OBSERVATIONS ON ENDOCYTOSIS IN TISSUE CULTURE

by

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ABSTRACT

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Endocytosis in Chinese hamster ovary fibroblasts was investigated by measuring the rate of uptake of sucrose-^{3}H, which is known to enter cells only by endocytosis. Serum, polyvinylpyrrolidone (PVP), adenosine triphosphate, insulin, and cyclic adenosine monophosphate, all of which are known to increase the rate of endocytosis by other cell systems, had no effect on Chinese hamster fibroblasts. However, medium in which these cells had been maintained for several days, referred to as conditioned medium, had a profound effect on endocytosis. These cells endocytosed 3 to 5 times as rapidly in conditioned medium as in fresh medium. A logarithimic inhibition of this effect was observed with increasing concentrations of D-glucose, however glucose free medium did not produce as great an effect as conditioned medium. This suggests that these cells may endocytose in response to their nutritional requirements.

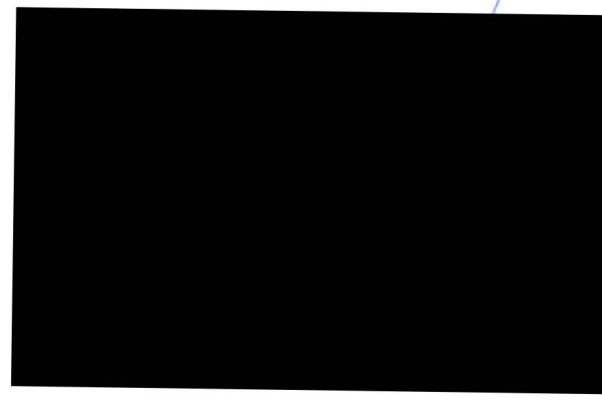
Cytochalasin B, which is known to cause disruption of microfilments, suppressed, but did not completely inhibit endocytosis by Chinese hamster fibroblasts. Cells derived from a benign human prostatic hypertrophy were effected similarily. This suggests both the involvement of microfilaments in endocytosis, and the possibility that there exists in these cells microfilaments which are relatively insensitive to the effects of cytochalasin B.

High resolution morphological studies on the effect of cytochalasin B showed that membrane ruffling, thought to be essential for endocytosis, is completely suppressed by cytochalasin B.

Morphological observations on the effect of the endocytosis of the polymers dextran and PVP by both the hamster and human prostatic cell lines demonstrated the profound effects these polymers can have on secondary lysosomes. In particular PVP was found to have a very marked deleterious effect on the human prostatic cell line, which presumably is a result of a disruption of the lysosomal-vacuolar system of these cells.

Insulin, 17-β-estradiol and testosterone were shown to have no effect on endocytosis by the hamster cell line, however insulin induced membrane ruffling with these cells, without however, a concomitant increase in endocytosis.

No correlation was observed between the endocytosis of the polymers dextran and PVP and the cryoprotective action these polymers exhibit with the hamster cell line. These polymers were also shown not to bind to the plasma membrane of these cells, indicating that their cryoprotective action is not related to either their intracellular presence or to binding to the plasma membrane of these cells.



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INTRODUCTION

Endocytosis is the term proposed by C. de Duve (26) to describe the processes variously known as pinocytosis, phagocytosis, micropinocytosis, Speicherung, cytopempsis, as well as numberous other terms, which describe the basic cellular mechanism of fluid and particulate engulfment and uptake, it being generally agreed upon that while differences exist between these processes, they are fundamentally similar (43). In general, endocytosis refers to the uptake into the cell of particles and solutes, to which the cell is impermeable, either by adsorption to the cell membrane, referred to by Jacques as adsorptive endocytosis, by fluid endocytosis, in which the particles in suspension, or solutes are taken up passively by the engulfment of droplets of extracellular fluid, or mixed type endocytosis, in which both adsorption and passive uptake are involved (43). In any case, endocytosis always involves the invagination of the plasma membrane, followed by the formation of a vesicle as the invaginated membrane pinches off from the plasma membrane. Vesicles formed in this manner, referred to an endocytotic vesicles, are thus always enclosed by a fragment of the plasma membrane. This distinguishes endocytosis from other forms of active transport, such as the well known sodium pump, in which ions are transported across the plasma membrane, without the membrane itself being transported.

The literature abounds with reports on endocytosis in amoebae and in cells in tissue culture. Since the introduction of electron microscopy endocytosis has also been demonstrated in the majority of animal and plant cell types, with the exception of mature erythrocytes and bacteria (43) (70). This suggests that endocytosis is of general importance in cell metabolism, since endocytosis can obviously provide a rapid means of uptake of essential nutrients, when these nutrients are present in the extracellular milieu. Thus an understanding of the factors which influence the rate of endocytosis, as well as an understanding of the mechanism of endocytosis, is of fundamental importance to cell biology. While many excellent studies have already been done, many were morphological studies, which although yielding much important information, lacked in many instances, sufficient sensitivity to quantitate the rate of endocytosis, and any factors which might change this rate. An obvious recourse would be to observe the rate of uptake of radioisotopically labelled molecules which can enter the cells under study only by endocytosis. Such studies have been done, as will be shown in the next section, and have been obtained by other methods.

Currently, studies are being undertaken in the laboratory of my supervisor, Professor M.J. Ashwood-Smith, to determine the cryoprotective action of various polymers on Chinese hamster fibroblasts, that is, the markedly enhanced ability of these cells to

survive freezing and thawing when these polymers are present in the medium in which the cells are frozen and thawed. Since these polymers can enter cells only by endocytosis, it was deemed desirable to investigate whether the endocytotic uptake of these polymers was related to their cryoprotective action, and hence whether their cryoprotective action was an extracellular, or an intracellular phenomenon. Once a suitable technique had been developed for quantitating endocytotic activity by cells in tissue culture, the technique was applied to the study of other biologically interesting compounds, apart from the cryoprotective polymers, in the hopes of contributing to the understanding of the mechanism of endocytosis and its metabolic regulation, as well as its inter-relationship with other metabolic events within the cell.

REVIEW OF LITERATURE

A comprehensive review of the wealth of literature that exists concerning endocytosis would be inappropriate here, particularly since excellent reviews already exist (38) (43) (70) (77) (85). Also, much of the work which I have read, is concerned with morphological studies in nonmammalian cells. In keeping with the scope of this study, only endocytosis in animal cells will be considered here, with emphasis on the studies involving mammalian cells in tissue culture. For convenience the literature on endocytosis will be divided into its four main aspects: the induction of endocytosis, the inhibition of endocytosis, the mechanism of endocytosis, and the fate of endocytosed materials.

The Induction of Endocytosis

The induction of the endocytosis of large particles, such as bacteria by macrophages, or ciliated protozoa by amoebae, usually referred to as phagocytosis, was first observed by Metchnikoff toward the end of the 19th century (57). This process involves the adsorption of the particle to the plasma membrane, which then engulfs the particle and takes it into the cell. Since bacteria are engulfed and destroyed in this manner by macrophages, this process, of obvious importance to immunology, has been extensively studied (36) (43) and will not be considered further here.

The endocytosis of fluids, frequently referred to as pinocytosis, was first observed by Lewis in 1931 (45) in tumor cells, and in amoebae by Mast and Doyle in 1934 (50). This type of endocytosis, in contrast to phagocytosis, does not require the presence of a specific inducer (21) (22) (37) (38) (43), as the conditions of the normal physiological medium in which the cells are maintained is normally sufficient to maintain a steady level of endocytosis (85) (87). The so-called induction of endocytosis may in fact be artifactual, rather than of true physiological significance (43). Nevertheless, the induction of fluid endocytosis has been extensively studied, primarily in amoebae, and reviewed by Chapman-Andresen (14). Amoebae form endocytotic vesicles, not directly, as first observed by Lewis (45), but indirectly by the formation of channels, which are themsevles invaginations of the plasma membrane, and from which the endocytotic vesicles pinch off into the cytoplasm (14). In amoebae, rapid endocytosis occurs only in the presence of inducers, and when induced occurs in cycles, during which endocytotic vesicles are formed at a rapid rate until as much as 50% of the plasma membrane is internalized, usually within 20-30 minutes, after which no further endocytosis occurs until after a period of quiesence, during which presumably the plasma membrane is renewed (14) (77).

Uncharged compounds do not induce endocytosis in amoebae (14). The charged compounds which do induce endocytosis in amoebae can be divided into three classes; firstly, salt solutions, whose ions show

the composition of this paper is extremely poor - very awkward sentence structure

no permanent binding to the plasma membrane, such as NaCl, and the basic or acidic amino acids, whose effects can be immediately discontinued by the replacement of the medium, secondly, the proteins and acidic dyes, which induce endocytosis only at the pH at which they are positively charged, and thirdly, certain phthalocyanine compounds, such as alcian blue, which bind irreversibly to the mucopolysaccharide coat of the plasma membrane, and exert their effect regardless of the pH of the solution (14). These findings, plus electron microscopic studies on the cell membrane of amoebae, which have demonstrated the presence of a homogenous amorphous layer on the surface of the membrane (49) (75), which biochemical analysis revealed to be an acid mucopolysaccharide rich in sulfate (49), ~~has~~ have led Holter (39), Stockem and Wohlfarth-Botterman (77) to believe that the first step in the induction of endocytosis in amoebae is the binding of the inducer to the mucoid layer of the plasma membrane, followed by the formation of endocytotic channels and vesicles.

