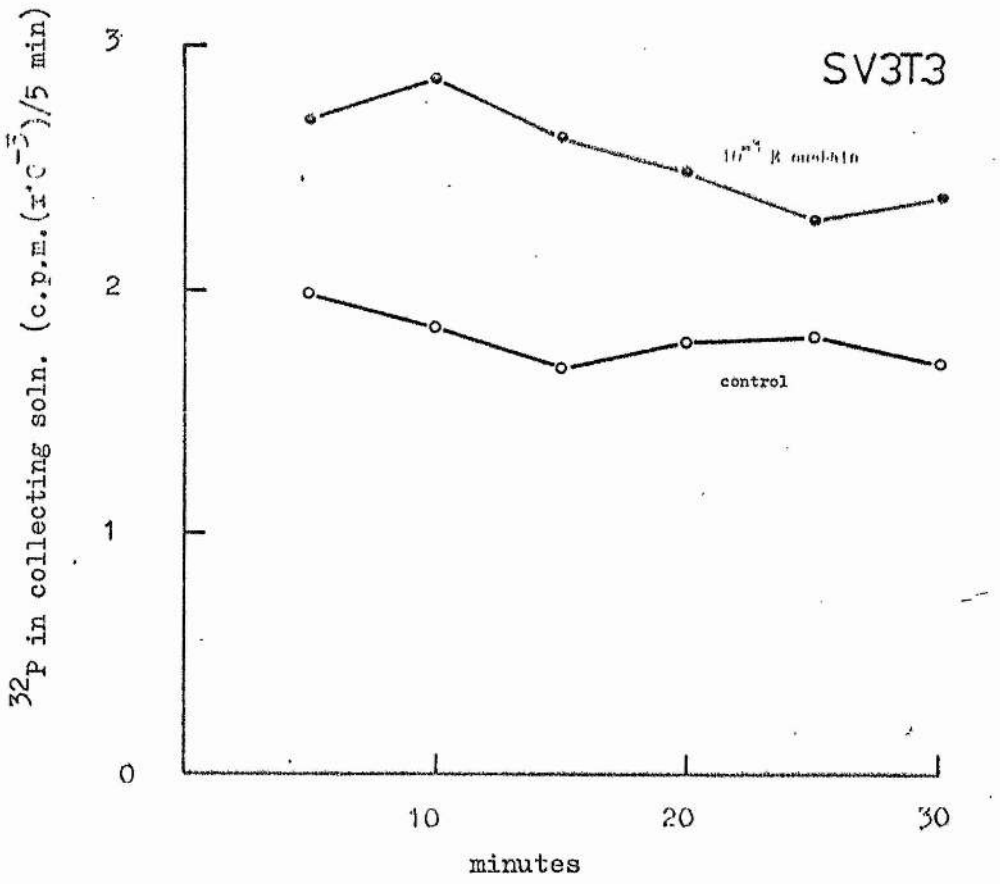


Fig. 32. Effect of ouabain on the washout of phosphate from SV3T3 cells. The cells were loaded with ^{32}Pi for 4 h. Ouabain (10^{-3} M final concentration) was added to one plate for the final 90 min of loading. The incubation was terminated by aspirating off the labelled Krebs and washing (x4) with Krebs at 2° and finally rinsing once with Krebs at 37°. Ten ml of inactive Krebs ($\pm 10^{-3}$ M ouabain) were added to the dish, which was incubated at 37°. After 5 min the solution was quickly poured from the dish into a scintillation vial for ^{32}P counting. A further 10ml of Krebs were added to the dish and the procedure was repeated for a total of 30 min (6x10ml samples). The cells were then trypsinised and the cell number, cell volume and residual radioactivity were determined as described in the METHODS. Each point represents the ^{32}P activity passing from the cells into extracellular solution during successive 5 min collecting periods. Flame photometric measurements showed that the $[Na^+]i$ rose from a value of 19 ± 4 m-mole/l in the control (○) cells to 61 ± 6 m-mole/l in the ouabain-treated (●) cells.

pre-loaded cells is exponential (Riggs, 1963). The amount of label in the bathing medium during a given period is a constant fraction of the label present at the start of each period. The data in Fig. 32 show that transport out of the cellular Pi pool did not conform to this simple behaviour. This point is considered in more detail in the next section. The rate of ^{32}Pi loss was significantly increased after ouabain treatment ($P<0.001$). Although other causes cannot be ruled out, the elevated $[Na^+]_i$ may ~~be~~ have caused ~~by~~ this increase. This conclusion is supported by the evidence from other Na-dependent transport systems. The inward movement of substrate is stimulated by the $[Na^+]_o$ and the outward movement by $[Na^+]_i$; (Eddy et al, 1967; Schultz & Curran, 1970). These effects have been termed cis-stimulation.

Effect of the extracellular Na^+ concentration on the Pi efflux. For some Na-dependent transport systems it has been found that increasing $[Na^+]_o$ inhibits the movement of substrate out of the cell. This effect is known as trans-inhibition (Schultz & Curran, 1970). The effect of $[Na^+]_o$ on the washout of ^{32}Pi from pre-loaded SV3T3 cells is shown in Fig. 33. In the cells exposed to decreased $[Na^+]_o$ the loss of ^{32}Pi during the first 5 minutes was approximately equal to the control. However, the loss of ^{32}Pi then declined to reach a new steady level which was below that of the control and depended on the $[Na^+]_o$. Thus, the effect of increasing $[Na^+]_o$ appears to be a stimulation of ^{32}Pi loss. This is the opposite of the expected trans-inhibitory effect. However, closer examination of the points at 5 minutes suggests that initially trans-inhibition of Pi loss by $[Na^+]_o$ may occur. All the values for ^{32}Pi loss when $[Na^+]_o$ was depleted were found to be greater than the control value. In a separate similar experiment the same effect was observed.

These seemingly contradictory results can be explained by supposing that in the control condition the

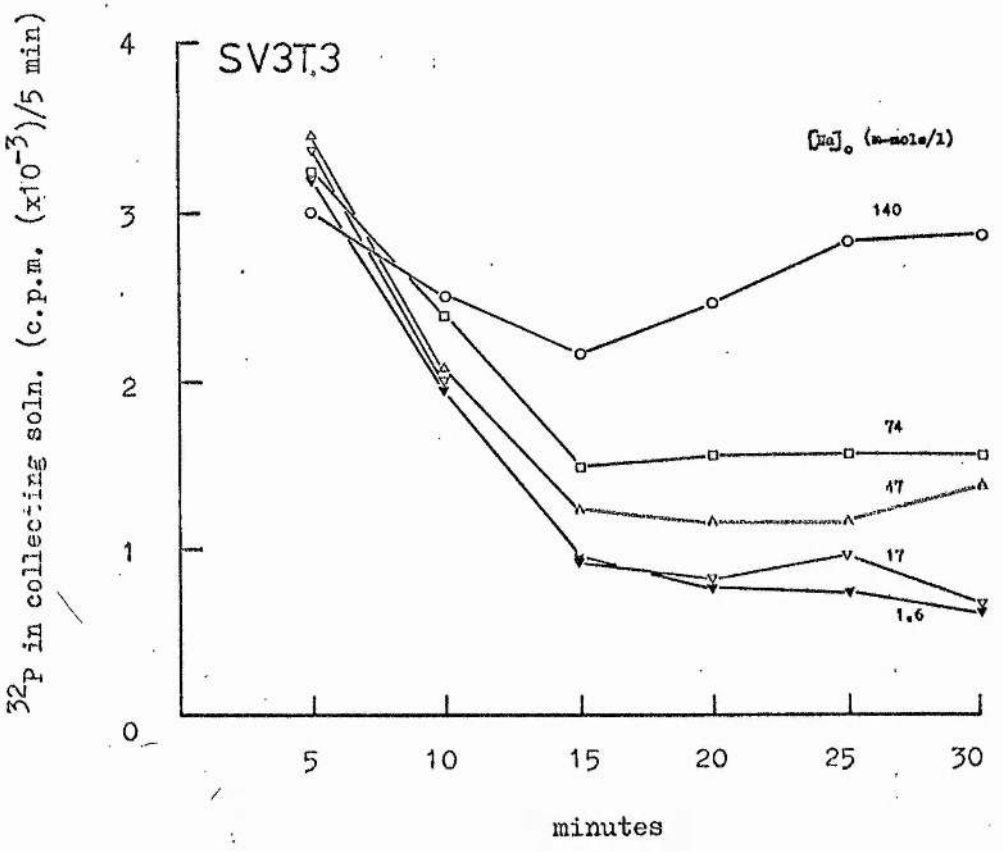


Fig. 33. Effect of the extracellular Na^+ concentration on the washout of ^{32}P from SV3T3 cells. The washout of ^{32}P from preloaded cells into Krebs of various $[Na^+]$ was followed as described in the legend to Fig. 32. Each point represents the ^{32}P activity passing from the cell into the extracellular solution during successive 5 min collecting periods. Choline was substituted for Na^+ to maintain isotonicity. The $[Na^+]$ of the Krebs were (○) 140; (□) 74; (△) 47; (▽) 17; (▼) 1.6 m-mole/l.

influx of Pi maintains the intracellular Pi pool at a constant size. The specific activity of this pool could also be maintained via rapid equilibration with the much larger organic phosphate pool. In this way approximately equal amounts of ^{32}Pi would be lost during successive intervals. This would continue until sufficient label was lost to cause a significant decrease in the specific activity of the organic pool. When the $[Na^+]_o$ was reduced the Pi influx would be decreased (Fig. 29). This could lead to a reduction in the size of the intracellular Pi pool which would decrease to a new steady level, the size of which would depend on the degree of inhibition of the Pi influx. The specific activity of this reduced pool would also be maintained via exchange with the large organic phosphate pool. A new decreased steady level of ^{32}Pi loss would then be observed.

On this basis the data in Fig. 33 show that inhibition of the Pi influx by decreasing $[Na^+]_o$ led to a reduction in the cellular Pi pool which was complete 15 minutes after the alteration of $[Na^+]_o$. After this period ^{32}Pi was again lost from the cells by a constant amount during each successive interval. The different steady levels of ^{32}Pi loss during the period 15-30 minutes thus reflect changes in the Pi pool size rather than a direct effect of $[Na^+]_o$. The direct effect of $[Na^+]_o$ on the outward transport of Pi is best judged from the values at 5 minutes which suggest that trans-inhibition occurs.

Trans-inhibition of substrate efflux should be reduced or abolished by raising the extracellular substrate concentration (Vidaver, 1964; Vidaver & Shepherd, 1968). This possibility has been examined for the ^{32}Pi efflux (Fig. 34). A similar procedure to that shown in Fig. 33 was carried out except that the Pi concentration of the collecting solution was raised 10-fold to 5.50 m-mole/l.

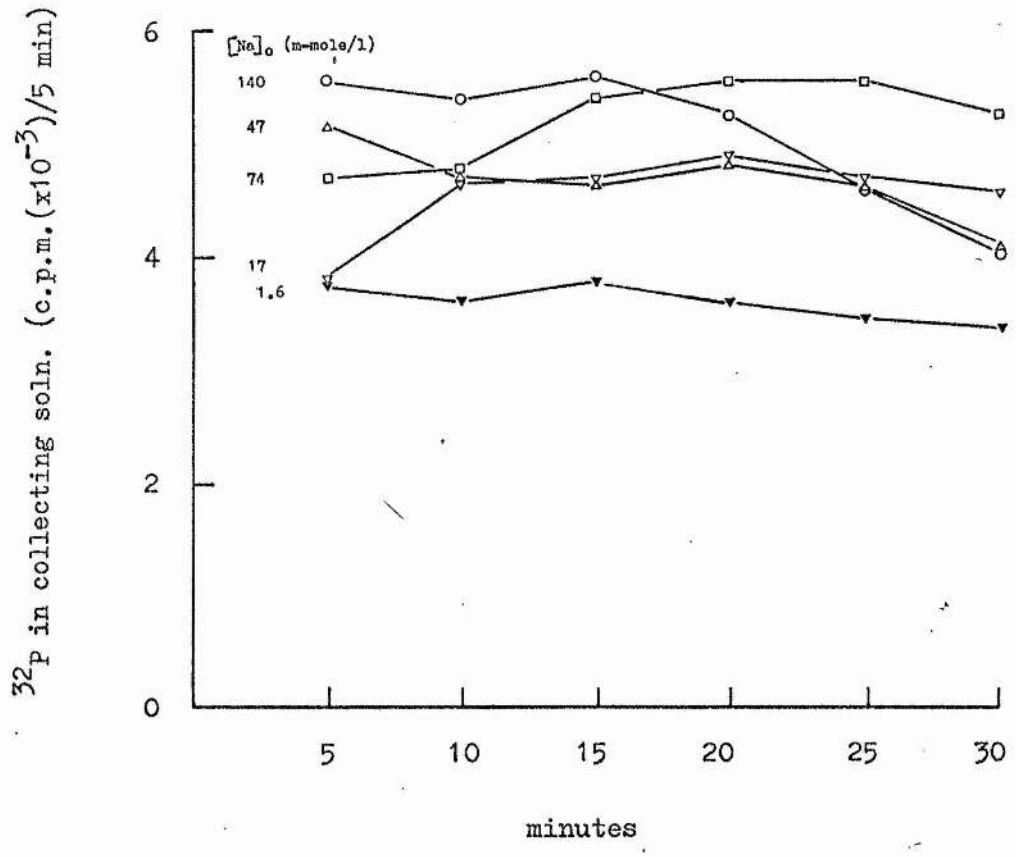


Fig. 34. Effect of the extracellular Na^+ concentration on the washout of ^{32}P from preloaded SV3T3 cells at elevated levels of extracellular Pi. The experimental details were identical with those described in the legend to Fig. 33, except that the [Pi] of the Krebs collecting solution was raised 10-fold from 0.55 to 5.5 m-mole/l.

Since the data for Figs. 33 & 34 are from separate parts of the same experiment the numbers of cells per plate and the specific activities of the loading solution were not significantly different. The ^{32}Pi counts are, therefore, quantitatively comparable. The increased $[Pi]_o$ caused an overall increase in the rate of ^{32}Pi loss presumably through an enlargement of the cellular Pi pool due to an increased Pi influx. The increased $[Pi]_o$ decreased the Na-dependency of ^{32}Pi loss. This would be expected since, at high $[Pi]_o$ the Pi influx is less inhibited by a reduction of the $[Na^+]_o$. (The effect of $[Na^+]_o$ is largely on the Km with less change in the Vmax for influx; Fig. 31). The values at 5 minutes suggest that raising the $[Pi]_o$ has abolished *trans*-inhibition of the Pi efflux by Na^+.

Serum-stimulation of phosphate transport in 3T3 cells

Stimulation of the phosphate influx. Quiescent 3T3 cells can be stimulated to divide by either a complete change of medium or by adding serum to the existing medium (Todaro et al, 1965, 1967). One of the earliest changes to occur following serum addition is an increase in the rate of sugar, uridine and phosphate transport (see INTRODUCTION). The serum-stimulation of phosphate influx has previously been measured only from solutions with a very low Pi concentration; in fact phosphate-free except for the ^{32}Pi label (Cunningham & Pardee, 1969; Jimenez de Asua et al, 1974; Jimenez de Asua & Rozengurt, 1974). The effect of serum-stimulation on the influx of phosphate from a Krebs solution containing 0.55 m-mole/l Pi is shown in Fig. 35 . In these 7-day-old quiescent cultures a change to complete fresh medium caused a 2 to 3-fold stimulation of the Pi influx ($P < 0.001$). Fresh medium without serum did not raise the Pi influx above the unstimulated controls ($P > 0.10$). The increased transport was only found when quiescent cells were used. Complete fresh medium did not significantly increase ($P > 0.10$) the influx of Pi into rapidly growing

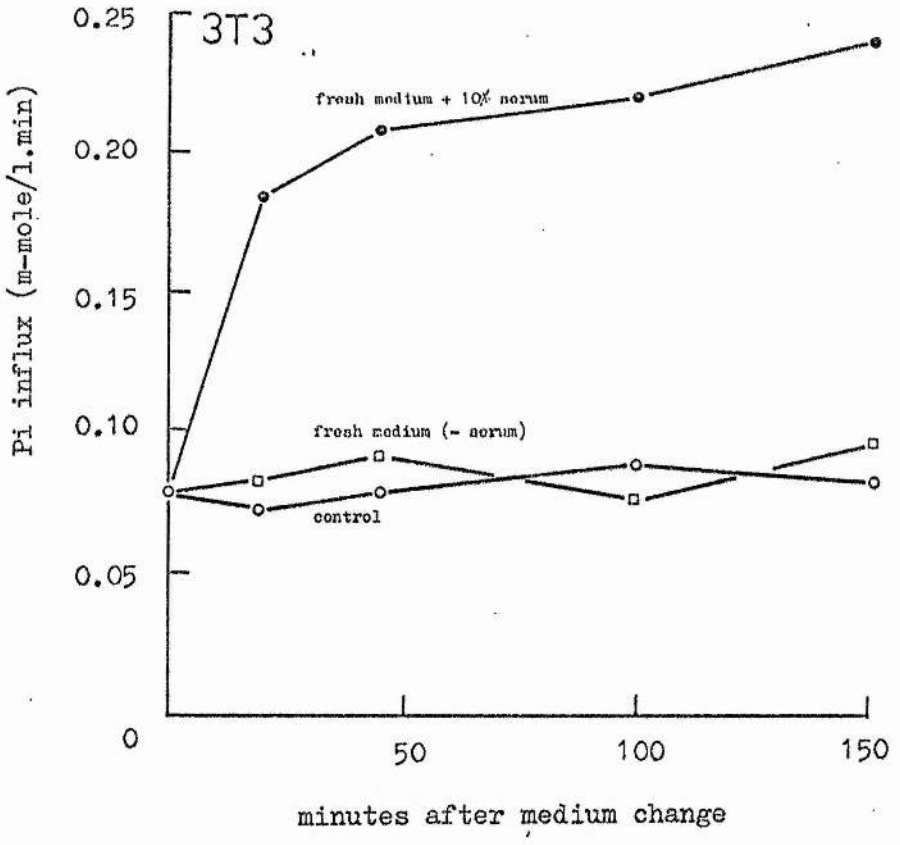


Fig. 35. The influx of Pi into quiescent 3T3 cells as a function of time after the addition of fresh medium with or without serum. The growth medium was removed from 7-day-old cultures and replaced by (○) the same medium, (◉) fresh medium + 10% calf serum and (□) fresh medium without serum. The influx was measured from Krebs (0.55 m-mole/l Pi) at the indicated times. Incubations with ^{32}Pi were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 20. Each point represents the value obtained for the phosphate influx from a single plate of cells. The lines were drawn by eye.

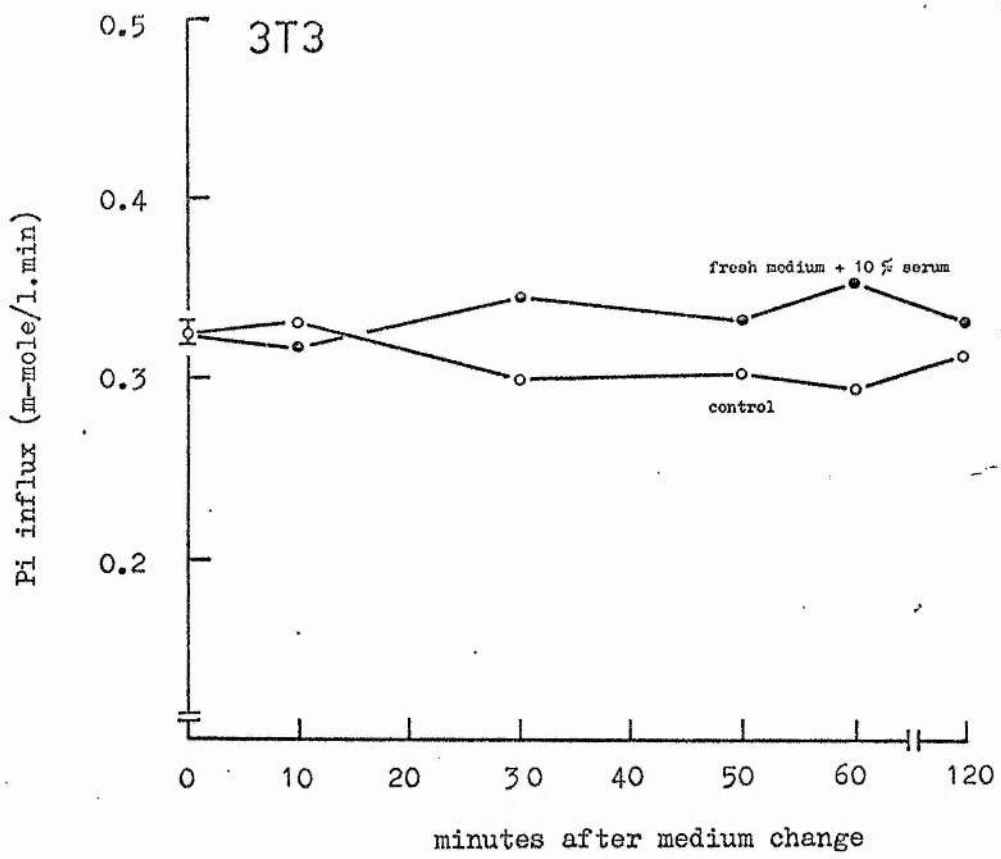


Fig. 36. The influx of Pi into growing 3T3 cells as a function of time after the addition of fresh complete medium. The growth medium was removed from 3-day-old, rapidly growing cells and replaced by (○) the same medium, (●) fresh medium + 10% calf serum. The influx was measured from Krebs (0.55 m-mole/l Pi) at the indicated times. Incubations with ^{32}Pi were carried out for 5 min according to the procedure described in the legend to Fig. 20. Each point represents the value obtained for the phosphate influx from a single plate of cells. The lines were drawn by eye.

3T3 cells (Fig. 36).

The degree of stimulation of the Pi influx shown in Fig. 35 is similar to that reported by Cunningham & Pardee (1969) and by Jimenez de Asua et al (1974). However, a 2 to 3-fold stimulation is lower than that most recently reported by Jimenez de Asua & Rozengurt (1974). They obtained a biphasic response with a 5-fold increase in the Pi influx during the first phase and 10-fold stimulation during the second phase when the response was followed for 5 hours after serum addition. The smaller stimulation shown here may have resulted from the higher $[Pi]_o$ used. In addition, after the stimulation period the cells in this study were transferred to a Krebs solution containing only 1% serum. It is possible that the increase in transport is rapidly reversed and that the full stimulation is only seen in the continued presence of 10% serum during the influx measurement. These possibilities have been checked by using the method of Jimenez de Asua & Rozengurt to measure the stimulation of Pi influx in these cells. Again a 2 to 3-fold increase in transport was found (Fig. 37). These results show that neither a Pi concentration effect nor a reversed response explain the observed difference. Since the magnitude of the response was not diminished by measuring the Pi influx from a Krebs solution this procedure was employed in further investigations of the serum-stimulation of Pi transport.

The combined results of several experiments are shown in Fig. 38 . The results show that the increased Pi transport was maintained for at least 24 hours after stimulation. When cells were grown in medium containing 10% serum prior to stimulation, a biphasic response never occurred. In an attempt to ensure complete quiescence, some cells were incubated in medium containing only 0.3% serum for 48 hours prior to stimulation. A biphasic response was found for the

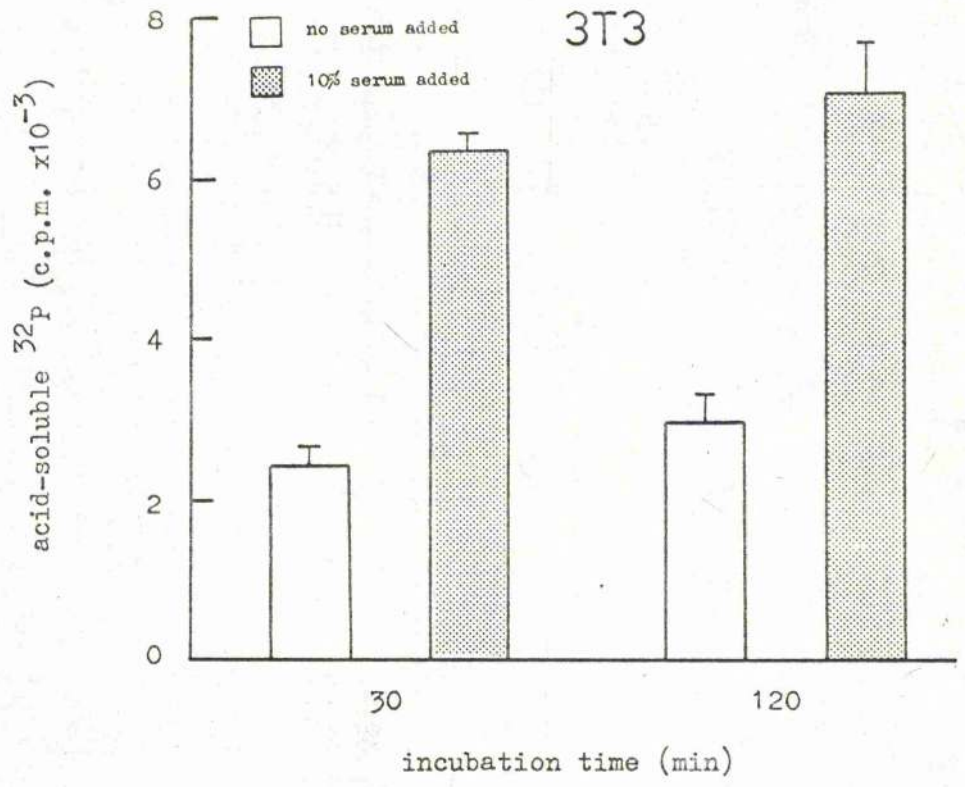


Fig. 37. The influx of Pi into quiescent 3T3 cells as a function of time after serum addition to the culture medium; method of Jimenez de Asua & Rozengurt (1974). Quiescent, 6-day-old cultures of 3T3 cells were washed (x2) with warm Pi-free medium and incubated with 9ml of this medium at 37°. After 10 min, 1ml of dialysed calf serum was added to some dishes and all the cultures were incubated for an additional 30 or 120 min. Cells were labelled with ^{32}Pi (1µCi/ml) during the final 5 min of incubation. The radioactive medium was aspirated off and the cells were washed (x4) with Krebs at 2°. Incorporation of ^{32}Pi into the acid-soluble fraction was measured by extracting washed cells for 30 min at 4° with 5% trichloracetic acid and counting a 1ml aliquot in NE250 scintillation fluid. The column height represents the mean of 3 determinations Pi influx. The error bars show the S.E.

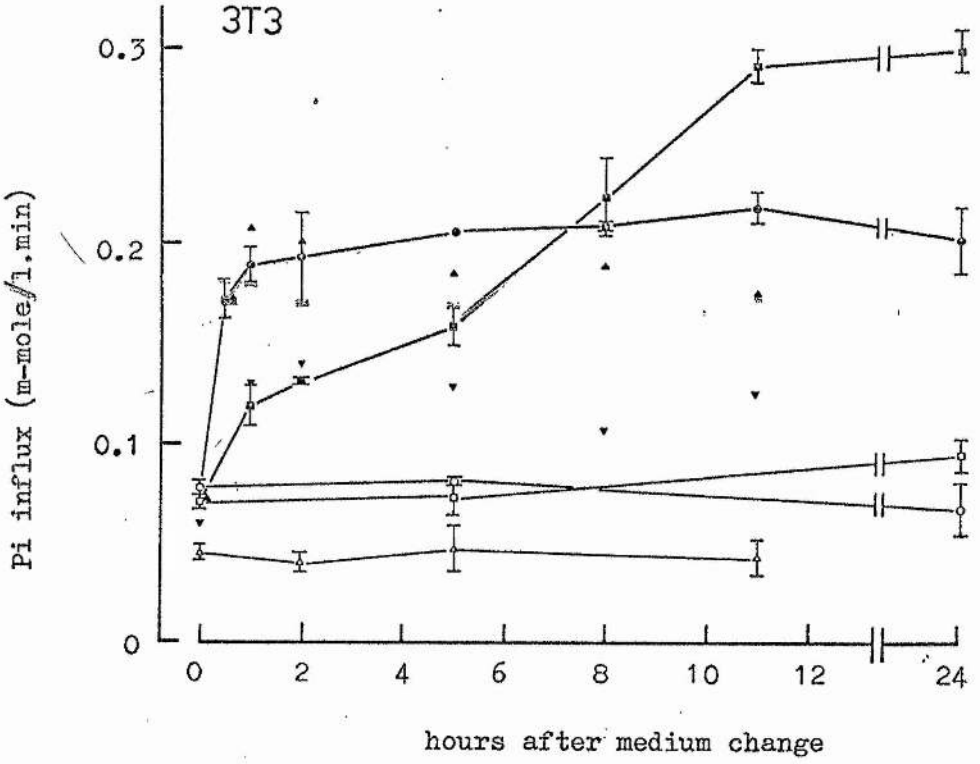


Fig. 38. The influx of Pi into quiescent 3T3 cells as a function of time after the addition of fresh medium and i) the serum concentration of the previous growth medium ii) the presence of cycloheximide in the fresh medium iii) the absence of Na^+ from the Krebs influx solution. All cultures had been growing for 6 days and were quiescent at the time of the experiments. Some of the cells had been exposed to medium containing reduced serum (0.3%) for the final 48 h of growth. At time 0, fresh medium (10% calf serum) was added to cells which had previously been growing in medium containing 10% serum (●-●) or 0.3% serum (■-■). At the indicated times the Pi influx was measured as described in the legend to Fig. 20. The corresponding untreated controls for cells previously exposed to 10% serum (○-○) and 0.3% serum (□-□) are shown. Fresh complete medium containing cycloheximide (10µg/ml) was added to some cells previously exposed to 10% serum (▲) or 0.3% serum (▽). Some cells received fresh complete medium for the indicated times after which the Pi influx was measured from Na-free Krebs (△). Where error bars are shown they indicate the S. E. of the mean for 2 or 3 determinations of the Pi influx. Single points represent the value for the Pi influx obtained from one plate of cells. The lines were drawn by eye.

serum-stimulation of the Pi influx into these cells. The early response (2h) was smaller than that found for the cells maintained in 10% serum but the maximum response (11h) was greater.

The response of the cells maintained in high serum and the initial response of cells maintained in low serum appears to be unaffected by cycloheximide-induced inhibition of protein synthesis. However, the second phase of the response of low serum treated cells was completely inhibited by cycloheximide. When the $[Na^+]$ of the Krebs solution was reduced to 1.6 m-mole/l no stimulation of the Pi influx was observed, indicating that serum stimulates Na-dependent Pi transport.

Effect of serum-stimulation on the kinetic constants for phosphate transport. Table 10 shows the values obtained for the Km and Vmax for Pi transport at different times after the addition of fresh medium to quiescent 3T3 cells. The data show that increased Pi transport was always associated with an increased Vmax. No comparable changes in Km were found. The significance of these results will be considered in the DISCUSSION.

Table 10. Effect of serum stimulation on the kinetic constants of phosphate transport

% serum prior to stimulation		Time after change of medium (h)		
		0	2	12
0.3	Km (m-mole/l)	0.26	0.34	0.28
	Vmax (m-mole/l. min)	0.12	0.21	0.56
10	Km (m-mole/l)	0.22	0.28	0.22
	Vmax (m-mole/l. min)	0.15	0.36	0.39

Kinetic constants for Pi transport by quiescent 3T3 cells as a function of time after the addition of fresh medium. The cultures were prepared as described in the legend to Fig. 38. The kinetic constants were measured as described in the legends to Figs. 25 and 26.