Endocytosis by mammalian cells in tissue culture has been most thorougly studied in mouse mononuclear phagocytes (macrophages) by Cohn and his colleagues (18) (19) (21) (22). Their studies indicate a direct relationship between the concentration of serum in the medium, and the rate at which endocytotic vesicles are formed (19). Concomitant with the serum induced increase in endocytosis was an increase in the rate of production of acid phosphatase, cathepsin, and β-glucuronidase, which are three hydrolytic enzymes known to be present in lysosomes (27),

thus a regulatory relationship between the rate of lysosomal enzyme synthesis and the rate of endocytosis is suggested (19).

Phase contrast observations on living macrophages demonstrated that the clear, recently formed, phase lucent endocytotic vesicles fuse with one another and migrate towards the perinuclear zone. The clear vesicles then gradually increase in phase density, begin to show a positive acid phosphatase reaction, and gradually decrease in size to become small phase dense granuales, with the entire transition taking place within 90 minutes of the initial formation of the endocytotic vesicle (19). Similar results have been reported by Lewis (45) (46) in macrophages and by Rose (69) in HeLa cells.

Subsequent studies by Cohn and his colleagues (21) demonstrated that proteins, such as albumin and fetuin, with isoelectric points of pH 5 or below, were strong stimulators of endocytosis, whereas basic proteins, such as lysozyme, histone and protamine had little effect. Interestingly, Cohn reported that the removal of bound fatty acids from bovine plasma albumin decreased its inducing effect, which could be reversed upon the addition of oleic or linoleic acid. In addition, the removal of sialic acid from fetuin reduced its binding effect (21). Other compounds found to have an inducing effect were polyglutamic acid and various acid mucopolysaccharides, such as heparin, hyaluronic acid and chondroitin sulfate (21). Dextran was found to have no effect, while dextran sulfate was found to have a very great effect. DNA and RNA both had some inducing effect, ~~as well as~~ N-acetylneuraminic acid.

Clearly anions are better inducers than either cations or neutral molecules in macrophages. This is in contrast to amoebae, in which positively charged basic proteins and amino acids are most effective, while the nucleic acids are ineffective (43). Additional studies by Cohn and Parks (22) demonstrated that adenosine-5'-phosphate (AMP), adenosine-5'-diphosphate (ADP), and adenosine-5'-triphosphate (ATP) were very effective, while the di- and triphosphates of the other nucleosides were less effective. Adenosine itself also had a marked effect, while the other nucleosides had little effect. A similar stimulation of endocytosis by ATP was observed by Gropp (36) with Walker tumor cells, a mouse breast carcinoma cell strain, and with HeLa cells.

Studies with mouse fibroblasts indicates that heparin, a sulfate containing polysaccharide, induced endocytosis in this tissue (30).

Ubukata (81) found that endocytosis was stimulated by the presence of increasing concentrations of serum with L-strain cells. In addition, both the number and the size of medium dense granules, which were shown to arise from endocytotic vesicles, were found to increase with increasing concentrations of serum. Polyvinylpyrrolidone (PVP) in concentrations of from 1 to 4% was found to induce endocytosis in a protein free medium (80).

While most inducers of endocytosis are charged molecules, there is some indication that hormones may influence the rate of endocytosis in some systems, i.e. with the toad urinary bladder (51) (52) (53) (54)

and with adipose cells (6). Studies by Masur *et al.*on the toad urinary bladder have demonstrated a marked increase in endocytosis induced by the neurohypophyseal hormones, mediated by cyclic 3', 5'-adenosine monophosphate (52) (53) (54). This, as Masur *et al.* explains (54), may only be a secondary effect, with the initial hormonal stimulation acting on the process of exocytosis, with the resulting increase of endocytosis serving as a means of recovering membrane from the plasma membrane which was deposited there as a result of the fusion of exocytotic vesicles with the plasma membrane. Ball and Barrett (6) have reported an induction of endocytosis by insulin in adipose cells *in vitro*. Similarly, Paul and Pearson (38) observed the induction of endocytosis by insulin in HeLa cells, but subsequent work by Paul (38) showed that this effect was difficult to reproduce, whereas the effect noted by Ball and Barrett (6) was very regular. Ball and Barrett's work was not, however, supported by the more quantitative work of Cushman (23) who studied the uptake of radioactive colloidal gold by isolated adipose cells *in vitro*. His results suggest that many of the vesicles presumed to be of endocytotic origin by Ball and Barrett may in fact be exocytotic vesicles. Insulin has also been shown to induce endocytosis in amoebae, but only in high, unphysiological concentrations (77). The effect in amoebae is therefore probably only a nonspecific protein induction, as opposed to the effect observed in adipose cells, which was observed at a very low (0.04 µg/ml) concentration, within the normal physiological range (6).

The only other clear case of hormone induction of endocytosis is the effect of the hormones thyrotropin, long acting thyroid stimulator, prostaglandin E_1, dibutyryl cyclic 3', 5'-AMP and cyclic 3', 5'-AMP on canine thyroid slices (11) (60) (61) (68) (73). The hormonal induction of endocytosis in this system is mediated by the activation of adenyl cyclase, and hence an elevated intracellular cyclic 3', 5'-AMP level (11) (67). This appears to be the only example in the literature in which the intracellular factors responsible for the induction and regulation of endocytosis are at all understood, with the possible exception of the work of Cushman (23), who reported that intracellular non esterified fatty acids may be factors involved in the regulation of endocytosis in adipose cells.

The Inhibition of Endocytosis

Extensive studies with metabolic inhibitors, particularly inhibitors of oxidative phosphorylation, have quite convincingly demonstrated the dependence of endocytosis on ATP as an energy source (13) (17) (29) (43). As endocytosis is an active cellular phenomenon, this is not surprising. More interesting are the results reported by Wagner *et al.* (82) in which a dose dependent inhibition of endocytosis was observed with the mold metabolite, cytochalasin B, a compound which is known to cause disaggregation of microfilaments (84). The importance of the requirement for ATP as an energy source, and intact microfilaments, will be apparent in the next section.

The Mechanism of Endocytosis

Much of the earlier work on the mechanism of endocytosis was concerned with the use of metabolic inhibitors, which established the requirement for ATP, and with the use of inducers, which established that inducing agents act by first adsorbing to the cell's plasma membrane.

Bennett (7), in his classic and frequently cited work on the role of membrane flow in active transport, was one of the first workers to discuss the importance of endocytosis as a means of selective uptake of particles and molecules which can adsorb to the cell's plasma membrane. The double labelling experiment of Chapman-Andresen and Holter (15) vividly demonstrated that molecules and particles which adsorb or bind to the plasma membrane would be taken up at a different rate than molecules or particles which do not bind to the membrane. In this experiment the uptake of ^{131}I labelled albumin and ^{14}C labelled glucose by amoebae were measured simultaneously. Since the plasma membrane of amoebae is impermeable to both glucose and albumin, these molecules could enter the cells only by endocytosis. The results demonstrated that albumin, because it binds to the plasma membrane, whereas glucose does not, is taken up at a rate of nearly ten times that of glucose, which enters the cell only passively, in solution, as fluid is taken into the cell in endocytotic vesicles. The importance of the differential kinetics of the endocytosis of materials which can

bind to the plasma membrane, and those which cannot, has been described elsewhere (35) (43).

More interesting, perhaps, than the study of the kinetics of endocytosis, has been the recent work attempting to elucidate the ultrastructural and biochemical mechanism of endocytosis. One sees innumerable accounts in the literature of morphological observations of endocytosis, beginning with Metchnikoff (57) in 1883, and later Lewis (45) in 1931, as well as observations of endocytosis in virtually every animal cell or tissue which has been observed with the electron microscope. However, it has only been since the discovery of some of the biological effects of the mold metabolites, the cytochalasins (12), followed by the later discovery of their effects on microfilaments (84), that real progress has been made.

The association between microfilaments and cellular motility has been clearly established in numerous cell types (3) (74) (84). Since microfilaments, which are 50-100 Å diameter fibers present in nearly all plant and animal cell types (84) are capable of binding with the muscle protein, heavy meromyosin, (3) (41), which is known to bind specifically with the muscle protein actin (4) (40), it is reasonable to assume that microfilaments are an actin-like protein (3).