2-deoxyglucose transport in 3T3 and virus-transformed 3T3 cells

Time-course of 2-deoxyglucose incorporation into total cell material. The uptake of 0.5 m-mole/l 2-deoxyglucose by 3T3, SV3T3 and Py3T3 cells over a 30 minute period is shown in Fig.39 . Since equilibrium was not reached during this time it was not possible to determine whether the total uptake of 2-deoxyglucose was altered by virus-transformation. Uptake in the transformed cells was linear out to 10 minutes and linear out to 30 minutes in the untransformed cells. This result indicates that 5 minutes would be a suitable incubation period for the measurement of 2-deoxyglucose influx in all cell lines. The data at 5 minutes in this experiment show that the influx was 0.43 m-mole/l. min in 3T3 cells, 1.53 m-mole/l. min in SV3T3 cells and 1.38 m-mole/l. min in Py3T3 cells. This suggests, in agreement with others (see INTRODUCTION), that the rate of 2-deoxyglucose transport is increased after virus-transformation. However, the cell population density has also been shown to affect 2-deoxyglucose transport (see INTRODUCTION) and the possibility of an interaction between cell density and virus-transformation should not be ignored. The effect of cell population density on the 2-deoxyglucose influx has therefore been examined in more detail for the 3 cell lines.

Effect of cell population density on the 2-deoxyglucose influx. Fig.40a shows the results of an experiment in which the 2-deoxyglucose influx in 3T3 cells was measured each day, for several days, after plating. The number of cells/dish at the time of measurement is also included in the figure. The 2-deoxyglucose influx increased for the first 2 days after plating to a maximum of 1.11 m-mole/l. min. The influx then steadily decreased to only 0.18 m-mole/l. min after 7 days growth. It can be seen that the reduction in 2-deoxyglucose transport precedes the cessation of cell

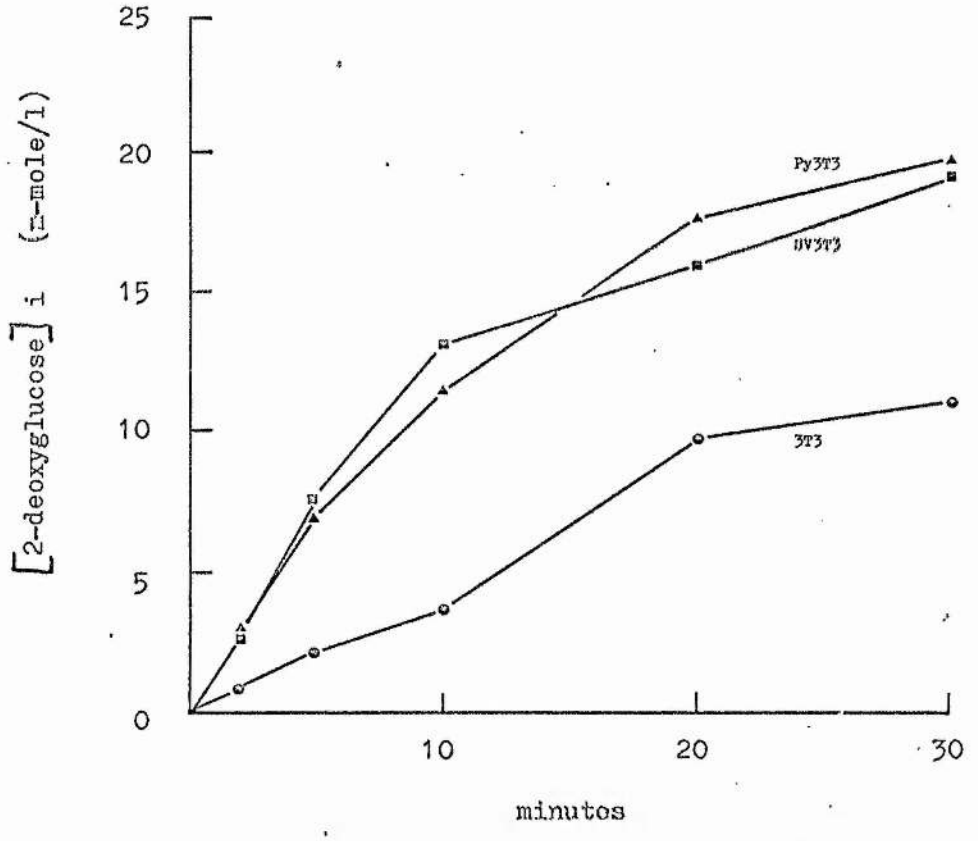


Fig. 39. Time-course of 2-deoxyglucose incorporation into total cell material of 3T3 and virus-transformed 3T3 cells. 3T3 (●) cells were seeded at 0.5×10^6 cells/9cm dish, SV3T3 (■) and Py3T3 (▲) cells were seeded at 0.8×10^6 cells/9cm dish, and the uptake was measured after 4 days growth. At zero time the growth medium was replaced with a glucose-free Krebs solution containing 0.50 m-mole/l of 2-deoxy-glucose and ^{3}H-2-deoxyglucose (~1μCi/ml). After periods ranging from 2 to 30 min the radioactive solution was removed, the cells washed (x4) with Krebs at 2°, detached from the dish with trypsin and analysed for ^{32}P activity, cell number and cell volume. Each point represents the value for [2-deoxyglucose]i obtained from a single plate of cells.

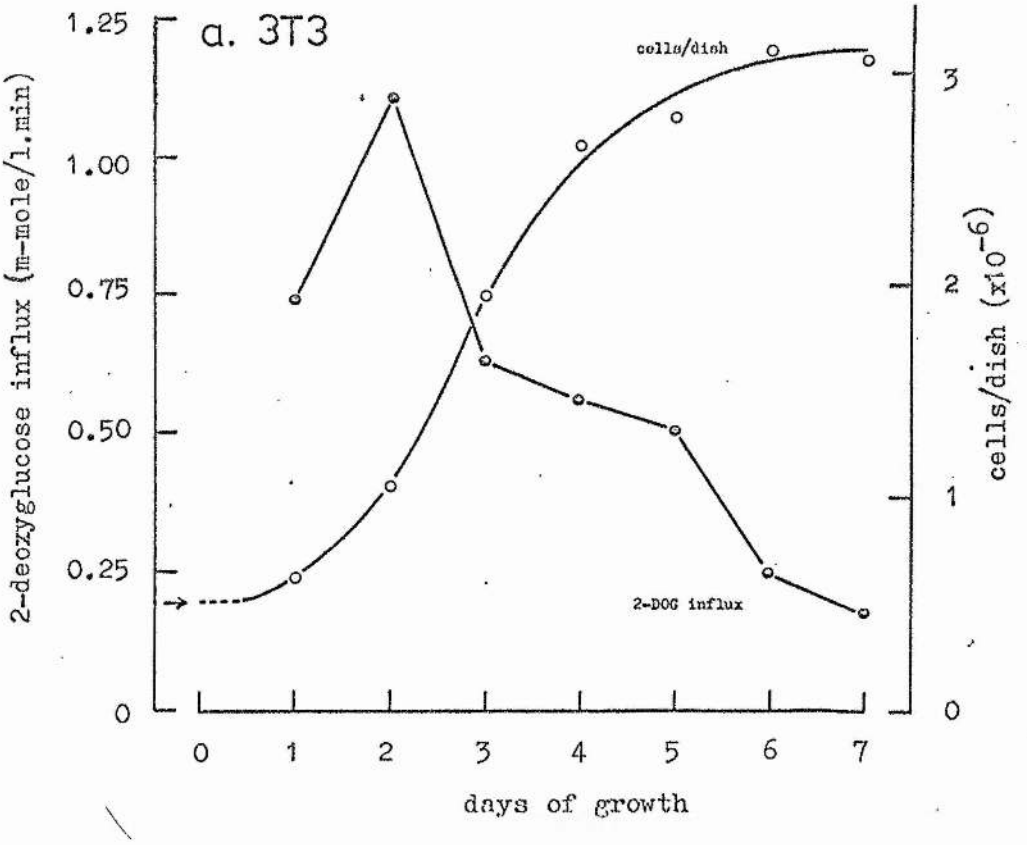
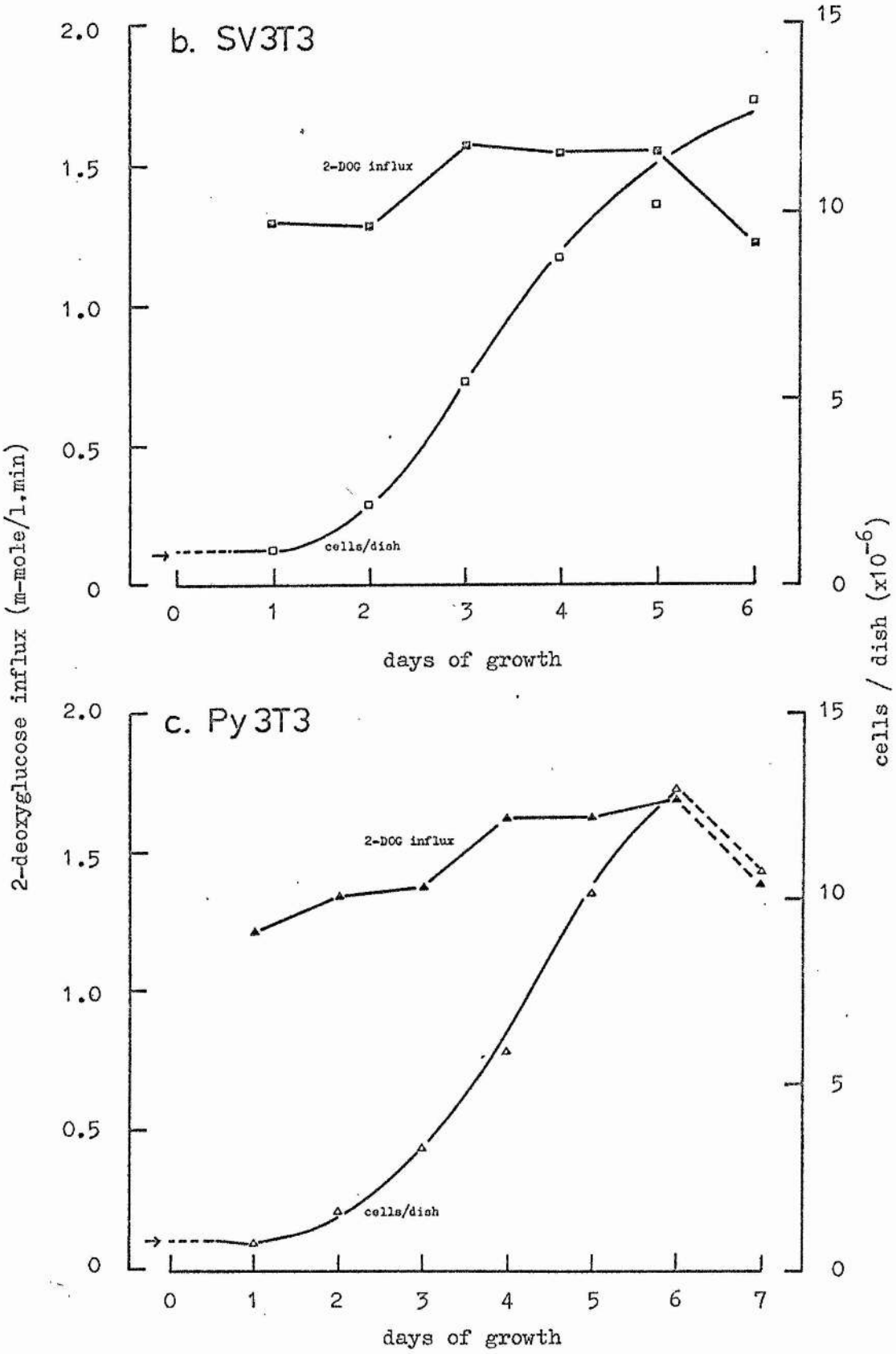


Fig. 40. Effect of the cell population density on the 2-deoxyglucose influx into 3T3 (a), SV3T3 (b) and Py3T3 (c) cells. Cells were seeded onto 9cm dishes at the indicated density (➡). The 2-deoxyglucose influx (closed symbols) was measured at the same time each day from 1 to 7 days after seeding. Incubations with 0.5 m-mole/l ^{3}H-2-deoxyglucose were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 39. The number of cells/dish (open symbols) at the time of the influx measurement has also been included in the figures. Each point represents a single determination of the influx or cell number.


b. SV3T3
2-DOG influx
cells/dish
2.0
1.5
1.0
0.5
0
15
10
5
0
0 1 2 3 4 5 6
days of growth
c. Py 3T3
2-DOG influx
cells/dish
2.0
1.5
1.0
0.5
0
15
10
5
0
0 1 2 3 4 5 6 7
days of growth
2-deoxyglucose influx (m-mole/l.min)
cells / dish (x10⁻⁶)

growth. A large reduction in the influx occurred between days 2 and 4, a period of almost exponential cell growth. The influx continued to decrease during the period from day 4 to day 7 when little cell growth occurred. The effect of cell density on the 2-deoxyglucose influx in SV3T3 and Py3T3 cells is shown in Fig.40b and 40c respectively. The results show that the transport of 2-deoxyglucose by virus-transformed cells was much less dependent on the cell population density. At day 7 no value was obtained for SV3T3 cells and the value for Py3T3 cells is unreliable due to the tendency of the thick cell layer to detach from the dish. In contrast to previous findings (see INTRODUCTION) these results indicate that polyoma and simian virus-transformation per se did not greatly increase the rate of 2-deoxyglucose transport. The maximum influx in the transformed cells was only slightly larger than the maximum influx in the untransformed cells. The major change after virus-transformation appears to be the abolition of the reduction in 2-deoxyglucose transport with increasing cell density. This observation will be considered in more detail in the DISCUSSION.