The cytochalasins, of which there are at least four; cytochalasin A, B, C, and D, are known to cause the disaggregation of microfilaments (84). Of these, cytochalasin B is the most readily available, and has been most widely used. Interestingly, the effects of

cytochalasin are reversible; normal microfilaments rapidly reappear in cytochalasin treated oviduct cells after the growth medium containing cytochalasin is removed and replaced with normal growth medium (84). This occurs even in the presence of cycloheximide, an inhibitor of protein synthesis (84). Cytochalasins are not known to have any cytotoxic effects, apart from their effect on microfilaments, and microfilament dependent processes (84).

Studies have been conducted which implicate the involvement of microfilaments with endocytosis, since cytochalasin B inhibited endocytosis in both macrophages (3) and in Chang liver cells (82). The drug colchicine, which disrupts microtubules but not microfilaments (8), when applied to macrophages in tissue culture, causes cessation of the normal directional gliding movement of these cells, with the induction of an amoeboid (flowing) type of movement (8). In addition, endocytosis is not effected by colchicine, except that endocytotic vesicles are not transported by long saltatory movements, as they are in normal untreated cells (3) (9). These results have caused some workers (3) to postulate the existence of an intracellular cytoskeleton system, formed of microtubules, to which microfilaments may be attached.

This is essentially the extent to which the mechanism of endocytosis is understood to date; microfilaments are probably attached to the plasma membrane, where by ATP dependent contractions they cause membrane ruffling and the formation of endocytotic vesicles (3) (82). The subsequent migration of the endocytotic vesicles to

the Golgi region probably depends upon the contraction of microfilaments attached to microtubules (3). The mechanism responsible for the initiation of the formation of endocytotic vesicles by microfilaments, and the organized control of microfilament contraction that must be necessary, is not at all known. Likewise, the mechanism by which inducers of endocytosis influence the formation of endocytotic vesicles by microfilaments is not known.

Fate of Endocytosed Materials

That endocytotic vesicles fuse with lysosomes to form secondary lysosomes, sometimes called phagosomes, has been known for some time now, and has been well reviewed by de Duve (27). The importance of this mechanism is obvious, as it provides a means by which materials can be taken up by the cells and subsequently digested by the hydrolytic enzymes of the lysosomes before being released into the cytoplasm, where the digested materials can be metabolised. The question of the fate of endocytosed materials becomes rather a question of their digestability, since materials which have not been digested to smaller molecules, i.e. simple sugars, amino acids and free fatty acids, cannot be used by the cell and may even be detrimental. In protozoa, endocytosed materials which cannot be digested are defecated by a reverse process of endocytosis (59). It has not been well proven whether this can occur in higher animals (25). Thus it can be expected that endocytosed materials which cannot be digested by the hydrolytic enzymes of the lysosomes, must

remain in the cells, and their presence would serve as a suitable marker of the endocytosis which has occurred.

One such compound which has been used to quantitate the endocytosis by cells in tissue culture is radioisotopically labelled sucrose (82). The plasma membrane of cells has been shown to be impermeable to sucrose (10) (20) (43) (55) (78). Therefore sucrose can enter the cell only by endocytosis. Sucrose, once inside the cell, cannot be digested (20) (28) (32) (42) (43) (44) (83), and hence can serve as a sensitive marker of endocytosis if it is radioisotopically labelled.

Once inside the cell, undigested endocytosed compounds remaining within the secondary lysosomes can exert osmotic effects on the secondary lysosomes. This has been clearly demonstrated with sucrose, which when added to the growth medium in which tissue culture cells are incubated, causes the endocytotic vesicles and secondary lysosomes to persist, since the fluid within these vesicles is hypertonic to the cytoplasm, and cannot be actively transported out of the vesicles against the osmotic barrier (20) (32) (58) (82). The same effect has been observed in renal proximal tubular epithelium when sucrose was administered intravenously to rats (72). Dextran, a polyglucose, also accumulates within secondary lysosomes (24). However, because of its high molecular weight, it exerts less osmotic pressure, with the result that dextran laden vesicles do not persist as large vesicles, but rather can be reduced in size by the cell as fluid is transported out of the vesicles. Dextran laden

vesicles do, however, persist (24). Polyvinylpyrrolidone (PVP), a polymer which has been used extensively as a plasma expander (33), has also been shown to prevent the normal transistion of endocytotic vesicles. PVP laden vesicles failed to become phase dense when observed with phase contrast microscopy, as do normal endocytotic vesicles as a result of water being actively transported out of the vesicles (80). In addition, these PVP laden vesicles persisted and accumulated (80). Although the failure of these PVP laden vesicles to become phase dense was attributed to the absence of serum proteins in the growth medium used in the experiment, it is possible that it may have been due to the osmotic effect of low molecular weight PVP known to exist in commercial undialysed preparations of PVP (4). In any case, the evidence quite clearly inducates that undigestable compounds such as sucrose, dextran, and PVP can profoundly alter the normal fate of secondary lysosomes.

OBJECTIVES OF THIS STUDY

A number of problems regarding endocytosis presented themselves, which warranted investigation. They are:

a. It has not been conclusively established whether cryoprotective polymers, such as dextran and PVP, exert their cryoprotective action intracellularly or extracellularly. If they act intracellularly, then factors which effect the cryoprotective action of these polymers, such as the presence of serum, would be expected to change the rate of endocytosis of the polymers, since they can enter the cell only by endocytosis.

b. A possible mechanism of the cryoprotective action of the polymers dextran and PVP would be the binding of these polymers to the plasma membrane. Such binding, if it occurred, could be detected by the kinetics of the endocytotic uptake of these polymers.

c. Cohn and Benson observed that the presence of serum in the growth medium induces endocytosis in mouse macrophages (19), an effect which clearly warrants investigation in other cell systems.

d. Although the accumulation of sucrose laden vesicles has been observed in a number of systems, as mentioned earlier,

it has been clearly established in only one cell system (82) that this is an osmotic effect, and is not due to an increase in the rate of endocytosis.

e. Munro (58) reported that the replacement of the growth medium in which cultures of Chinese hamster fibroblasts of Puck's Clone A are being maintained, with fresh medium, stimulates endocytosis by these cells. This effect should be investigated more thoroughly for its possible significance.

f. Endocytosis in canine thyroid slices appears to be regulated by the intracellular levels of cyclic 3' 5'-AMP (52) (53) (54). This, as well as other possible hormone effects should be investigated in other systems.

g. The relationship between microfilaments and endocytosis has been discussed earlier. Although cytochalasin B, which disrupts microfilaments, has already been shown to inhibit endocytosis in the two cell systems investigated previously (3) (82), verification of this effect in other cell systems would more conclusively establish the relationship between microfilaments and endocytosis, and the inhibition of endocytosis by cytochalasin B.

h. While numerous morphological observations of endocytosis in cells in tissue culture have already been done, additional

observations concerning the effects of cytochalasin B, sucrose, dextran and PVP might yield interesting results, particularly with regard to any observable correlations between endocytotic activity as measured by the uptake of radioactively labelled compounds and any observable morphological changes.

These problems were investigated with the aid of dextran-^{3}H, PVP-^{14}C, sucrose-^{14}C and sucrose-^{3}H. Sucrose-^{3}H was used extensively throughout this study as a general marker of the rate of endocytosis, for reasons already described (see page 15). Morphological studies were carried out using phase contrast and Zeiss interference (after Nomarski) optics. Details of the experimental procedures follow in the next section.

MATERIALS AND METHODS

Cell Lines

Serially propagated Chinese hamster ovary fibroblasts, designated as Puck's Clone A, were obtained at the 351st passage as a gift from Dr. D.M. Robinson of the American National Red Cross Blood Research Laboratory, Bethesda, Maryland. These cells will hereafter be referred to as CHA cells.

Serially propagated cells derived from a beneign human prostatic hypertrophy, cell line MA-160, were obtained at the 357th passage from Microbiological Associates, Inc., Bethesda, Maryland. These cells will be referred to as PCC cells.

Sterility

All sterile work was performed on an Envirco Laminar Flow Bench (Envirco Division of Belton, Dickonson and Company, Albuquerque, New Mexico).

Culture Medium

Both the CHA and PCC cells were maintained in Eagle's minimum essential medium with Earle's balanced salt solution (31) obtained from Biocult Laboratories Ltd., Glasgow, Scotland. Except where otherwise indicated, this medium was supplemented with 10% fetal

calf serum, obtained from Microbiological Associates, and 50 µg/ml of kanamycin, obtained from Biocult Laboratories Ltd. The addition to the medium of 0.05 M Tricine (N-tris (hydroxymethyl) methyl glycine) obtained from the Sigma Chemical Corp., St. Louis, Missouri, and adjusted to pH 7.2, made it unnecessary to gas the cell cultures with a 5% CO_2 and air mixture. In the earlier experiments with PCC cells, this culture medium was supplemented with 10^{-3} M sodium pyruvate (Sigma), but this was later found to be unnecessary. This medium will be referred to as CTM.

Glucose free medium, referred to as GFM, was prepared in the same manner except that Eagle's minimum essential medium was prepared directly from the constituent amino acids, obtained from Nutrional Biochemicals, Cleveland, Ohio, the vitamins, obtained from Vitamins Ltd., Great Britain, and salts (all standard analytical reagent grade) according to the method of Paul (62).

Method of Culturing

Stock cultures of either PCC or CHA cells were harvested from the culture flask, on whose bottom glass surface they were attached, by removing the CTM in the flask by vacuum suction, and then immediately replacing the medium with a 0.1% trypsin solution (Bacto-Trypsin 1:250, Difco Laboratories, Detroit, Michigan) in a Ca^{++} and Mg^{++} free Earle's balanced salt solution. The cell

cultures were then incubated in the trypsin solution for 2-3 minutes until the cells became detached from the glass surface on which they were growing. The resulting suspension of cells was then centrifuged at 75 g for 3 minutes to cause the cells to form a pellet at the bottom of the centrifuge tube. The supernatant was then removed and replaced by several mls. of CTM in which the cells were resuspended. The resulting suspension of cells was then added to a larger volume of CTM which was then distributed to the vessels in which subsequent experiments were to be performed.

Uptake of Radioisotopically Labelled Compounds

For the studies on the uptake of various radioisotopically labelled compounds, cell cultures were grown directly in sterile scintillation vials (Beckman low-potassium borosilicate liquid scintillation vials, part no. 161641, Beckman Instrument Co., Fullerton, California), which had previously been cleaned with Microsolve tissue culture detergent (Microbiological Associates, Inc., Albany, California). 3.0 ml, or in later experiments 1.5 ml of cells suspended in CTM were placed in each vial, after which a polyethylene cap liner, sterilized by boiling in water, was placed on the top of each scintillation vial and screwed down tightly with an ordinary scintillation vial cap. Usually 100 such cultures were set up for each experiment, with each culture being innoculated with about 20,000 cells. The cultures were then placed in an

incubating room, kept at 36.5°C, and allowed to incubate until the cell density at the bottom of the vials had just reached confluency, after which the experiments were performed as soon as possible.

For each experiment the vials were removed from the incubating room, placed on the laminar flow bench, and opened. The old medium was then removed from each vial and replaced with 3.0 or 1.5 ml of the experimental medium as quickly as possible. The cell cultures were then allowed to adjust to their new medium for about 15 minutes before the radioisotopically labelled isotope was added, usually in 0.1 ml of physiological saline.

As soon as possible after the addition of the radioisotopically labelled compound, the caps were screwed back on the vials (without the polyethylene liners) and the vials were replaced in the incubating room. Sample vials were then removed from the incubating room at regular intervals, typically 20, 90, 200, 360, and 480 minutes after the incubation with the isotope had begun, and were immediately washed. The washing procedure involved first pouring off the experimental medium, and then repeatedly filling the vials with physiological saline (made by dissolving 8.5 g/litre of sodium chloride in tap water) and pouring off the saline four times.

The sample vials were air dried overnight. The next day 0.1 ml of distilled water was added to each vial, followed by 0.5 ml of a mixture 50% Protosol tissue solubilizer (New England Nuclear, Pilot Chemicals Division, Boston, Massachusetts) and toluene (analytical reagent grade). The scintillation vials were then allowed to incubate with the tissue solubilizer for about 30 minutes, after which 12 ml of a solution of 5 g/litre of PPO (2, 5-diphenyloxazole, scintillation grade, Amersham/ Searle Corporation, Arlington Heights, Illinois) in toluene (analytical reagent grade, Mallinckrodt Chemical Works, St. Louis, Missouri) was added to each vial. The vials were again sealed with polyethylene liners and plastic caps, and then incubated for 30 minutes at 60^{o}C to allow the cells to become completely solubilized. The samples were then counted in a Beckman LS-133 Liquid Scintillation Spectrometer, at 38-41% efficiency for ^{3}H and 92% efficiency for ^{14}C labelled compounds.

For each experiment, at least four sample vials were not digested, but were instead treated with 2 ml of trypsin solution to detach the cells, which after shaking produced a uniform cell suspension, which was counted in a hemacytometer to estimate the average number of cells per vial.

Details of each experiment, as well as any variation from the above procedure, will be given for each experiment in the next section.

Radioisotopically Labelled Compounds

The radioisotopically labelled compounds used in this study were as follows: Sucrose-6, 6'-^{3}H, with a specific activity of 1.8 Ci/mmole was obtained from Amersham/Searle. Dextran-^{3}H, universally labelled, with a specific activity of 406.5 mCi/g and a molecular weight distribution of 60,000 to 90,000 Daltons was obtained from New England Nuclear, and was subsequently dialysed against distilled water. Sucrose-^{14}C, with a specific activity of 420 mCi/mmole, was obtained from Schwarz/Mann, Orangeburg, New York. Polyvinylpyrrolidone-^{14}C, K 17.8, with a specific activity of 11.24 mCi/g was a gift from The General Aniline and Film Corp., New York, New York.

Oxygen Consumption Studies

Identical cultures of CHA cells were grown in medicine bottles until confluent. The medium in the bottles was then removed and replaced with 5 ml of the test medium. The cells were then scraped off the surface of the bottle on which they were growing, suspended in the test medium and removed from the bottle. The cell suspension was then placed in the stirring chamber of a Yellow Springs Instrument Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). The Clark

polarographic electrode was then inserted into the stirring chamber and the depletion of oxygen from the medium by the cells was recorded as percent saturation of oxygen as a function of time. Cell counts were determined after each run using a hemacytometer. Eosin exclusion viability tests were performed on the cells both before and after each run, to determine the extent to which the cells had been damaged by scraping them off the glass, and the extent they had been damaged by being stirred in the stirring chamber. The eosin exclusion viability test consisted of placing a few drops of the cell suspension in 2 ml of a solution of 0.1% eosin in phosphate buffered physiological saline for three minutes, after which the cells were examined microscopically to determine the percent viability, the assumption being that eosin cannot penetrate living cells (71).

Electrophoresis

Polyacrylamide disc gel electrophoresis was performed on samples of fresh medium and medium in which cell cultures had been maintained for 4 or more days, using the method of Clarke (16). 25 μ litres of a 1:1 mixture of test medium and a 40% by weight per volume solution of sucrose was layered on the top of each gel. 0.04 M tris-glycine buffer (tris (hydroxymethyl) aminomethane, (Sigma) and reagent grade glycine, (Fisher Scientific Co., Fair Lawn, New Jersey) pH 8.3 was used. Twelve tubes were run

simultaneously with the current set at 25 milliamperes, voltage of 250 volts and the temperature maintained at 0°C. After each run the gels were stained for protein for 30 minutes in a 0.2% amidoschwartz in 7% acetic acid solution, and destained overnight with several changes of 7% acetic acid.

Light Microscopy

Cells were grown on glass coverslips in petri dishes in normal CTM. After several days, when there were sufficient numbers of cells, the CTM in which they were growing was removed by vacuum suction and replaced by the test medium. The cultures were then returned to the incubating room for the desired length of time, after which the coverslips were removed and mounted with several drops of the test medium on a microscope slide on which a ring of petrolatum had been made, which sealed the coverslip. The slides were then examined and photographed with phase contrast or Nomarski differential interference optics on a Zeiss Ultraphot II (Carl Zeiss, Oberkochen, West Germany) using a 100 X oil immersion objective and a 1.4 NA oiled condensor, illuminated with a high pressure mercury lamp. During observations the microscope stage temperature was maintained between 32° and 37°C with a Sage Air Curtain Incubator (Sage Instruments Inc., White Plains, New York).

Cells were also grown in Falcon plastic tissue culture flasks (Falcon Plastics, Oxnard, California), fixed with 1% glutaraldehyde, followed by 1% OsO_4 by the method of Goldman (34), except that following alcohol dehydration the cells were not scraped off the plastic surface on which they were growing, but rather Epon (48) was poured directly into the Falcon flasks, without the usual preliminary propylene oxide equilibrations. The Epon was allowed to remain in the flasks overnight at room temperature, after which it was poured off and replaced with fresh Epon. The flasks were then incubated at 60°C for 18 hours to allow the Epon to polymerize. The Falcon flasks containing the Epon embedded cells were then shattered with a sharp blow from a hammer in order to obtain chips of Epon containing cells, which were detached from the plastic substrate on which they had been growing. Chips of Epon obtained in this manner contained cells on surface of the chip, which when observed under the microscope appeared to be of normal morphology. The Epon chips were cut to a suitable shape, mounted in a chuck and sectioned on a Reichert OM U2 Ultramicrotome (Reichert, Vienna, Austria). 1.0 μ thick sections were cut and stained with azur 2 and methylene blue according to the method of Richardson *et al.* (64) and photographed with bright field optics on a Zeiss Ultraphot II, using an orange (540 nm) interference filter to improve contrast.