The effect of the cell population density on the influx of 2-deoxyglucose in 3T3 cells was also examined using an alternative procedure. Cells were plated at different population densities in the range 0.2- 1.0 (x 10^6) cells/dish. The influx was measured 4 or 5 days after plating. In this way a range of population densities was obtained in cultures of the same age. The results show that, once again, the influx of 2-deoxyglucose decreased with increasing cell densities. The values at 4 and 5 days have been represented by different symbols and can be seen to lie on the same curve (○ and □ ; Fig. 41). The data shown previously for 3T3 cells in Fig. 40a has been plotted as influx against cell number and included in Fig. 41 (⊙). The different experimental procedures appear to produce quantitatively

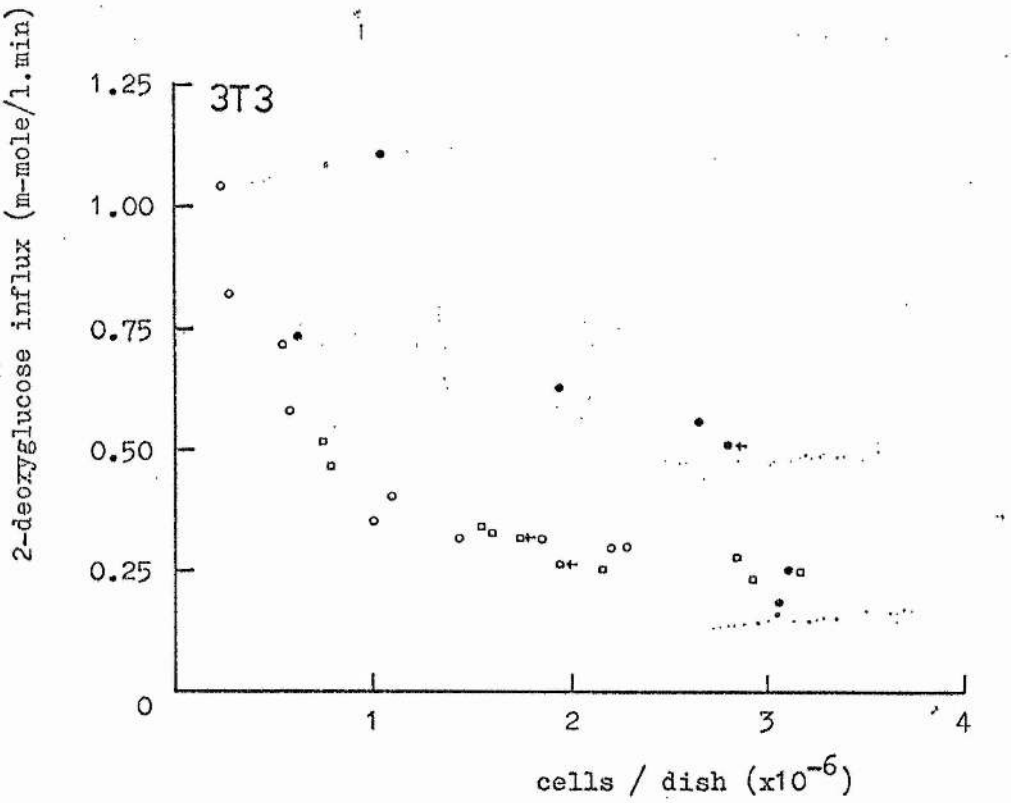


Fig. 41. Effect of the cell population density on the 2-deoxyglucose influx into 3T3 cells. Four dishes of cells were seeded at each of the following densities: 0.2, 0.4, 0.5, 0.7 and 1.0 ($x10^6$) cells/9cm dish. After 4 days growth the 2-deoxyglucose influx was measured using 2 plates from each batch. The remaining plates were used for influx measurements 1 day later. Incubations with 0.5 m-mole/l ^{3}H-2-deoxyglucose were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 39. Each point represents the value for the 2-deoxyglucose influx obtained from a single dish of cells. The results show that the values obtained after 4 (○) or 5 (□) days growth lie on the same curve. The data presented for 3T3 cells in Fig. 40a has been included in this graph (⊙) to facilitate a direct comparison of the two sets of results.

different results for the effect of cell density on 2-deoxyglucose influx (compare curves in Fig. 41). When cells were seeded at different densities and examined after a fixed growth period the initial rise in 2-deoxyglucose transport was not observed and the reduction in transport occurred at lower cell densities. This may have been a real effect caused by an interaction of cell density and culture age. Alternatively, the difference could have been caused by changes in cell growth characteristics during the several-week interval between these experiments. In this respect it should be noted that the cells showing the higher rate of transport increased from 0.5×10^6 to almost 3×10^6 cells/dish during 5 days growth (●←). The cells seeded at 0.5×10^6 cells/dish in the other experiment reached only 2×10^6 cells/dish after 5 days growth (□←). The observed experimental differences may, therefore, have been caused by a difference in cell growth rates between the two experiments.

Effect of the cell population density on the kinetic constants for 2-deoxyglucose transport. The effect of cell density on the kinetics of 2-deoxyglucose transport by 3T3 cells is shown in Fig. 42 . The influx from a range of extracellular 2-deoxyglucose concentrations (0.2-10.0 m-mole/l) was measured 2 and 6 days after seeding. The data is shown after linear transformation (v against v/ S) together with a summary of the calculated kinetic constants. As expected, the kinetics of 2-deoxyglucose transport were dependent on cell density. The reduction of 2-deoxyglucose transport associated with increased cell density can be attributed to a reduction in Vmax with little change in the Km of the system (Fig. 42).

In a similar experiment, SV3T3 cells (Fig. 43) showed a much smaller reduction of Vmax, again with no change in the Km, when 2 and 6 day-old cultures were compared. There was little difference in the Km for 2-deoxyglucose

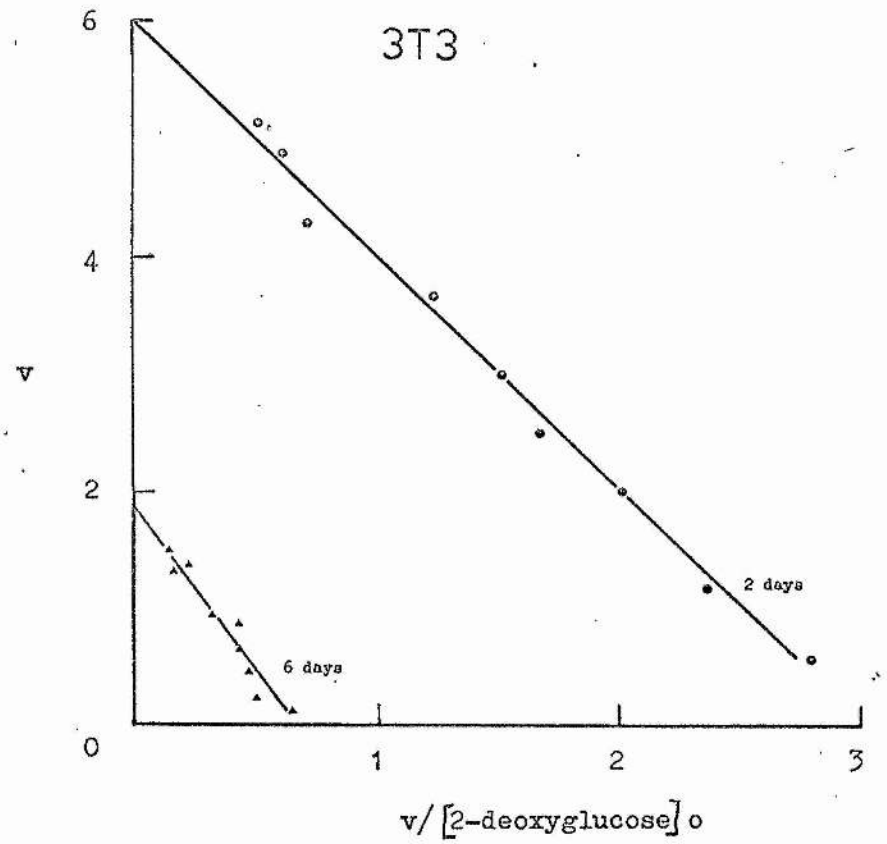


Fig. 42. Effect of the cell population density on the kinetic constants for 2-deoxyglucose transport by 3T3 cells. The influx of 2-deoxyglucose from Krebs with [2-deoxyglucose] in the range 0.2 to 10.0 m-mole/l was determined for 2 (●) and 6 (▲)-day-old cultures of 3T3 cells. Incubations with ^{3}H-2-deoxyglucose were carried out for 5 min at 37° as described in the legend to Fig. 39. The data is shown after linear transformation (v against v/S; APPENDIX B). Each point represents a single value for the influx (v) which is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km and Vmax, are shown in the attached table.

Days of growth	mean cell no./ dish ($\times 10^{-6}$)	Km m-mole/l	Vmax m-mole/l. min
● 2	0.56 ($\pm$0.07)	2.01	6.00
▲ 6	2.43 ($\pm$0.11)	2.84	1.90

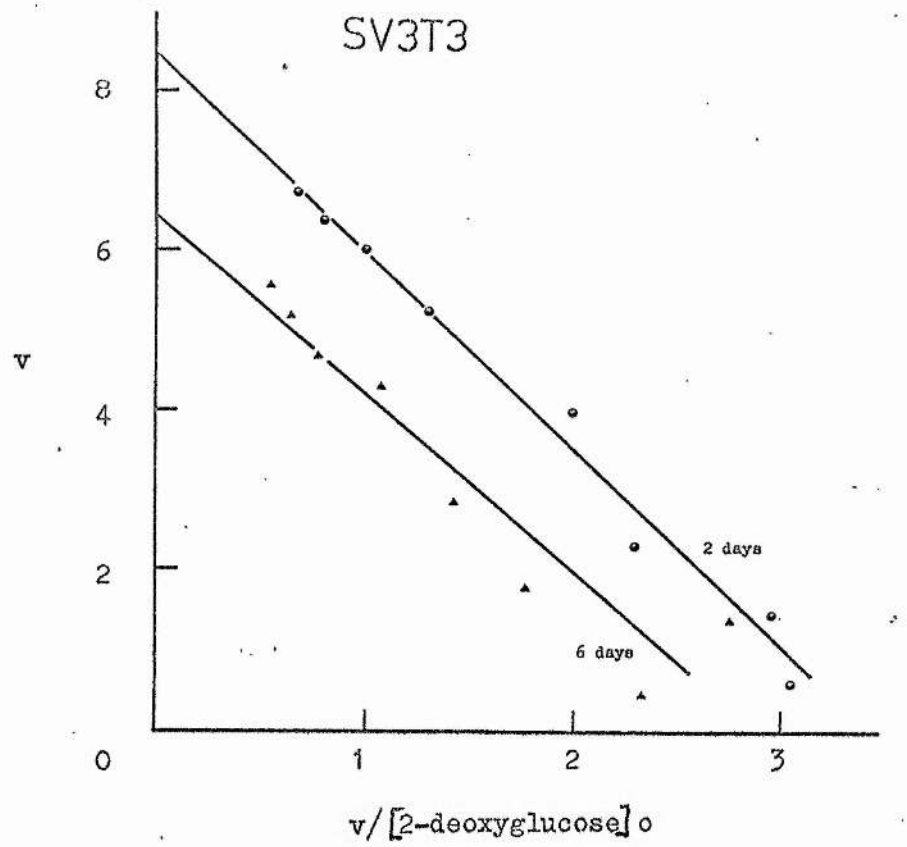


Fig. 43. Effect of the cell population density on the kinetic constants for 2-deoxyglucose transport by SV3T3 cells. Experimental details were identical to those described for 3T3 cells in the legend to Fig. 42. The calculated kinetic constants, Km and Vmax, are shown in the attached table.

Days of growth	mean cell no./ dish ($\times 10^{-6}$)	Km m-mole/l	Vmax m-mole/l. min
○ 2	1.89 ($\pm$0.10)	2.48	8.46
▲ 6	11.21 ($\pm$0.46)	2.23	6.44

transport between 3T3 and SV3T3 cells. The Vmax was slightly higher (41%) in low-density SV3T3 cells than in low-density 3T3 cells and much higher (338%) when high-density cells were compared. This result emphasises that, for these cells at least, virus-transformation did not greatly increase the maximum transport capacity of the cells. The good fit obtained for the straight line transformations (Figs.42 &43) indicates that, in both cell lines, the transport of 2-deoxyglucose "follows Michaelis-Menten kinetics". The decreased Vmax associated with increasing cell density suggests that as the cell density increases the number of functional transport sites decreases. The absence of any change in the Km suggests that cell density does not influence the affinity of the transport carrier for the substrate.

Inhibition of 2-deoxyglucose transport by D-glucose and cytochalasin B. The observation that D-glucose competitively inhibits the influx of 2-deoxyglucose (Renner, Plagemann & Bernlohr, 1972) suggests that both substrates are transported by a single system. The mould metabolite cytochalasin B has been shown to be a potent competitive inhibitor of both substrates in several cell lines (Estensen & Plagemann, 1972; Mizel & Wilson, 1972; Plagemann, 1973; Kletzein & Perdue, 1973). The kinetics of 2-deoxyglucose transport in 3T3 (Fig. 44) and SV3T3 cells (Fig. 45) have been measured in the presence of these inhibitors. The data is shown after linear transformation (v against v/S) and the calculated kinetic constants are shown in Table 11 . In both cell lines both D-glucose and cytochalasin B competitively inhibited 2-deoxyglucose transport. The presence of the inhibitor caused an increase in the apparent Km of the system with no great change in Vmax. The Ki's (Table 11) were similar in both cell lines. The significance of this result will be considered in more detail in the DISCUSSION.

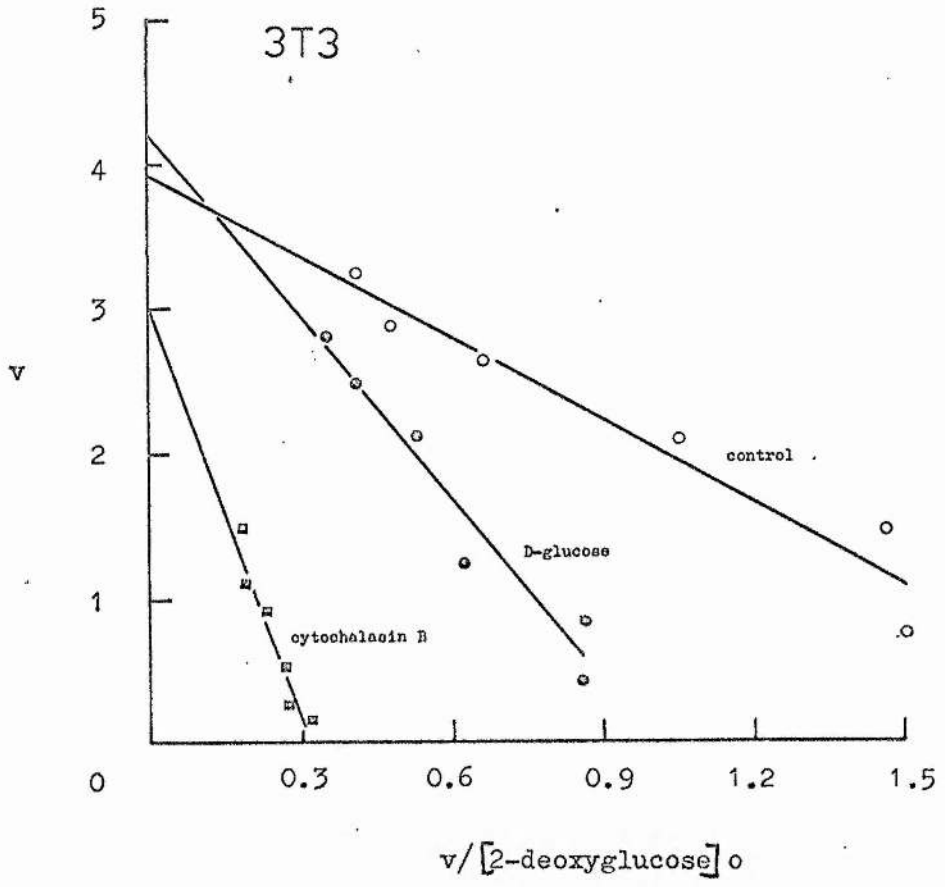


Fig. 44. Inhibition of 2-deoxyglucose transport by D-glucose and cytochalasin B in 3T3 cells. The influx of 2-deoxyglucose from Krebs (O) was determined over the concentration range 0.2 to 8.0 m-mole/l. Where indicated D-glucose (⊙; 2.0 m-mole/l) or cytochalasin B (▣; 0.5 µg/ml final concentration) were included in the Krebs. The data is shown after linear transformation (v against v/S). Each point represents a single value for the influx (v) which is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km (Ka), Ki and Vmax are shown in Table 11.

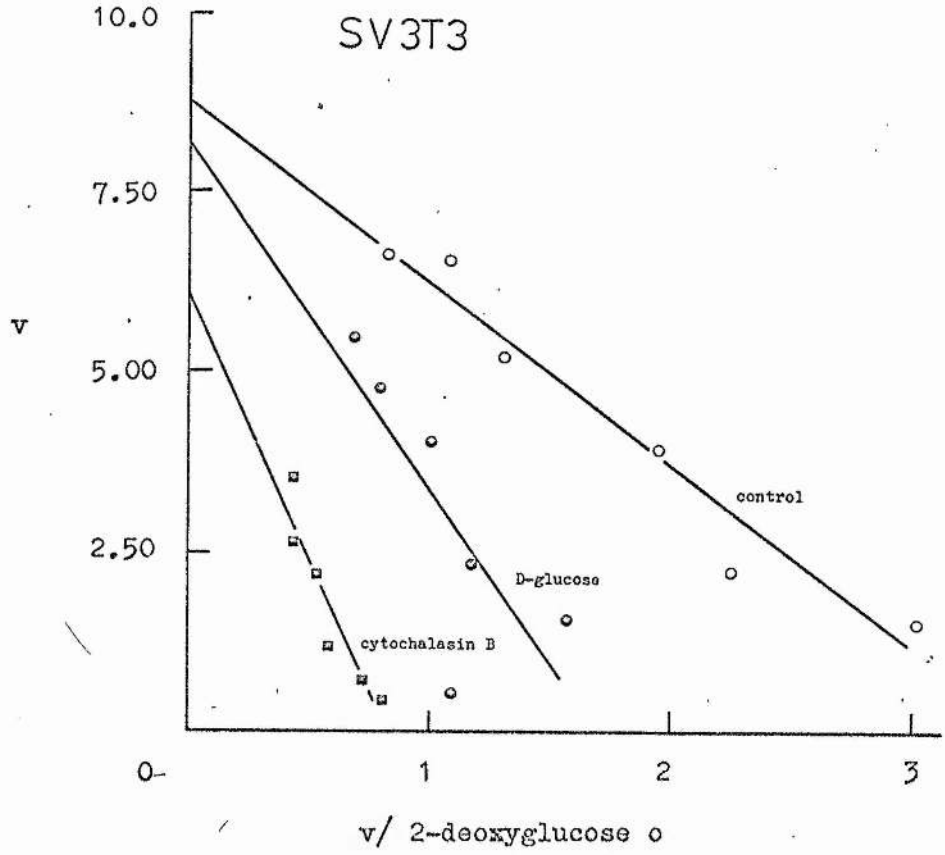


Fig. 45. Inhibition of 2-deoxyglucose transport (O) by D-glucose (⊙) and cytochalasin B (⊡) in SV3T3 cells. Experimental details were identical to those described for 3T3 cells in the legend to Fig. 44. The calculated kinetic constants Km (Ka), Ki and Vmax are shown in Table 11.

Table 11. Kinetic constants for the inhibition of 2-deoxyglucose transport by D-glucose and cytochalasin B

		Vmax (m-mole/l. min)	Km (m-mole/l)	Ka (m-mole/l)	*Ki (M)
3T3	control	3.94	1.89	---	---
	D-glucose	4.23	---	4.25	1.60×10^{-3}
	cytochalasin B	3.02	---	9.30	0.27×10^{-6}
SV3T3	control	8.81	2.57	---	---
	D-glucose	8.22	---	4.83	2.27×10^{-3}
	cytochalasin B	6.08	---	7.26	0.57×10^{-6}

Calculations of the kinetic constants were made from the data shown in Figs. 44 and 45 .

* Calculations using $Ki = [i]/((Ka/Km)-1)$; where Ki is the Michaelis constant of the inhibitor, $[i]$ is the inhibitor concentration, Km the Michaelis constant of the substrate in the absence of the inhibitor and Ka the apparent Michaelis constant of the substrate in the presence of the inhibitor.

Serum-stimulation of 2-deoxyglucose transport in 3T3 cells

Stimulation of the 2-deoxyglucose influx. When quiescent 3T3 cells are exposed to fresh serum they restart DNA synthesis and cell division (Todaro et al, 1965). Within minutes of serum addition the influx of inorganic phosphate, uridine and glucose increase several fold (see INTRODUCTION). However, there are differences in the literature as to the time-course and magnitude of this transport stimulation. The effect of the addition of fresh medium on the influx of 2-deoxyglucose into quiescent 3T3 cells was investigated. The rate of 2-deoxyglucose transport was found to increase rapidly after the switch to fresh medium ($P<0.001$) reaching a maximum within 40 minutes after the change. Thereafter, the influx remained constant at a value some 2-fold greater than the untreated controls. The influx remained at this elevated level for at least 5 hours after the change of medium (Fig. 46).

A much larger (5-10 fold) biphasic response has been reported (Jimenez de Asua & Rozengurt, 1974; Bradley & Culp, 1974). While the first phase was found to be independent of protein synthesis, the development of the second phase was inhibited by cycloheximide. The effect of cycloheximide on the single phase stimulation of 2-deoxyglucose transport reported here has been investigated (Fig. 47). The presence of cycloheximide in the fresh medium did not alter the response at 2 hours after the medium change. The slight reduction of the response in the presence of cycloheximide 5 hours after the change of medium is probably not due to a specific inhibition but to a more general effect brought about by the prolonged inhibition of protein synthesis. In this experiment some cells received fresh medium for 2 or 5 hours and the 2-deoxyglucose influx was then measured in the presence of cytochalasin B. The results (Fig. 47) show that the increase in 2-deoxyglucose

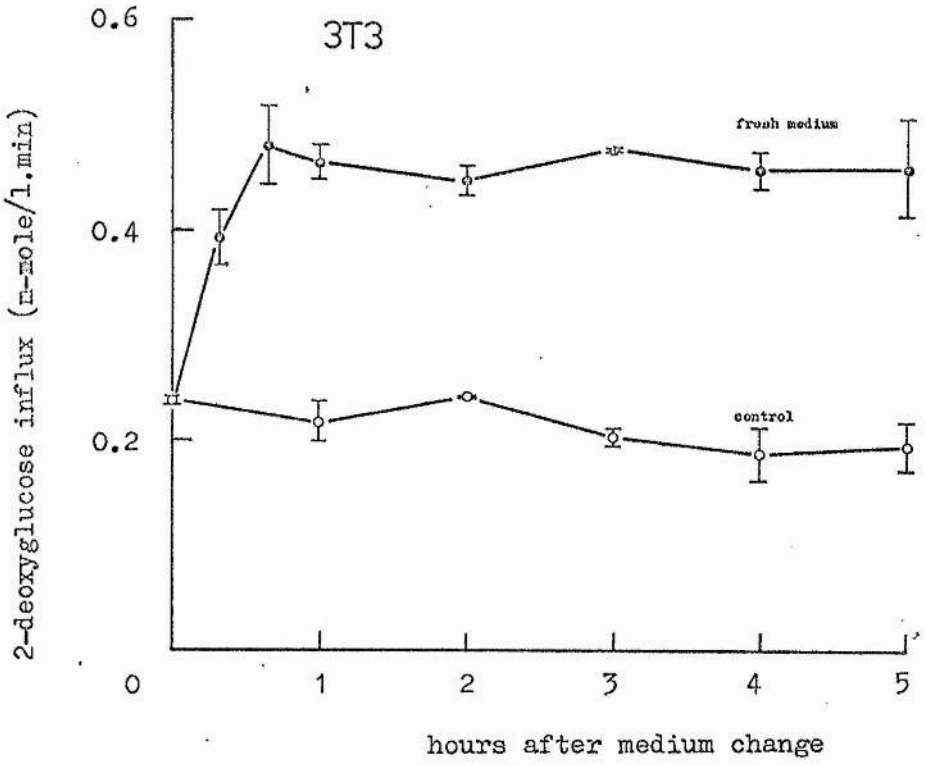


Fig. 46. The influx of 2-deoxyglucose into quiescent 3T3 cells as a function of time after the addition of fresh medium. The growth medium was removed from 6-day-old quiescent cultures and replaced by the same medium (○) or fresh complete medium (●). The influx was measured from glucose-free Krebs (0.5 m-mole/l 2-deoxyglucose) at the indicated times. Incubations with ^{3}H-2-deoxyglucose were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 39. Each point represents the mean for 2 determinations of the influx. The error bars show ± S.E.

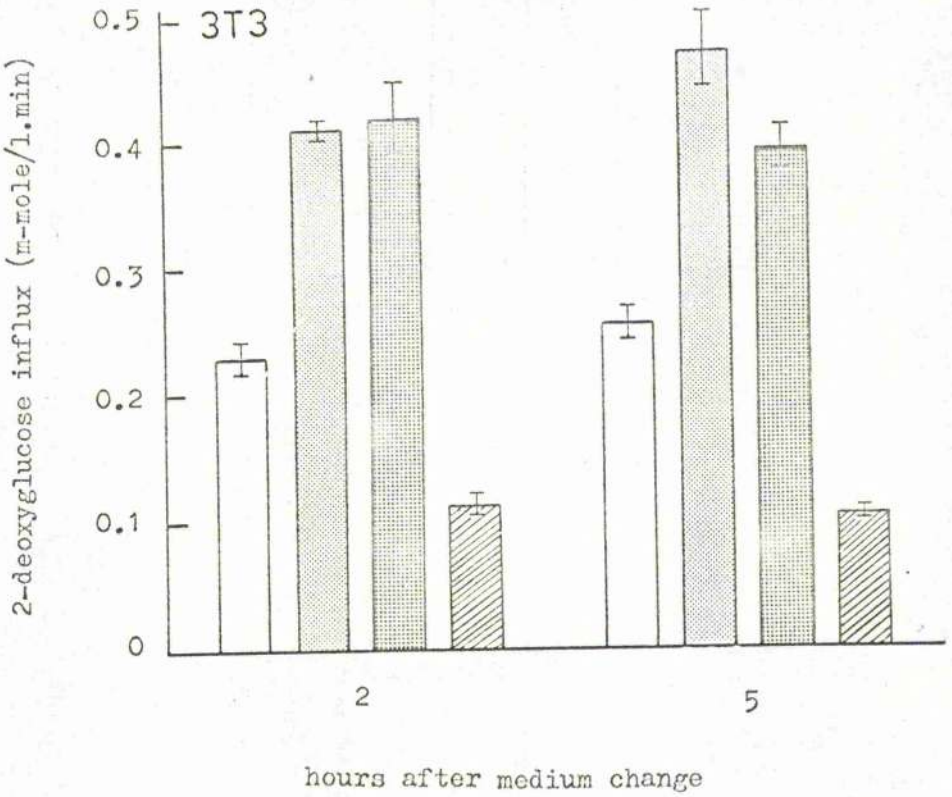


Fig. 47. Effect of cycloheximide and cytochalasin B on the stimulation of 2-deoxyglucose transport by fresh medium. The growth medium was removed from 6-day-old quiescent 3T3 cells and replaced by the same medium, fresh medium, or fresh medium + 10µg/ml cycloheximide. After 2 or 5 hours the 2-deoxyglucose influx was measured from glucose-free Krebs. Incubations with ^{3}H-2-deoxyglucose were carried out for 5 min at 37° according to the procedure described in the legend to Fig. 39. Cytochalasin B (0.5µg/ml) was included in the Krebs solution used for some cells treated with fresh medium. The column height represents the mean value for 2 determinations of the 2-deoxyglucose influx. The error bars show ± S.E.

control

fresh medium + cycloheximide

fresh medium

fresh medium/cytochalasin B in Krebs

transport was completely inhibited by this drug. The degree of transport inhibition (75%) was very similar to that shown previously for unstimulated control cultures of 3T3 cells (79% ; Fig. 44).

Effect of serum-stimulation on the kinetic constants for 2-deoxyglucose transport. Fresh medium was added to quiescent 3T3 cells. After 2 hours the kinetic constants for 2-deoxyglucose transport were measured and compared to untreated controls. The results (Fig. 48) show that the serum-induced increase in 2-deoxyglucose transport was associated with an increased Vmax with no comparable change in Km.

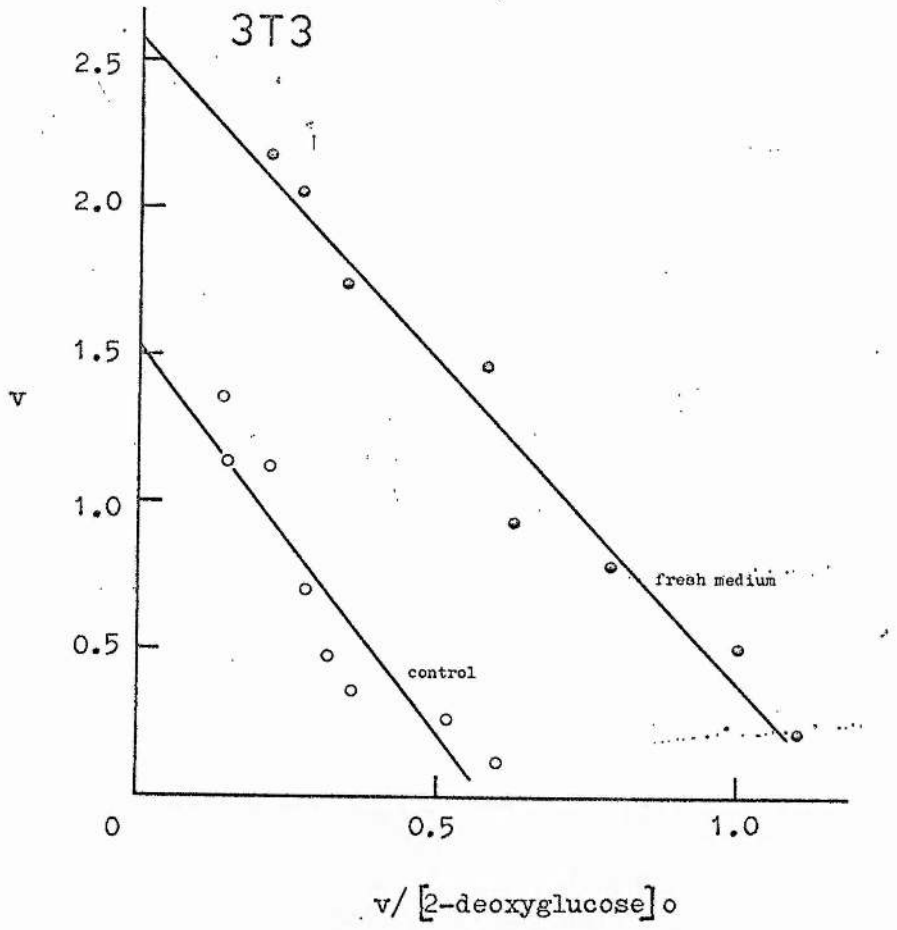


Fig. 48. Alterations in the kinetic constants for 2-deoxyglucose transport following the addition of fresh medium. The growth medium was removed from 6-day-old quiescent cultures of 3T3 cells and replaced by the same medium (O) or fresh complete medium (⊙). After 2 hours the influx of 2-deoxyglucose was measured from Krebs with [2-deoxyglucose] in the range 0.2 to 8.0 m-mole/l. Incubations with ^{3}H-2-deoxyglucose were carried out for 5 min at 37° as described in the legend to Fig. 39. The data is shown after linear transformation (v against v/S). Each point represents a single value for the influx (v) which is expressed as m-mole/l. min. Slopes and intercepts were obtained from a linear regression programme without weighted factors. The calculated kinetic constants, Km and Vmax, are shown in the attached table.

medium	Km (m-mole/l)	Vmax (m-mole/l. min
Ocontrol	2.59	1.53
⊙fresh	2.17	2.58

DISCUSSION

Effects of the cell population density on membrane transport in 3T3 cells

3T3 cells were seeded at a single population density and the influx of K^+, inorganic phosphate and 2-deoxyglucose was measured each day during growth to the stationary phase. The results indicate that the transport of all three substrates decreased with increasing cell population density. A possible objection to this conclusion is that the reductions in influx were related, not to the cell density, but to the age of the culture. However, this does not appear to be the case. When cells were seeded at different densities and incubated for a fixed growth period, transport could be measured over a range of cell densities in cultures of the same age. This procedure demonstrated that the observed reduction in transport was, for K^+ and 2-deoxy-glucose at least, directly related to the cell population density. Although the latter procedure was not used for measurements of Pi transport it seems likely that a similar conclusion applies.

There was one noticeable difference between the results obtained using the two procedures outlined above. With the former procedure (fixed seeding density, variable incubation time) an increase in transport was observed during the first 2 to 3 days after seeding. The increase was followed by a marked decline in the rate of substrate uptake during the next few days. When the alternative procedure (variable seeding density, fixed incubation period) was used the initial increase in uptake rates was not observed. The most likely explanation for this difference is related to the state of the cultures at the time of seeding. The cells were obtained from 4-5 day-old cultures growing in Roux flasks and direct measurements on the cells in the Roux flasks produced influx values similar to those for 5 day-old dish cultures. Thus, at the time of seeding, the transport rates of the

cells were below maximum. Although transfer into fresh medium produces a rapid increase in transport activity it is possible that cells need some time to fully establish the higher rates of transport "appropriate" to the new lower density. The results indicate that 2 or 3 days may be required for this process. Thus an initial time-dependent response would be superimposed on a density-dependent response when using the fixed-density procedure. When using the variable-density procedure, where all cultures received at least 3 days incubation, the time-dependent response would not be observed. Under these circumstances results similar to those obtained might be expected.