Adsorptivity of Polyvinylpyrrolidone (PVP)

To compare the relative adsorptivity of PVP and sucrose to CHA cells, large cultures of CHA cells were grown in 250 ml medicine bottles until confluent. The old medium was then poured off and replaced with 5 ml of fresh CTM, which was just sufficient to cover all the cells. The cultures were then replaced in the incubating room for one hour, after which they were removed and innoculated with 0.25 ml of either sucrose-^{14}C or PVP-^{14}C (K 30), both with a specific activity of 0.01 mCi/ml and a concentration of 0.889 mg/ml. The cultures were then allowed to incubate for 5 minutes at room temperature.

The medium containing the labelled sucrose or PVP was then poured off, followed by the addition of 5 ml of saline (prepared as described earlier).

Two of the cultures, one which had been incubated with sucrose-^{14}C and the other which had been incubated with PVP-^{14}C received no further washing. The cells in these cultures were then scraped off with a rubber policeman and withdrawn in suspension in saline with a pasteur pipette, and spun at 200 g in a small clinical centrifuge until the cells pelleted, about 3 minutes. The supernatant was then drawn off, except for 0.2 ml in which the pellet of cells was resuspended, placed in a scintillation vial, digested with Protosol and counted in a liquid scintillation counter as described

earlier. These two samples were designated as sucrose-^{14}C and PVP-^{14}C Wash 1. Samples designated as sucrose-^{14}C and PVP-^{14}C Washes 2, 3, 4, and 5 were treated as above except that prior to the addition of 5 ml of saline, followed by scraping off the cells with a rubber policeman, the bottles were completely filled with saline, which was then poured off, 1, 2, 3, and 4 times respectively. The results were then plotted as counts per minute of radioactivity remaining as a function of the number of washes.

RESULTS

General Morphology

CHA cells grew in monolayers, with some piling up of cells noticeable both in phase contrast observations of living cells (figs. 1, 3, and 6) and in fixed and sectioned cells (fig. 4). CHA cells displayed a well spread morphology, typical for fibroblasts. Areas of membrane ruffling are visible in many of the cells (figs. 1, 3, 5, 6, and 7), which frequently exhibit endocytotic activity. Not all areas of membrane ruffling exhibited endocytosis, however.

CHA cells were occasionally multinucleate (not shown), with each nucleus containing 3 or more nucleoli (fig. 1).

Abundant mitochondria were seen occupying regions of the cytoplasm outside of the perinuclear zone, and extending at times into the numerous pseudopodia of these cells (figs. 1 and 3).

PCC cells were also well spread, but were not observed to pile up on top of each other (fig. 8, 9, and 10), as did CHA cells. Membrane ruffling, like that seen in CHA cells, was not observed with PCC cells, nor was endocytotic activity visible. Mitochondria, although visible, were not so apparent as in CHA cells.

Phase lucent vacuoles, frequently seen in CHA cells, were generally absent in PCC cells, however numerous phase dense granules, probably primary and secondary lysosomes, were frequently seen in the perinuclear zone (figs. 9 and 10).

Figures 1-3

Figure 1. Phase contrast micrograph of normal Chinese hamster cells. Phase lucent endocytotic vesicles (EV) can be seen in the zone surrounding the nucleus (Nu), referred to as the perinuclear zone. Areas of membrane ruffling can be seen at the periphery of the cells. Numerous mitochondria (M) are also visible. X 1500.

Figure 2. Phase contrast micrograph of normal Chinese hamster cells. Dark phase dense masses in each nucleus are nucleoli. X 1500.

Figure 3. Nomarski interference micrograph of normal Chinese hamster cells. Note that phase dense structures seen in phase contrast, i.e. nucleoli, mitochondria and nuclear membrane, appear as elevations or raised structures with Nomarski optics. Similarly the phase lucent endocytotic vesicles seen with phase contrast appear as hollowed out depressions (EV) with Nomarski optics. X 1500.

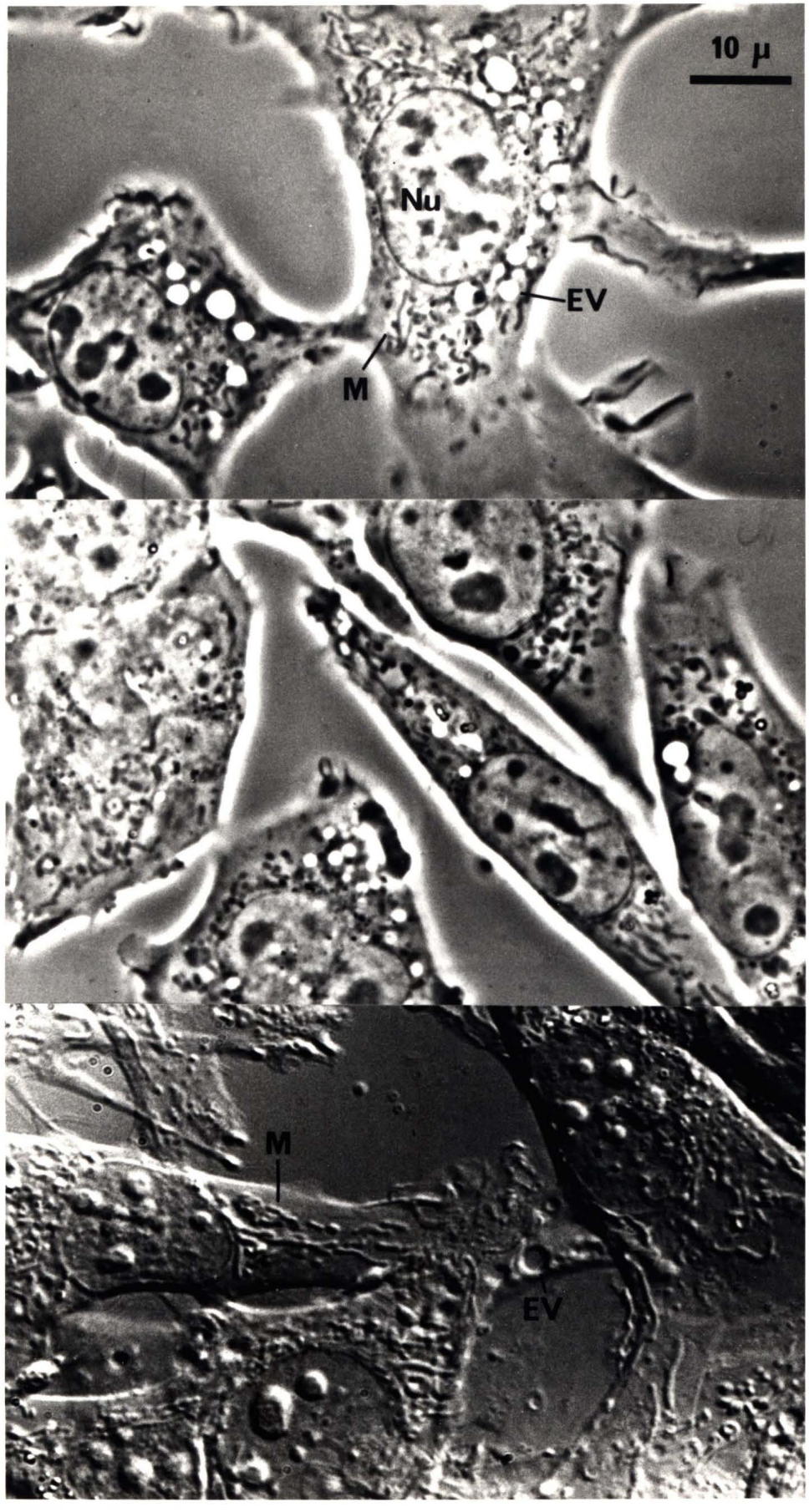

10 μ
Nu
EV
M
M
EV

Figure 4. 1.0 μ thick sections of Chinese hamster cells, stained with azur 2 and methylene blue. Cells are shown in cross section as they were growing on the plastic substrate (PS). Piling up of the cells is evident in all the micrographs. Nuclei (Nu) and clear endocytotic vesicles (EV) are also seen in all the micrographs. In D are visible numerous small pseudopodia (P). Bright field optics with a 540 nm interference filter. X 1500.