Influx and efflux

Potassium. The results show a 4-fold reduction in the K^+ influx over cell densities from 0.2 to 4 ($x10^6$) cells/9cm dish (Fig. 6). In addition, actively growing cells were found to have a markedly higher $[K^+]_i$ than quiescent cells (Table 5). The decreased K^+ transport was shown to be due to a reduction in "Na-pump" activity with no change in the passive fluxes across the membrane (Fig. 8). In this respect it is interesting that Elligsen et al. (1974) found a 2 to 4-fold reduction in membrane (Na^+-K^+)-ATPase activity when growing and quiescent 3T3 cells were compared. These results suggest that the lower $[K^+]_i$ of quiescent 3T3 cells resulted from the reduced "Na-pump" activity. This would also be expected to give rise to an increase in $[Na]_i$ and this was in fact observed (Table 5).

Over the range of cell densities investigated the reduced K^+ influx was shown to be balanced by a similar reduction in the K^+ efflux (Fig. 6 and Table 6). This decrease in the K^+ efflux was due to a reduction in $[K^+]_i$ with no change in the "fractional loss" of K^+. However, the "fractional loss" was not measured at densities much above

$2x10^6$ cells/dish. At this density the calculated efflux value was 2.58 m-mole/l. min and similar values were obtained for the K^+ influx. At higher cell densities the influx was shown to fall to about 1 m-mole/l. min and unless the "fractional loss" decreased the $[K^+]_i$ would need to fall as low as 60 m-mole/l to maintain a steady-state. It seems unlikely that the $[K^+]_i$ would be reduced to such a low level and the minimum values observed in this work were around 110 m-mole/l. It is possible that at the highest cell densities a reduced "fractional loss" of K^+ might become apparent.

Few other results are available on the effect of cell density on K^+ transport. Raab & Humphreys (1974) found no density-dependent effects on $[K^+]_i$ in chick embryo fibroblasts. Flame photometric analysis produced values of 105 m-mole/l for both growing and density-inhibited cells. This value was claimed as typical for K^+ levels in higher vertebrate cells. In fact much higher values (150-200 m-mole/l) have been generally reported for cultured mammalian cells (eg. Lamb & Mackinnon, 1971; McDonald et al., 1972; Quissell & Suttie, 1973; Boardman et al., 1974; Prigent et al., 1975; Cuff & Lichtman, 1975). Raab & Humphreys found a 30% decrease in the rate of K^+ exchange in density-inhibited cells compared to growing cells. They argue that this change was probably not significant. Kimelberg & Mayhew (1975) found a reduction in the K^+ "uptake" (measured over 1 hour) in density-inhibited 3T3 cells. In contrast to the results presented here, the reduction in uptake was not apparent until after the cessation of cell growth. The growth curve for 3T3 cells presented by these workers shows that the cells reached a density of $2.5x10^5$ cells cm^{-2} after 6 days growth. This is equivalent to about $15x10^6$ cells/9cm dish and is far too high a saturation density for "true" 3T3 cells in standard culture conditions. It seems possible that Kimelberg & Mayhew were working with a line of 3T3 cells which had become "spontaneously transformed". I have

observed that "3T3" cells with a greatly increased saturation density, exhibited a smaller density-dependent reduction in the K^+ influx than normal and the reduction occurred at high cell densities (unreported observation).

Phosphate. The rate of uptake of inorganic phosphate into total cell material will accurately represent Pi transport only if movement across the membrane is the rate-limiting step for the process. No unequivocal evidence is available on this question. However, the small size of the intracellular free Pi pool (6-12% of total cell phosphate) indicates that Pi, on entering the cell, is rapidly incorporated into organic compounds (Table 8). This finding suggests that transport, not intracellular metabolism, is the rate-limiting step in phosphate uptake.

The influx of Pi into 3T3 cells was shown to increase during the first 3 days after seeding and then decrease during growth to the saturation density (Fig. 22). An overall 5-fold reduction of the influx was observed. The equilibrium value for total intracellular phosphate was apparently independent of cell density (Tables 7 & 8). However, the size of the small intracellular Pi pool showed some reduction with increasing cell density (Table 8).

Previous experiments have indicated that phosphate transport in L cells (Brown, 1971) and Erlich ascites tumour cells (Levinson, 1972) is carrier-mediated. In this study the curve for Pi influx as a function of extracellular Pi concentration showed clear evidence of saturation (Fig. 25). This is consistent with carrier-mediation. The Km, or substrate concentration for half of the maximal influx, was 0.25 m-mole/l and the Vmax for growing cells was 0.50 m-mole/l. min. For Erlich ascites cells Levinson (1972) obtained Km and Vmax values of 0.33 m-mole/l and 0.73 m-mole/l. min. respectively.

The intracellular concentration of free Pi in growing 3T3 cells was shown to be 2.40 m-mole/l (Table 8). The extracellular concentration of Pi in Krebs was 0.55 m-mole/l. Chloride distribution studies across the 3T3 cell membrane indicated that these cells have a membrane potential of about -15mV (unreported observation). It is clear that Pi is accumulated against a substantial electrochemical gradient. It is difficult to test whether the transport of Pi requires a direct supply of metabolic energy. The usual test for this possibility is to measure substrate transport in the presence of metabolic inhibitors. Incubation with DNP+IAA was shown to reduce the Pi influx to about 5% of control values. However, this result is difficult to interpret, since, in the presence of the inhibitors, intracellular phosphorylation would be inhibited and transport will no longer be the rate-limiting step in phosphate uptake. The kinetics of uptake could be expected to change under these circumstances.

The demonstration of a Na-dependency of Pi transport suggests that some of the energy for phosphate accumulation may be provided through an Na-coupled transport system. Such systems have been extensively demonstrated for the transport of organic solutes, particularly amino acids (Schultz & Curran, 1970). In a coupled system, the entry of Na^+ into the cell down its concentration gradient furnishes at least part of the energy requirement for the accumulation of a co-substrate against a concentration gradient. No major differences were found between 3T3 and SV3T3 cells for Na^+ effects on Pi transport. Using the combined evidence from 3T3 and SV3T3 cells the following conclusions may be made concerning the Na-dependency of Pi transport:

1) It is specific for Na^+ since neither choline$^+$ nor Li^+ were able to maintain Pi transport levels when $[Na^+]_o$ was reduced.

2) Although the Vmax for Pi influx was reduced by decreasing $[Na^+]_o$ by far the greater effect was on the Km which increased markedly with decreasing $[Na^+]_o$ (Table 9). This

result suggests that the main effect of Na^+ on Pi transport is to facilitate binding of Pi to a phosphate-carrier molecule.
3) The slight sigmoid shape of the curves for Pi influx against $[Na^+]_o$ (Figs. 28 & 29) suggests that the phosphate carrier may require more than one Na^+ for each Pi molecule. A plot of the Na-dependent Pi influx (v) against $v/[Na^+]_o^2$ produced a reasonable straight line ($r=0.91$; $P<0.01$) whereas a plot of v against $v/[Na^+]_o$ did not produce a good fit ($r=0.07$; $P>0.05$).
4) Efflux of Pi was shown to be complicated by the complex compartmentalisation of phosphate within the cell. When cells were treated with ouabain the Pi efflux increased suggesting that the outward movement of Pi is also Na-dependent (Fig. 32). Preliminary evidence suggests that the efflux of Pi from preloaded cells was inhibited by increasing $[Na^+]_o$. When the $[Pi]_o$ was raised this inhibition was eliminated (Figs. 33 & 34).

These characteristics are very similar to those shown for glycine transport in pigeon erythrocytes by Vidaver who has proposed a detailed kinetic model for that system (Vidaver & Shepherd, 1968). A model for Na-dependent Pi transport, based on the scheme of Vidaver, is presented in Fig. 49. The model is speculative and will no doubt require modification when further experimental evidence is obtained. One immediate difficulty is that the model predicts that in the absence of extracellular Na^+ no influx of Pi will occur. The data shown in Figs. 28 & 29 indicate that a small Pi influx would be present. It is possible that this influx represents entry by simple diffusion. However, the kinetic studies presented in Fig. 26 show that a good fit to Michaelis-Menten kinetics was obtained without applying a correction for simple diffusion. This suggests that at a low $[Pi]_o$ the contribution of simple diffusion to the total Pi influx was negligible. An alternative possibility for the residual Pi entry in the absence of Na^+ is that EPi (Fig. 49)

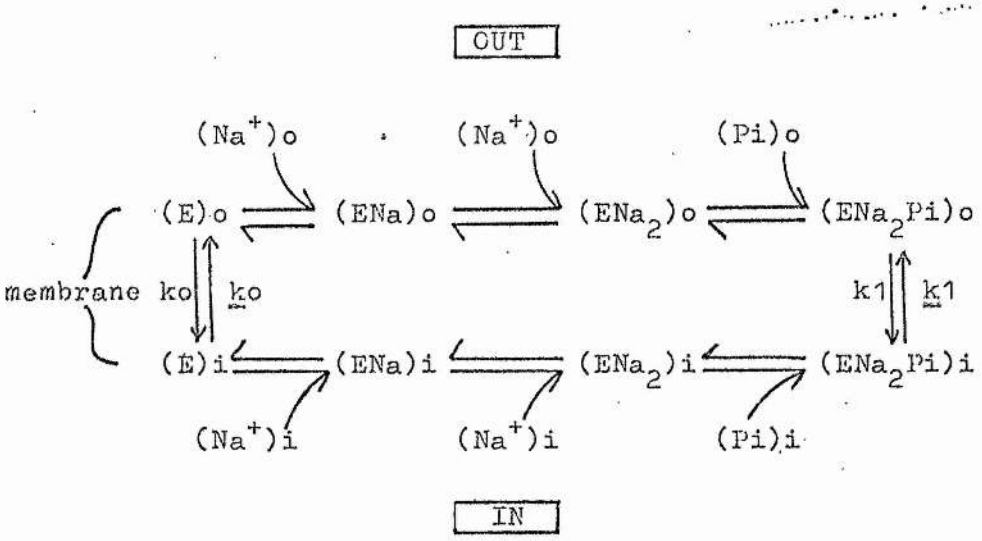


Fig. 49. Kinetic model to illustrate a possible mechanism for the Na-dependency of Pi transport. The scheme is based on the model of Vidaver & Shepherd (1968) for glycine transport in pigeon red blood cells. Both inward and outward movement movement of Pi is mediated by a carrier-complex containing 2 Na^+. It is proposed that the Na ions must bind to the carrier before the Pi and that (ENa) and (ENa_2) do not cross the membrane.

can be formed and cross the membrane, though perhaps more slowly than ENa_2Pi. This might also account for the small effect of $[Na]_o$ on the Vmax for Pi influx.

The data in Fig. 38 show that the Na-independent influx into quiescent 3T3 cells was not significantly different from the Na-independent influx into growing 3T3 cells (Fig. 28). The density-dependent reduction in the total Pi influx appears, therefore, to be due to a reduction in Na-dependent transport. This is supported by the observation that, although the total Pi influx increased rapidly after the addition of fresh medium to quiescent cells, the Na-independent component did not increase (Fig. 38). If the Na-independent transport is carrier-mediated this observation suggests that the Na-dependent and Na-independent carriers are qualitatively different since the activity of the former decreased with increasing cell density whereas the activity of the latter did not. If Na-independent transport is the result of simple diffusion of Pi into the cell its independence of cell density might be expected since the results for K^+ transport indicate that the permeability of the membrane does not alter with increasing cell density.

2-deoxyglucose. Previous work on the effects of cell density on 2-deoxyglucose transport in 3T3 cells has yielded conflicting results. Bannai & Sheppard (1974), using variable-density seeding, found an exponential decrease in the 2-deoxyglucose influx with increasing cell density. The reduction in transport began at cell densities as low as $2x10^3$ cells cm^{-2} ($0.13x10^6$ cells/9cm dish). Rose & Zlotnick (1973) used both fixed-density and variable-density seeding procedures and, in both cases, found a similar exponential reduction in transport with increasing cell density. In contrast, Schultz & Culp (1973) using fixed-density seeding, found that 2-deoxyglucose transport did not decrease until after the cessation of cell growth on the formation of a confluent monolayer. At confluency the 2-deoxyglucose influx decreased sharply.

The results reported here are more consistent with the former observations. Using variable-density seeding the decline in 2-deoxyglucose influx with increasing cell density (Fig. 41) fits an exponential reasonably well ($r=0.953$; $P<0.01$). When the fixed-density procedure was used the decline in transport began at slightly higher densities but still well before the cessation of growth. A close analysis of the data of Bose & Zlotnick (their Fig. 2) shows that they also obtained a small increase in uptake rate during the initial period of cell growth. This is in agreement with the result shown here.

Kinetic constants for transport

With the exception of the data of Schultz & Culp (1973) there is general agreement that the density-dependent reduction in the rate of 2-deoxyglucose transport is due to a decreased Vmax with no change in Km. The results presented in this study support and extend this view since the decreased transport of 2-deoxyglucose, inorganic phosphate and K^+ were all shown to be attributable to changes in Vmax with no change in Km. The results for K^+ transport are particularly convincing since the kinetic constants were measured over a wide range of cell densities. The results show a clear correlation between cell density and Vmax and no correlation with Km (Fig. 12). Since the measurement of cell density effects on K^+ transport is not complicated by possible effects on intracellular metabolism, the reduction in transport reported here is clear evidence of a direct membrane effect.

The kinetic studies suggest that the membrane alteration is quantitative rather than qualitative. Since transport Km's were independent of cell density, reduced transport cannot be due to a reduction in the affinity of carrier sites for the substrate. The reduction in Vmax suggests that the decreased transport was associated with a

reduction in the number of functionally active transport sites. This could result from one (or a combination) of the following conditions:

1) Reduction in the number of transport sites in the membrane.
2) Complete inactivation of sites which remain in the membrane.
3) Reduced transport turnover at sites.
4) A reduction in the cell's surface area/volume ratio.

For K^+, one possible way of distinguishing between these possibilities would have been to use [^{3}H]-ouabain to measure the number of "Na-pump" sites directly. Unfortunately, these cells require high ouabain concentrations (10^{-3}) to inhibit Na-K exchange. Ouabain-binding studies cannot be accurately performed because of the high level of non-specific binding which occurs at this concentration. However, the observation that the membrane (Na^+-K^+)-ATPase level decreases with increasing 3T3 cell density (Elligsen et al., 1974) indicates that at least part of the reduced transport of K^+ was due to condition 1.

Effects of virus-transformation on membrane transport properties

Virus-transformation appears to reduce or abolish the density-dependent reduction in membrane transport which was observed in untransformed cells. The influx of K^+ and 2-deoxyglucose into Py3T3 and SV3T3 cells was shown to be independent of cell population density. The influx of Pi into SV3T3 cells did show a reduction with increasing cell density but this was smaller and occurred at much higher cell densities than the Pi transport reduction in 3T3 cells.

It is widely believed that transformed cells transport 2-deoxyglucose 2 to 4-fold more rapidly than the corresponding untransformed cells (see INTRODUCTION). The results presented here do not support this view. The maximum

influx of Pi and 2-deoxyglucose into transformed 3T3 cells was only some 30% and 50% higher than the maximum influx into 3T3 cells. The maximum influx of K^+ was no higher in the transformed cells; indeed, the data at very low cell densities suggest that the untransformed cells may have the higher rate of K^+ uptake. It is possible that many previous results were obtained using untransformed cells at a population density which exhibited density-reduced transport rates. Much of the observed transport difference between the cell lines could thus be due to a failure to measure maximum transport rates in the untransformed cells. Of course, some workers were aware of this problem and ensured that they used preconfluent growing cultures for comparison. However, it has been shown here that a density-dependent decrease in transport becomes apparent at very low cell densities. Thus, the fact that cells are preconfluent and growing would not ensure that maximum uptake rates were measured.