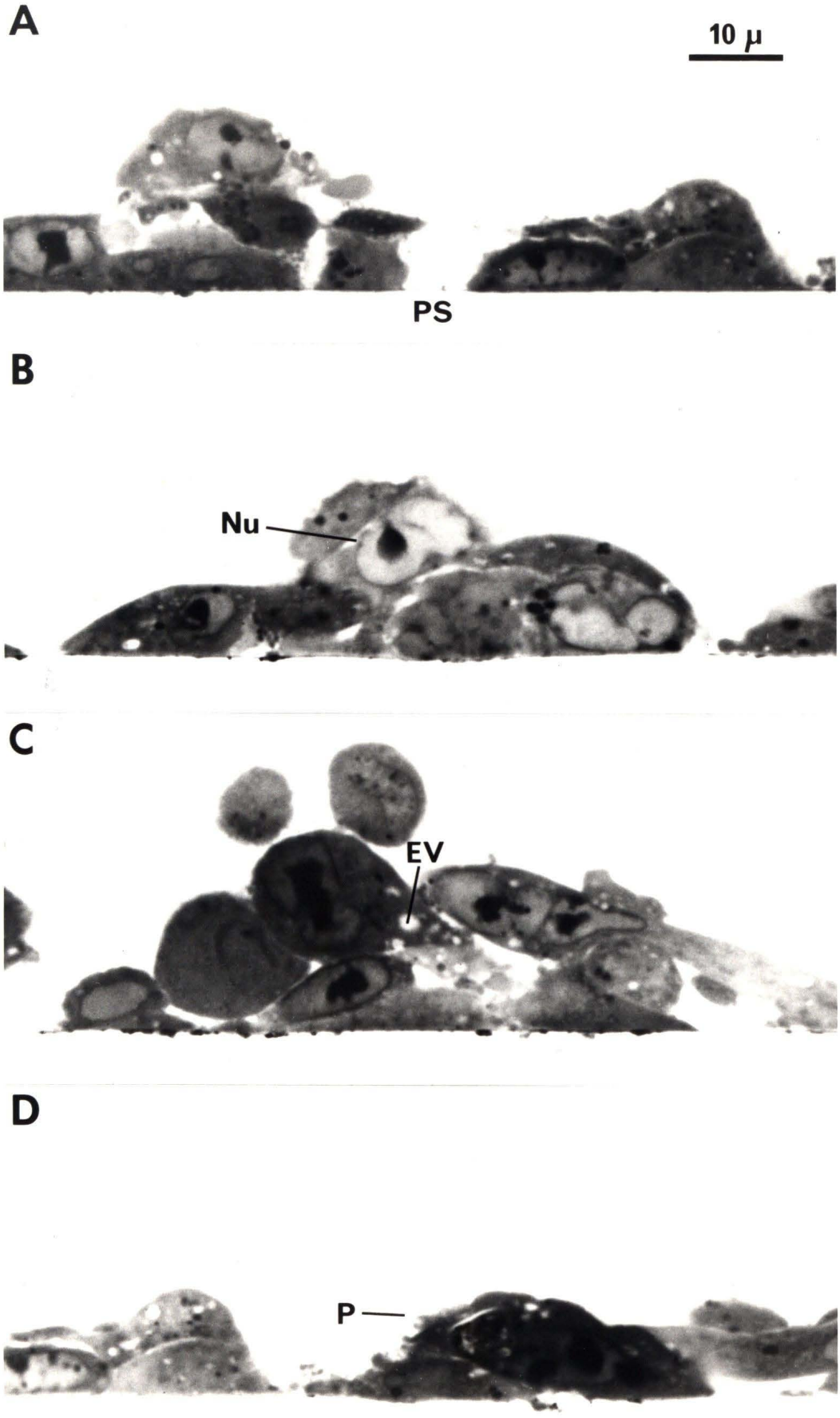

A
10 μ
PS
B
Nu
C
EV
D
P

Figure 5. Time-lapse series of endocytotic activity in Chinese hamster cells. In A can be seen the formation of an endocytotic vesicle in an area of membrane ruffling (arrow). In C and D the newly formed vesicle is seen to migrate to the perinuclear zone. 30 second time interval between micrographs. Nomarski optics. X 1600.

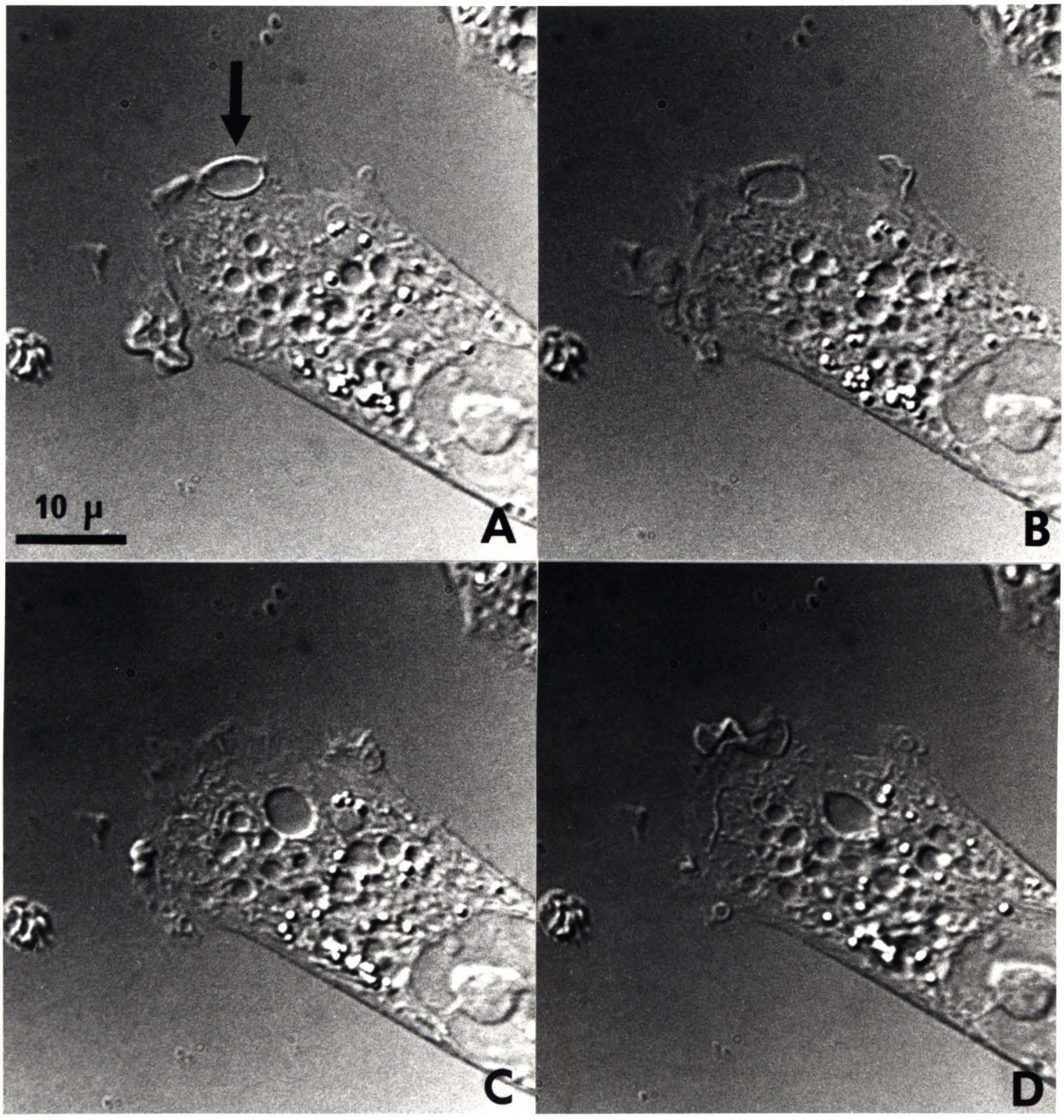

10 µ
A
B
C
D

Figure 6. Time-lapse series of endocytotic activity in Chinese hamster cells. An area of membrane ruffling (arrow) can be seen in A which gives rise to an endocytotic vesicle B and C. The newly formed vesicle can be seen to migrate towards the perinuclear zone D through H. In H can also be seen the formation of another endocytotic vesicle in approximately the same place where the vesicle was formed in A. 30 second time interval between micrographs. Nomarski optics. X 1350.

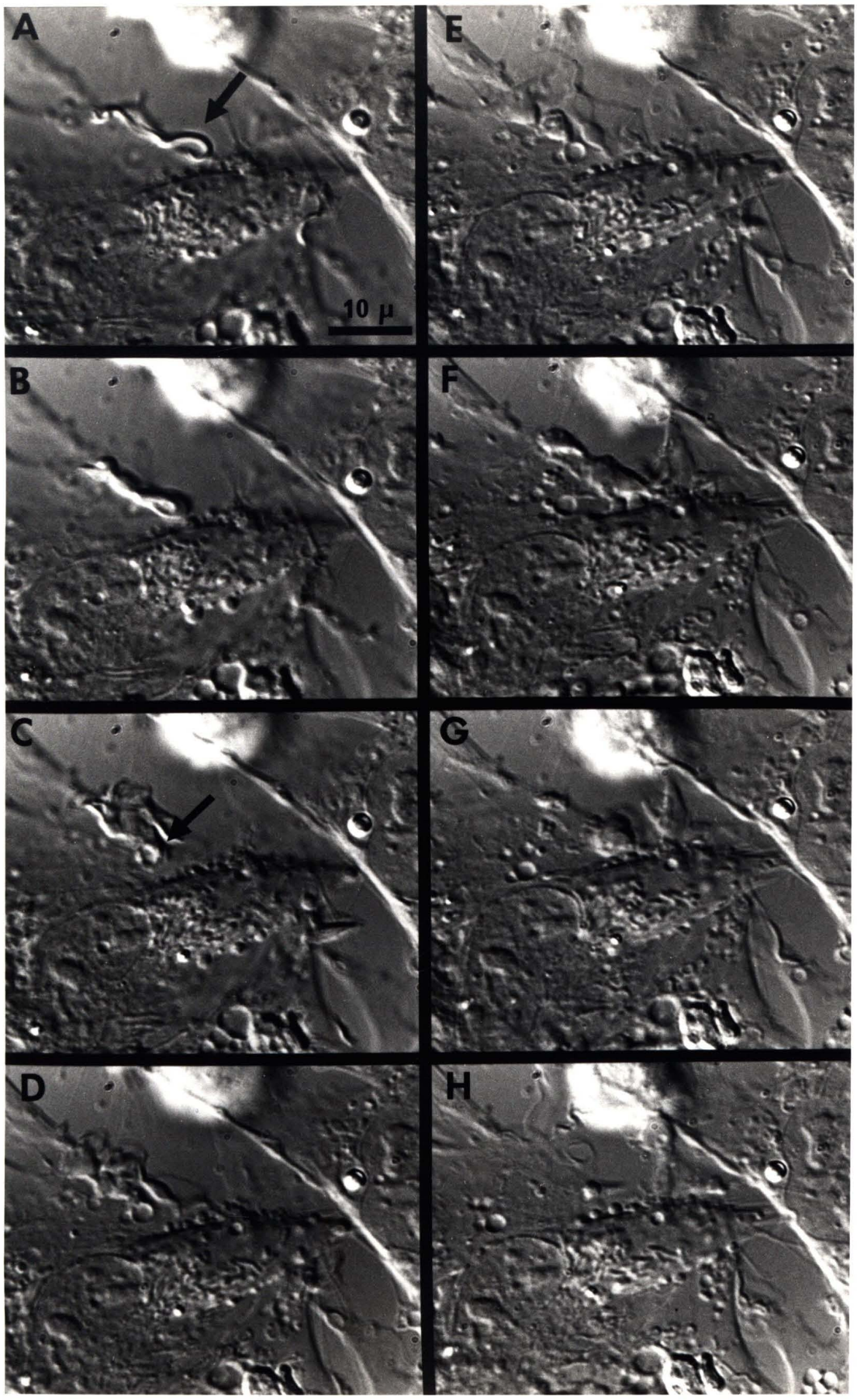
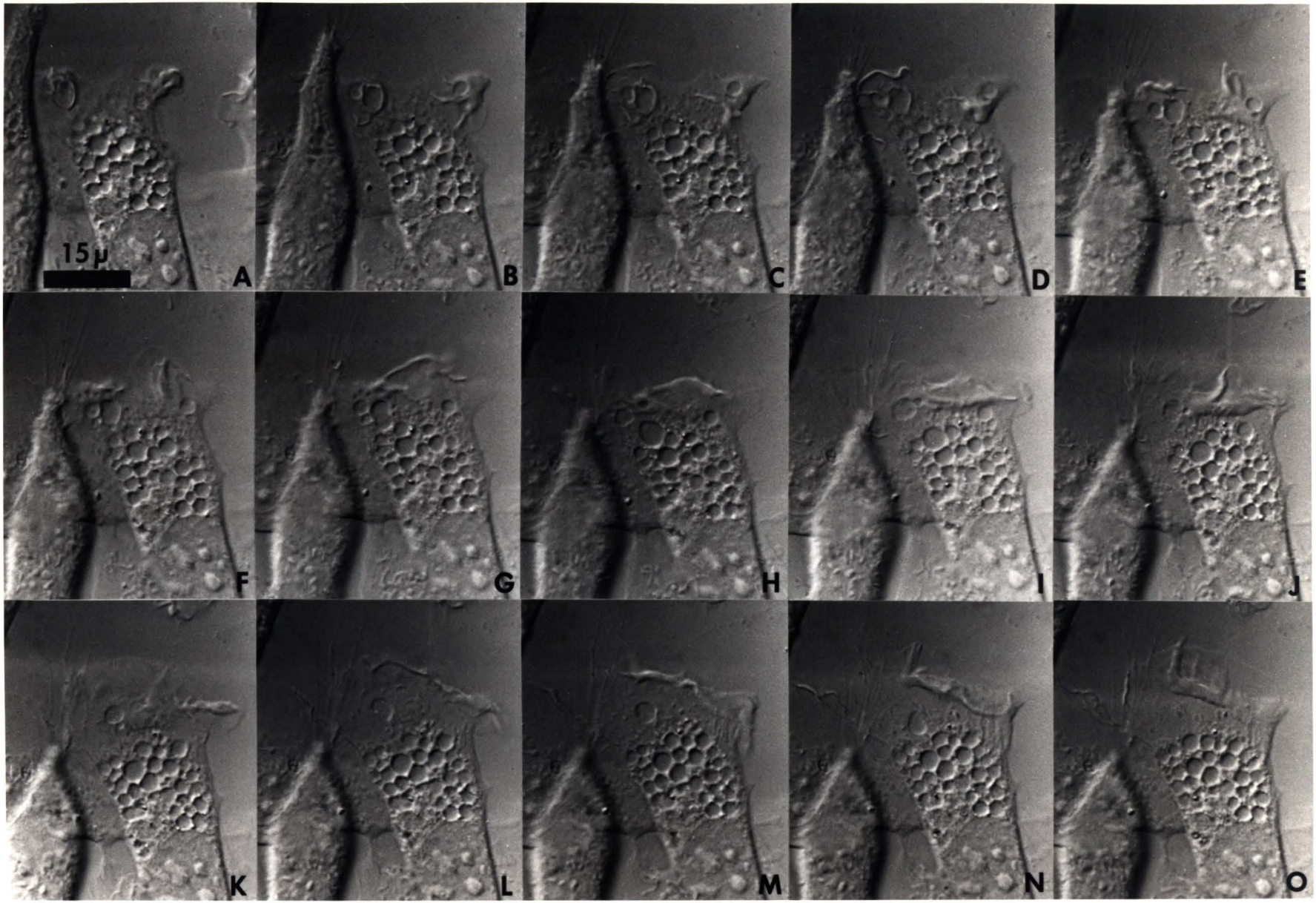

A
10 μ
B
C
D
E
F
G
H

Figure 7. Time-lapse series of endocytotic activity in a Chinese hamster cell. Intense membrane ruffling can be seen in the top of the cell in the center of A. The formation of a large endocytotic vesicle can be seen in A through D. In E through H this vesicle can be seen to migrate towards the perinuclear zone to join the large number of previously formed endocytotic vesicles. In G it appears as though this vesicle is fusing with another vesicle. In I through N another newly formed vesicle can be seen migrating inward. In L it appears as though the vesicle is being pulled towards its destination, as evidenced by its distorted shape. 30 second time interval between micrographs. Nomarski optics. X 1000.


15 μ
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Time-Lapse Observations of Endocytotic Activity

Several areas of endocytotic activity in CHA cells were photographed at 30 second time intervals with Nomarski optics to demonstrate the formation and migration of endocytotic vesicles (figs. 5, 6, and 7). In general, the formation of endocytotic vesicles occurred only at areas of membrane ruffling, where the vesicles were observed to be formed from foldings of the membrane, followed by a nonrandom migration towards the perinuclear zone. Endocytotic vesicles, once formed, appeared to be pulled by what were sometimes quite rapid movements, referred to as long saltatory movements (9). Endocytotic vesicles were sometimes seen to fuse with one another, but the actual fusion occurred too rapidly to be photographed.

Morphological Effects of Sucrose

Both PCC and CHA cells showed profound morphological alterations when incubated in CTM containing 0.08 M sucrose (figs. 11 through 15). These effects first became apparent in both cell lines after about three hours. In CHA cells, sucrose treatment resulted in the formation of large vesicles which accumulated in the perinuclear zone, and in some cells nearly filled the entire cytoplasm.