Other workers have obtained results similar to those reported here. Bose & Zlotnick (1973) found that the maximum rate of 2-deoxyglucose uptake by 3T3 cells (measured at very low cell densities) was similar to that in mouse sarcoma virus-transformed 3T3 cells. Plagemann (1973) showed that the influx of 2-deoxyglucose into mouse embryo fibroblasts at low densities approached the level measured in virus-transformed MEF. These results support the proposal that virus-transformation abolishes the density-dependent transport reduction of untransformed cells but does not cause an additional increase in transport capacity.

Most workers were unable to find differences in the Km for 2-deoxyglucose transport when normal and transformed cells were compared (see INTRODUCTION). In this study no differences in Km were observed between 3T3 and SV3T3/Py3T3 cells for K^+, Pi and 2-deoxyglucose transport. In addition the Ki's for inhibition of 2-deoxyglucose transport

by glucose and cytochalasin B were similar in the untransformed and transformed cell lines. These results indicate that, for a variety of transported substrates, virus-transformation does not qualitatively alter the membrane carrier sites.

The altered response of virus-transformed cells to ouabain

The altered response of the virus-transformed cells to ouabain suggests that their plasma membrane is in some way different to that of 3T3 cells. The mechanism of the ouabain-induced K_i -K exchange in Py3T3 and SV3T3 cells remains unknown. Ouabain-inhibition of Na -K exchange is brought about by the binding of the drug to specific membrane sites. This was shown to occur rapidly and was very slowly reversed, whereas the ouabain-induced K-K exchange developed and was reversed with similar half-times of about 20 minutes (Figs. 14 & 18). The markedly different time-courses for the two effects, together with the fact that both can exist simultaneously, suggests that K-K exchange was not caused by ouabain-binding to "Na-pump" sites. It is possible that K-K exchange was not caused by binding of the drug to any membrane sites but by a more general interaction with membrane lipids. This might account for the slower development but more rapid reversal of the response.

Barnett et al. (1974) have demonstrated that the plasma membrane of SV3T3 cells is more "fluid" than the membrane of 3T3 cells at 37°. This is thought to indicate a decrease in the fraction of membrane lipids existing in an ordered state in the transformed cell membrane. It is possible that the altered response of transformed cells to ouabain is due to this change in the properties of membrane lipids. At 20° ouabain did not induce K-K exchange in the

transformed cells (Fig. 19). Since a reduction in temperature might be expected to reduce membrane fluidity (Chapman, 1975) this result is consistent with the previous proposal. Further information needs to be obtained by examining the effect of ouabain at several temperatures between 4° and 37°.

Kimelberg & Mayhew (1975) measured the uptake of K^+ into 3T3 and SV3T3 cells in the presence and absence of ouabain. They concluded that the SV3T3 cells showed increased ouabain-sensitive K^+ "uptake" measured over 60 minutes. I have observed (unreported observation) that, in the presence of ouabain, transformed cells show anomalous, non-exponential uptake curves. The curve shows a marked flattening or even a decrease after the first 30 minutes, presumably due to the delayed effect of ouabain on the cells. The K^+ uptake curve of Kimelberg & Mayhew (their Fig. 1) also shows this effect which has been mistakenly interpreted as an increased ouabain-sensitive "uptake". It is not known whether the induction of K-K exchange by ouabain is general after virus-transformation. The experiments described here need to be repeated using other cell lines, preferably more sensitive to ouabain so that lower drug concentrations can be employed.

Serum-stimulation of membrane transport in quiescent 3T3 cells

Previous reports have indicated that the addition of fresh medium to quiescent cultures of 3T3 cells produces a rapid increase in the rate of uptake of several substrates (see INTRODUCTION). I have confirmed that the influx of Pi and 2-deoxyglucose increased 2-fold within 30 minutes of the medium change. Fresh medium without serum did not cause an increase indicating that ~~that~~ factors in the serum are responsible for the stimulation. Preliminary results (unreported) have indicated that the influx of K^+ did not

increase during the first 5 hours after a medium change but was usually some 50-100% increased 24 hours after the change. The time at which the increased K^+ transport begins to appear is not yet known.

The results for Pi and 2-deoxyglucose transport demonstrate that the increased influx was attributable to an increase in Vmax with no change in Km. This result would be expected if the effect of fresh medium is to reverse the density-dependent reduction in transport seen in 3T3 cells. However, in these experiments fresh medium did not stimulate transport to the maximum levels measured during the growth of preconfluent cultures. This could be explained by supposing that cells at high densities have a higher requirement for serum factors or that the factors are more rapidly destroyed/ inactivated by dense cultures. That this is a possibility is indicated by the results in Fig. 38. Cells which had previously been grown in 0.3% serum exhibited a biphasic increase in Pi transport after the addition of fresh medium (10% serum). The maximum transport levels reached in this experiment were virtually as high as those seen during preconfluent growth. However, these cultures contained fewer cells (mean 1.72×10^6) than the cultures continuously maintained in 10% serum (mean 3.34×10^6). It is possible that the enhanced response was due to this lower cell density.

The early transport increase in cells maintained in either high or low serum concentrations was independent of protein synthesis. The second phase of the response of cells exposed to low serum was inhibited by cycloheximide. These results indicate that serum factors cause a rapid increase in transport perhaps by directly stimulating existing transport sites in the membrane. Serum was shown to produce an additional delayed increase in the transport capacity of cells at lower densities. This increase, which requires protein synthesis, may be due to the insertion of new

transport sites into the membrane. This interpretation appears consistent with the increase in transport observed during the first few days after reseeding cells in fresh medium at reduced densities.

Rôle of membrane transport changes in cell growth control

The existence of growth-related changes in membrane transport is now well established, though their role in the control of cell proliferation remains to be determined. It has been shown that, for untransformed cells, conditions of rapid growth are accompanied by high transport rates whereas quiescence is associated with decreased rates of transport. In view of the role of glucose and inorganic phosphate in the cell's energy metabolism it seems advantageous that their entry into the cell should be closely regulated. One might expect this regulation to involve changes similar to those described here; a plentiful supply during cell growth with a limited supply to resting cells. Lubin (1967) has shown that depletion of cell K^+ by amphotericin was paralleled by a depression of the rate of synthesis of protein and DNA. If the amount of K^+ in the medium was increased nearly normal levels of K^+ could be maintained in the leaky cells and the depression of macromolecular synthesis could be largely prevented. This suggests that high cell K^+ levels are also favourable to growth.

It is tempting to speculate that the apparent correlation between transport and growth represents a causal effect of transport on growth. Increased or decreased transport could directly modify the intracellular environment in such a way as to initiate or inhibit cell division. Alternatively, the transport alterations could be secondary to a more basic and, as yet, unknown change. It has been shown that density-dependent transport reductions precede the cessation of cell growth and that an increased influx of Pi

and 2-deoxyglucose is measurable very soon after the addition of fresh medium to quiescent 3T3 cells. These results suggest, but by no means prove, that transport rates may have a causal control over cell division.

As described in the INTRODUCTION, the availability of serum factors is the most important external regulator of density-dependent inhibition of cell growth. In a recent review Holley (1975) concluded that these factors are probably polypeptide hormones or hormone-like materials. In Fig. 50 a model is proposed which attempts to explain the mode of action of these polypeptide factors in terms of transport regulation. However, the possibility of other actions is accepted and included in the model.

Although the proposed model is mostly speculative it has been shown that serum produces a rapid increase in some transport activities. Evidence has also been presented for a later, additional, increase in the number of transport sites under certain conditions. On the basis of this model growth restriction could be caused by a depletion of growth factors from the medium, an increased rate of factor-inactivation, or by a reduction in the number of receptor sites in the membrane. Transformed cells would escape growth restriction through a reduced requirement for growth factors. The cells could inactivate the factors less rapidly or they could have an increased number of receptor sites at the membrane.

Dulbecco (1975) has stressed the importance for the organism that the growth of each cell type is controlled independently of other cell types. He states "the evidence suggests that a given growth promoter can affect a spectrum of cell types and that a cell type responds to a spectrum of promoters. In addition, there is evidence for the existence of growth inhibitors, again with some degree of specificity.

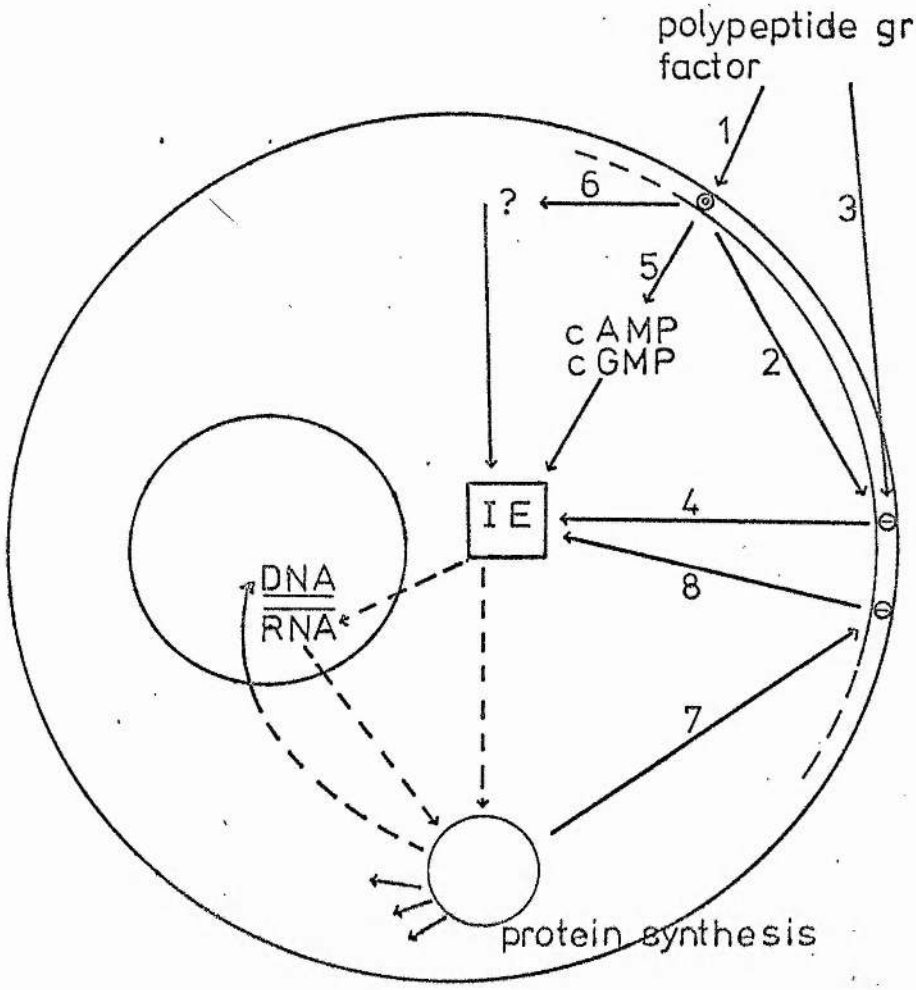


Fig. 50. Hypothetical model which explains the growth regulatory function of serum polypeptide factors in terms of their action on membrane transport. It is proposed that serum factors interact with specific receptor sites (⊙) at the cell membrane. This interaction produces an increased activity of membrane transport sites (⊖) via routes 1 & 2. Alternatively the factors could interact directly with the transport sites (3). In either case the increased transport would alter the"intracellular environment", IE (4). It has been shown that serum also causes rapid changes in cellular cyclic nucleotide levels (Kram & Tomkins, 1973) and the possible involvement of cAMP and cGMP is included (5). Other unknowm effects are represented (6). It is suggested that the summation of these effects produces a change in IE which is favourable to RNA synthesis, protein synthesis, the initiation of DNA synthesis and ultimately mitosis (dotted lines). One specific effect of the increased protein synthesis is the insertion of new transport sites into the membrane producing a positive feedback on IE (7 & 8).

Growth regulation of a cell type is likely to depend on the balance between all the various regulators; specificity may arise from the summation of many relatively small differences of affinities of regulators, or their effectors, for constituents of different cells." The scheme shown in Fig. 50 could easily be extended to include this type of multi-factorial regulation.

The model suggests several directions for further investigation. What is the nature and origin of the polypeptide factors? How many factors exist? Are these factors the component of serum which stimulates transport? Are the various transport systems controlled by different factors or by substances produced in the cell in response to a single factor? Finally, and perhaps most importantly, what components in the intracellular environment are ultimately responsible for the control of DNA synthesis?

Although the past five years have produced a great deal of valuable information, the major problems remain unsolved.

APPENDIX A

Measurement of glucose transport in cultured cells

$[^3H]$ and $[^{14}C]$ -glucose were used as a tracer in most of the early studies on glucose transport in cultured cells (Hatanaka et al., 1969). The value of results obtained using labelled glucose as a tracer has been questioned (Renner et al., 1972). Mammalian cells in culture metabolise glucose, particularly at high concentrations in the medium, very rapidly to CO_2 and lactate which are released into the medium. Renner and co-workers showed that almost immediately on adding $[^{14}C]$ -glucose to a suspension of Novikoff rat hepatoma cells, significant amounts of glucose began to be converted to extracellular, radioactively-labelled, lactate and CO_2. Thus the intracellular label represented only a proportion of the total glucose transported into the cell. The relative amounts of glucose converted to lactate and CO_2 and assimilated into cell material varied markedly with the glucose concentration of the medium. The ratio was higher for higher glucose concentrations. The error can thus be minimised by using very low glucose concentrations (10μ-mole/l) and very short labelling times. Even then significant errors could be expected to arise.

A better solution to the problem is to use a non-metabolisable glucose analogue eg. 2-deoxyglucose or 3-O-methylglucose. The former is rapidly phosphorylated by mammalian cells to deoxyglucose-6-phosphate; a trace of which is then oxidised to 6-phosphodeoxygluconate (Renner et al., 1972). Providing that cellular ATP pools are sufficient the phosphorylation is rapid and the 2-deoxyglucose is effectively "trapped" inside the cell. Under these conditions the transport reaction proceeds linearly for an extended period of time enabling relatively easy determinations of

initial rates of uptake to be made.

3-O-methylglucose is a nonphosphorylatable glucose analogue. Because it is transported into the cells but is not phosphorylated this analogue is useful in isolating direct transport alterations from alterations which may be due to changes in cellular metabolism. A major disadvantage however, is the very rapid equilibration across the cell membrane exhibited by 3-O-methylglucose. Equilibration is reached within 1-2 minutes (Weber, 1973). Estimations of initial rates of entry are difficult to obtain because of the very short influx times (15-30 sec) which must be employed.

Glucose, 2-deoxyglucose and 3-O-methylglucose are apparently transported by the same system in cultured cells (Renner et al., 1972; Weber, 1973; Plagemann, 1973; Kletzein & Perdue, 1974a).

APPENDIX B

Evaluation of the kinetic characteristics of carrier-mediated transport systems

In enzyme kinetic studies the starting point for the determination of the characteristics of the system is to measure the initial reaction rate over a range of substrate concentrations. The data is then analysed to determine whether they conform to simple Michaelis-Menten kinetics (1913) and, if so, what values are obtained for the kinetic constants Vmax and Km. Since transport reactions often "follow Michaelis-Menten kinetics", they can be analysed in a similar way. In the case of transport into a cell the reaction rate is taken as the initial rate of uptake (ie. influx) and the substrate concentration corresponds to the extracellular concentration of the substance being followed.

In the absence of a significant contribution to the uptake rate from simple diffusion the graph of influx (v) against substrate concentration (S) produces a rectangular hyperbola (Fig. B1) which is described by the Michaelis-Menten equation:

$$v = Vmax.S/(S + Km) \qquad B1$$

where Vmax is the maximal rate of unidirectional carrier transport; Km is the Michaelis constant for carrier transport which has a value equal to the substrate concentration at which half the maximal rate of transport is attained ie. the value of S when $v = Vmax/2$.

When a significant amount of substrate also enters the cell by simple diffusion the curve is altered to that shown in Fig. B2 In this case the total transport can be represented by the following equation:

$$v = \underbrace{(Vmax.S/(S + Km))}_{\text{(carrier)}} + \underbrace{(K_D\ S)}_{\text{(diffusion)}} \qquad B2$$

where K_D is the diffusion constant.

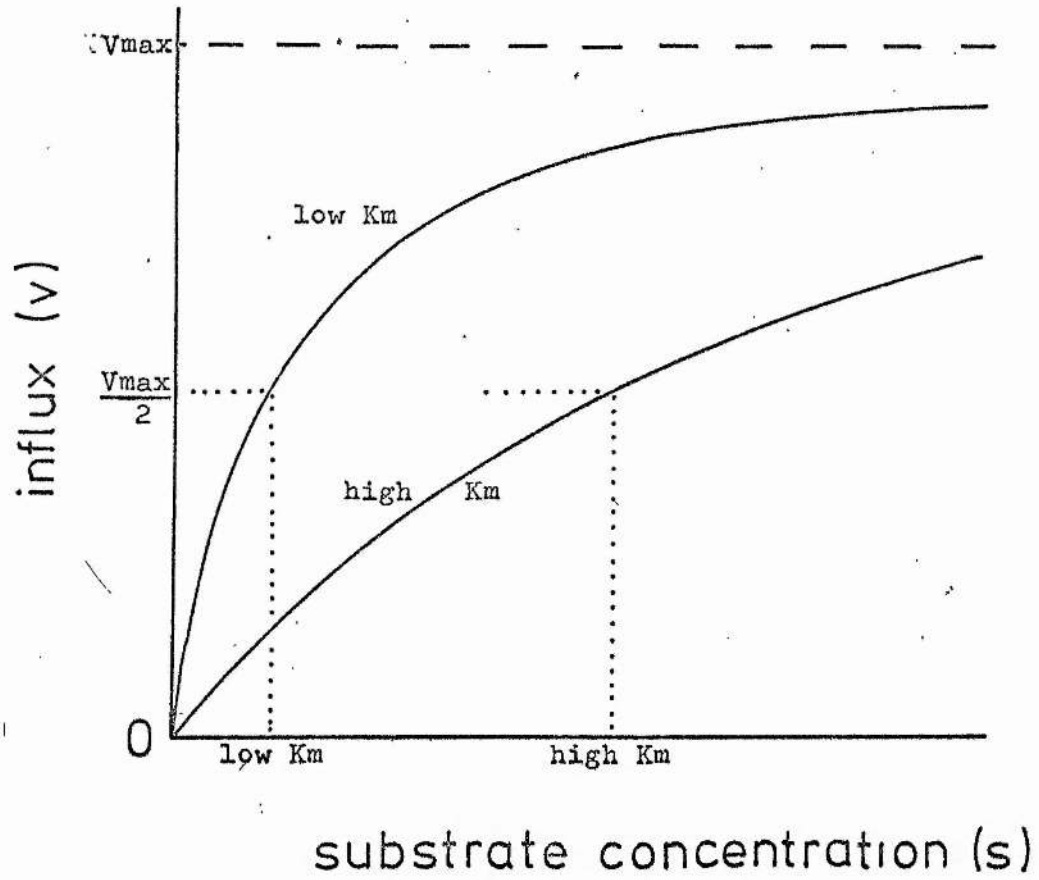


Fig. B1. Effect of the extracellular substrate concentration (S) on the initial rate of substrate uptake (v) in a carrier-mediated system. Characteristic curves for substrates with low or high Km and the same Vmax are shown. The process is saturatable.

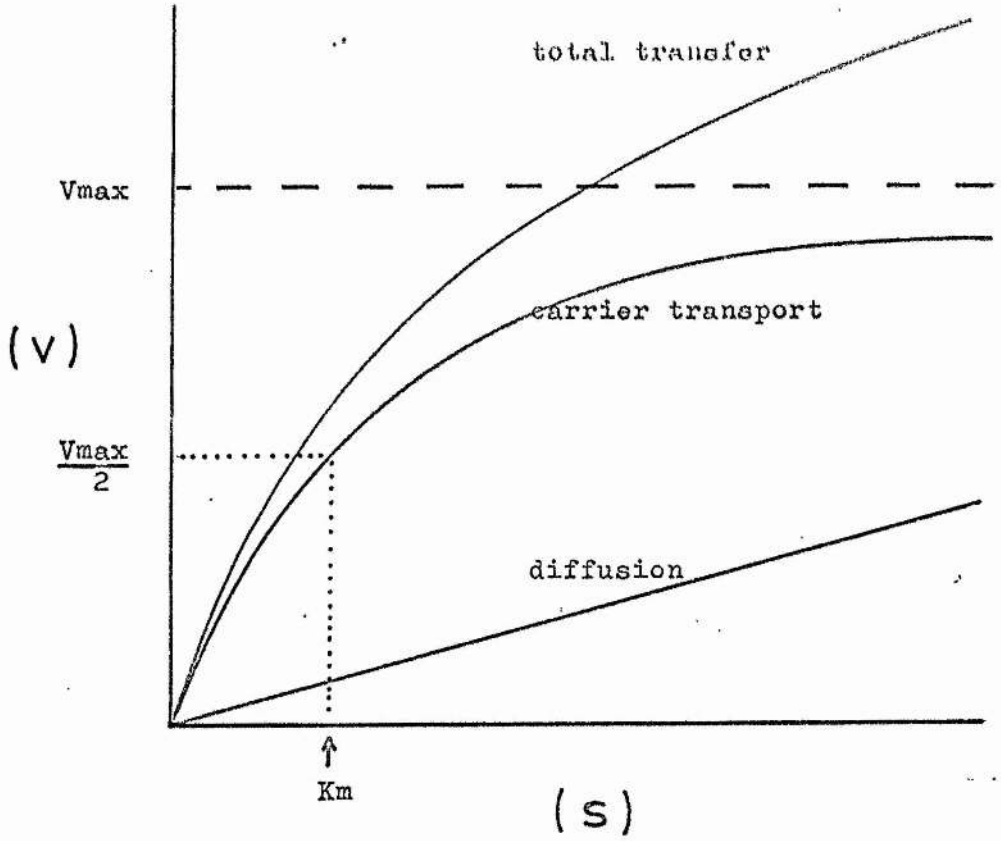


Fig. B2. Effect of diffusion on the kinetics of carrier mediated transport. The total transfer is the sum of the saturable carrier component and the non-saturable diffusional component.

Since the relationship between S and v is curvilinear the transport characteristics, Km and Vmax, are usually estimated from plots of one of the following linear transformations of equation B1.

$$(1/v) = (1/Vmax) + (Km/Vmax)\ (1/S) \qquad B1\ (a)$$

$$v = Vmax - Km\ (v/S) \qquad B1\ (b)$$

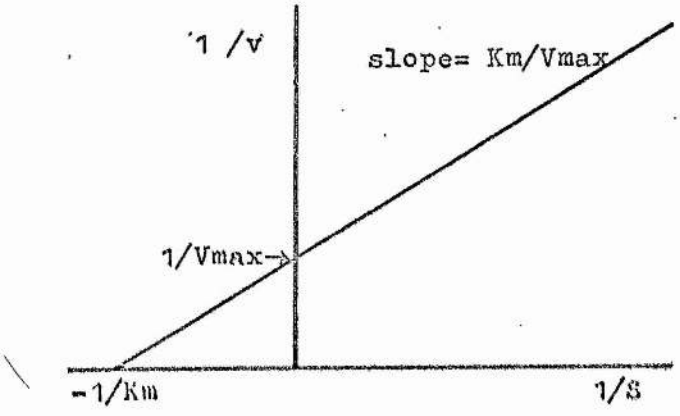
$$S/v = (Km/Vmax) + (1/Vmax)S \qquad B1\ (c)$$

From equation (a) a plot of 1/v against 1/S is linear. The slope of the line corresponds to Km/Vmax and 1/Vmax is obtained from the y intercept (Fig. B3a).
From equation (b) a plot of v against v/S is also linear. The slope of the straight line obtained corresponds to -Km and the y intercept to Vmax (Fig. B3b).
From equation (c) a plot of S/v against S is linear. The slope of this line corresponds to 1/Vmax and the y intercept to Km/Vmax (Fig. B3c).

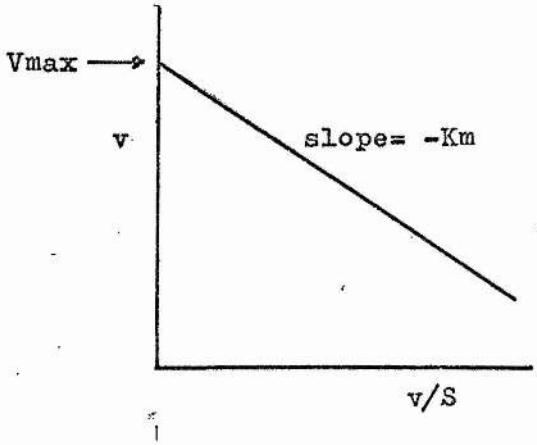
In addition to these graphical procedures for the determination of kinetic constants, algebraic methods are also available. Wilkinson (1961) has presented one method in detail. Neame & Richards (1972) have suggested an alternative method, based on simultaneous equations which is simpler in approach and in the calculations required. The advantage of algebraic methods lies in their ability to furnish not only values for the kinetic constants, but also an estimate of their accuracy (eg. standard error). The advantage of the graphical method is that, used correctly, deviations from Michaelis-Menten kinetics will be observed as deviations from linearity after transformation. For this reason even when algebraic methods are employed the data should always be examined graphically as well.

The Lineweaver-Burk, or double-reciprocal plot (B3a) is, by far, the most widely used of the available graphical methods. For example, in the 1971 issue of The Journal of Biological Chemistry 97.3% of all the transformed

(a) Plot of 1/v against 1/S (Lineweaver-Burk plot)



(b) Plot of v against v/S (Hofstee plot)



(c) Plot of S/v against S (Eadie plot)

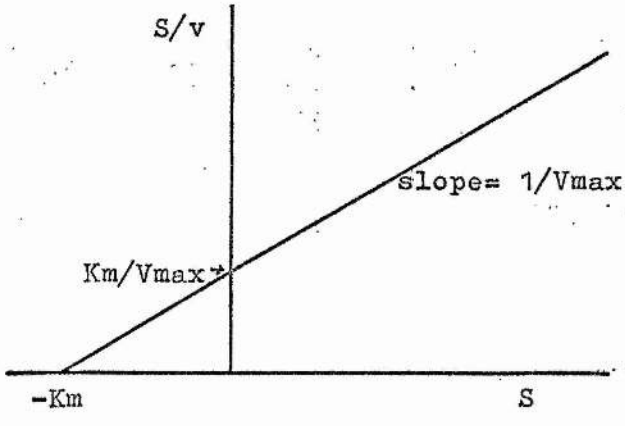


Fig. B3. Graphical procedures for the estimation of the kinetic characteristics of transport systems

plots were of this type (Walter, 1974). Virtually 100% of the transport studies cited in this thesis also employed this transformation. The widespread use of double-reciprocal plots is surprising since it is generally considered the least satisfactory of the transformations.

Dowd & Riggs (1965) conducted a thorough analysis of the relative merits of the various linear transformations. A digital computer was used to generate random samples from populations of simulated data and to estimate Km and Vmax from each sample for each of the linear transformations. The distribution of the sample estimates of the kinetic constants could then be compared with the "true" values. When using unweighted observations, Dowd & Riggs found 1/v against 1/S to be the least satisfactory under all the conditions examined. It proved more difficult to choose between the other two transformations, but, except when the error in v was very small, v against v/S generally proved more satisfactory in accurately estimating the kinetic constants. In addition a plot of v against v/S was most sensitive to deviations from the linear relationship expected on the basis of Michaelis-Menten kinetics. A similar conclusion has been reached by Walter (1974). Lineweaver-Burk plots on the other hand gave apparently good straight line fits even to data subject to a large error. Dowd & Riggs conclude, "We are thus confronted with the paradox of obtaining the best estimates from the 'worst-fitting'line and the worst estimates from the 'best-fitting' line! The undeserved popularity of the Lineweaver-Burk method may well be based upon just this ability to provide what seems a good fit even when the experiments are poor". In this work the plot of v against v/S fitted by the least-squares method to unweighted data has been used.

APPENDIX C

Derivation of equations for exponential substrate uptake and efflux

Uptake from a constant source. Let a cell of surface area A (cm^2) and volume V (cm^3) with an internal concentration Ci (mole/cm^3) of a substrate, s, be suspended in a solution containing the same substrate at a concentration Co (mole/cm^3). Some of the external substrate is then replaced by labelled substrate s^*; the specific activity of the bathing solution is then Co^*/Co (usually expressed as so many counts per minute per mole of total substrate present). The rate of accumulation of s^* inside the cell is given by the equation

$$\frac{dSi^*}{dt} = - PA (Ci^* - Co^*) \qquad C1$$

where Si^* represents the amount of intracellular labelled substrate and P is the permeability constant (cm/sec). Dividing eqn. C1 by the cell volume, V

$$\frac{1}{V}\frac{dSi^*}{dt} = - P\frac{A}{V} (Ci^* - Co^*)$$

$$\frac{dCi^*}{dt} = - k_1 (Ci^* - Co^*) \qquad C2$$

where k_1 is the uptake rate constant (sec^{-1}). Integration of eqn. C2 between $Ci^*=Ci^*(0)$ when t=0 and $Ci^*=Ci^*$ when t=t gives

$$\int \frac{1}{Ci^* - Co^*} dCi^* = -k_1 \int dt$$

$$\left[\ln(Ci^* - Co^*)\right]_0^t = -k_1 \left[t\right]_0^t$$

$$\ln \frac{Ci^* - Co^*}{-Co^*} = -k_1 t$$

$$\frac{Ci^* - Co^*}{-Co^*} = e^{-k_1 t}$$

$$Ci^* = Co^* - Co^* e^{-k_1 t}$$

$$Ci^* = Co^* (1 - e^{-k_1 t}) \qquad C3$$

Ci may be obtained by dividing Ci^* by Co^*/Co. In practice, Ci is measured at several values of t and the data are plot

as $(1-Ci/Ci(\infty))$ against t on semi-log graph paper. The resulting straight line will have a slope equal to $-0.4343k_1$.

Efflux. The cell is allowed to accumulate radioactive substrate until an equilibrium state is reached and then transferred into an unlabelled bathing solution.

$$\frac{dSi^*}{dt} = - PA\ (Ci^* - Co^*)$$

Dividing through by V gives

$$\frac{dCi^*}{dt} = -k_2(Ci^* - Co^*) \qquad C4$$

where k_2 is the efflux rate constant (sec^{-1})
If the volume of the bathing solution is sufficiently large in relation tp cell volume, Co^* will approximate to zero and can be neglected. Eqn. C4 can then be written as

$$\frac{dCi^*}{dt} = -k_2(Ci^*) \qquad C5$$

Integration of eqn C5 between $Ci^*=Ci^*(0)$ when t=0 and $Ci^*=Ci^*$ when t=t gives

$$\int \frac{1}{Ci^*}\, dCi^* = -k_2 \int dt$$

$$\left[\ln Ci^*\right]_0^t = -k_2 \left[t\right]_0^t$$

$$\ln \frac{Ci^*}{Ci^*(0)} = -k_2 t$$

$$\frac{Ci^*}{Ci^*(0)} = e^{-k_2 t}$$

$$Ci^* = Ci^*(0)e^{-k_2 t} \qquad C6$$

It is often convenient to express eqn.C6 in terms of $\log_{10}$, so that it becomes

$$\log Ci^* = \log Ci^*(0) - 0.4343k_2 t$$

In practice Ci^* is obtained at various times and a plot of $\log Ci^*$ against t gives a straight line of intercept $\log Ci^*(0)$ and slope $-0.4343k_2$.

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