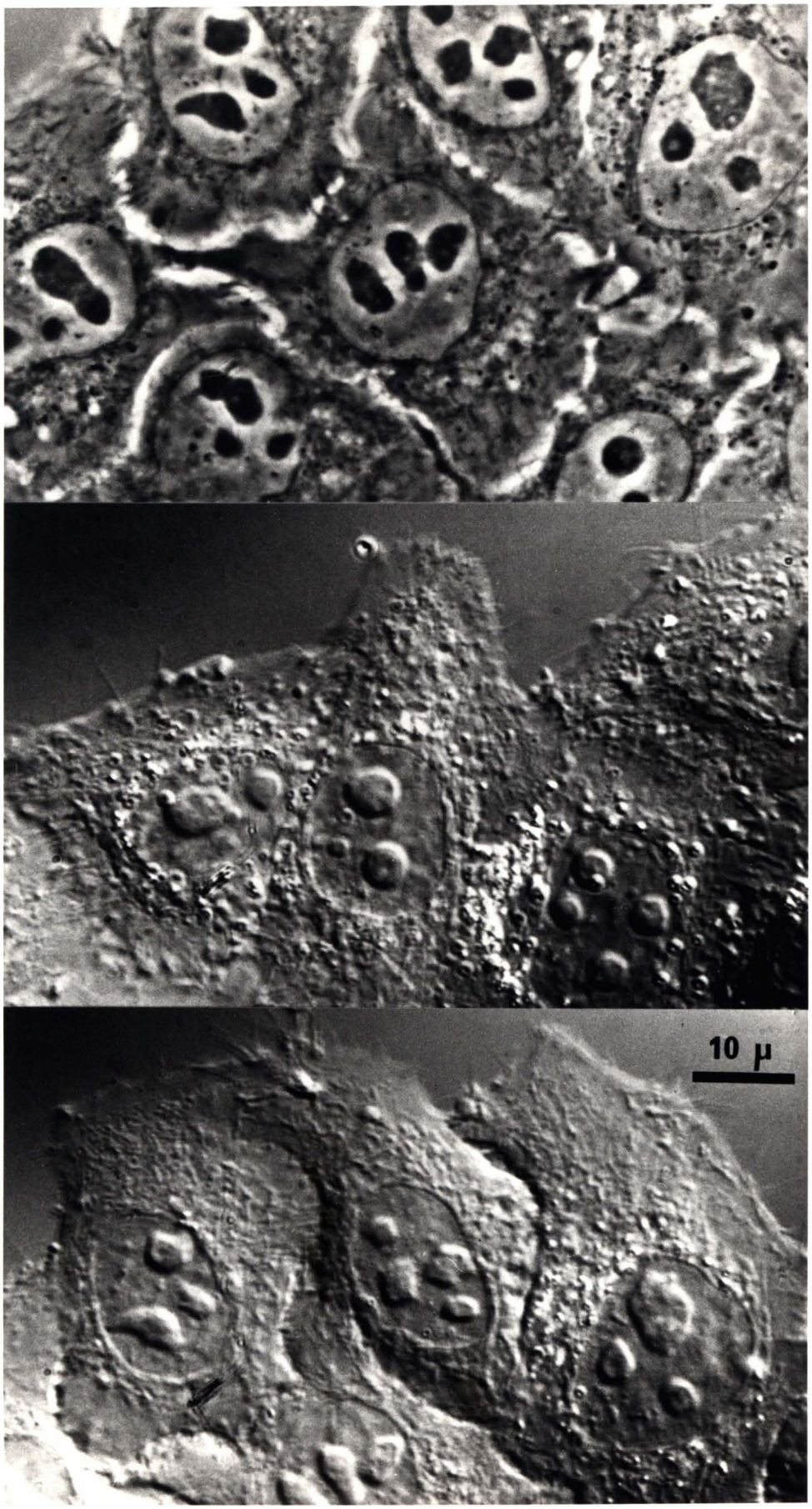
In PCC cells, large vesicles were not observed, but very numerous, phase dense vesicles, 0.8-1.5 μ in diameter accumulated in the perinuclear zone, which were seen only occasionally in normal, untreated cells.

Figures 8-10

Figure 8. Phase contrast micrograph of normal prostatic cancer cells. Note the well spread morphology and the lack of piling up of the cells. Several nucleoli are visible within each nucleus. X 1500.

Figure 9. Nomarski interference micrograph of normal prostatic canc cells. Note the absence of large endocytotic vesicles su as were normally seen in Chinese hamster cells. Smaller vesicles are, however, seen the perinuclear zone. These appear as small white bumps. X 1500.

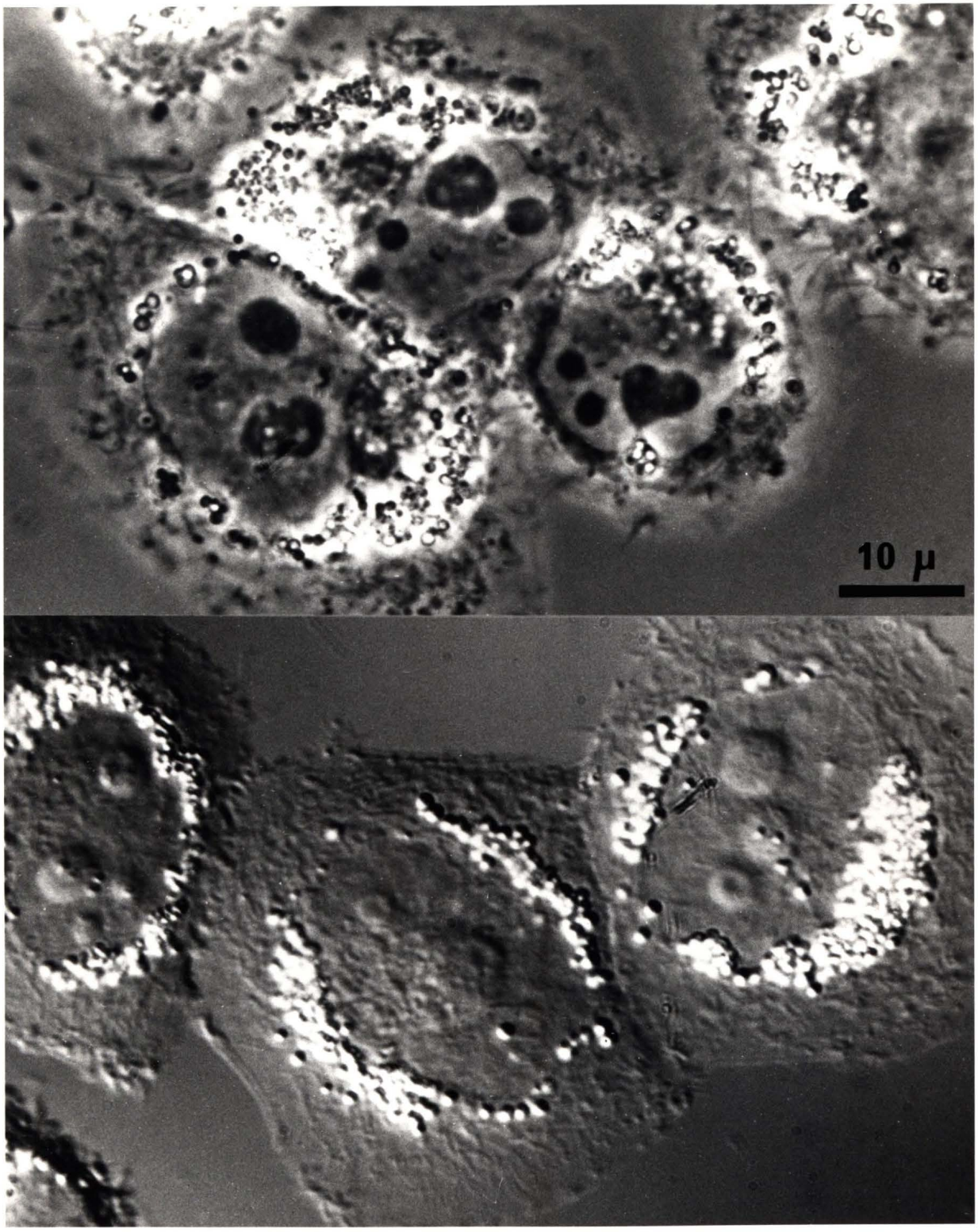
Figure 10. Nomarski interference micrograph of normal prostatic cancer cells. X 1500.


10 µ

Figures 11-12

Figure 11. Prostatic cancer cells incubated with 0.08 M sucrose for 9 hours. Note the accumulation of large numbers of phase dense vesicles in the perinuclear zone of each cell. Phase contrast. X 1900.

Figure 12. Prostatic cancer cells incubated with 0.08 M sucrose for 9 hours. The phase dense vesicles seen in the above figure appear as white bumps surrounding each nucleus when observed with Nomarski optics. X 1900.


10 μ

Morphological Effects of Dextran

Dextran T 70 (Pharmacia Fine Chemicals, Uppsala, Sweden) when present in CTM at a concentration of 10 % weight per volume, produced morphological changes in CHA and PCC cells similar to the changes observed with sucrose. However intense vesiculation did not begin so rapidly in CHA cells treated with 10 % dextran as it did with 0.08 M sucrose, nor did as many vesicles accumulate (figs. 16 through 18). In addition the vesicles were not phase lucent, as were the sucrose induced vesicles, but were grey in phase. This difference in density is also seen in the Nomarski micrographs (figs. 17 and 18) in which two classes of vesicles are seen; those which appear as depressions, which correspond to normal endocytotic vesicles, which appear phase lucent in phase contrast (fig. 16), and those which appear as elevations, which correspond to the vesicles which appear grey in phase in fig. 16.

Nearly all of the grey phase vesicles have a ring, which appears phase dense with phase contrast optics, that partially encircles the vesicles, and is presumably located on or in the membrane of the vesicles. This dark ring appears as a crust on the edge of endocytotic vesicles with Nomarski optics (figs. 17 and 18).

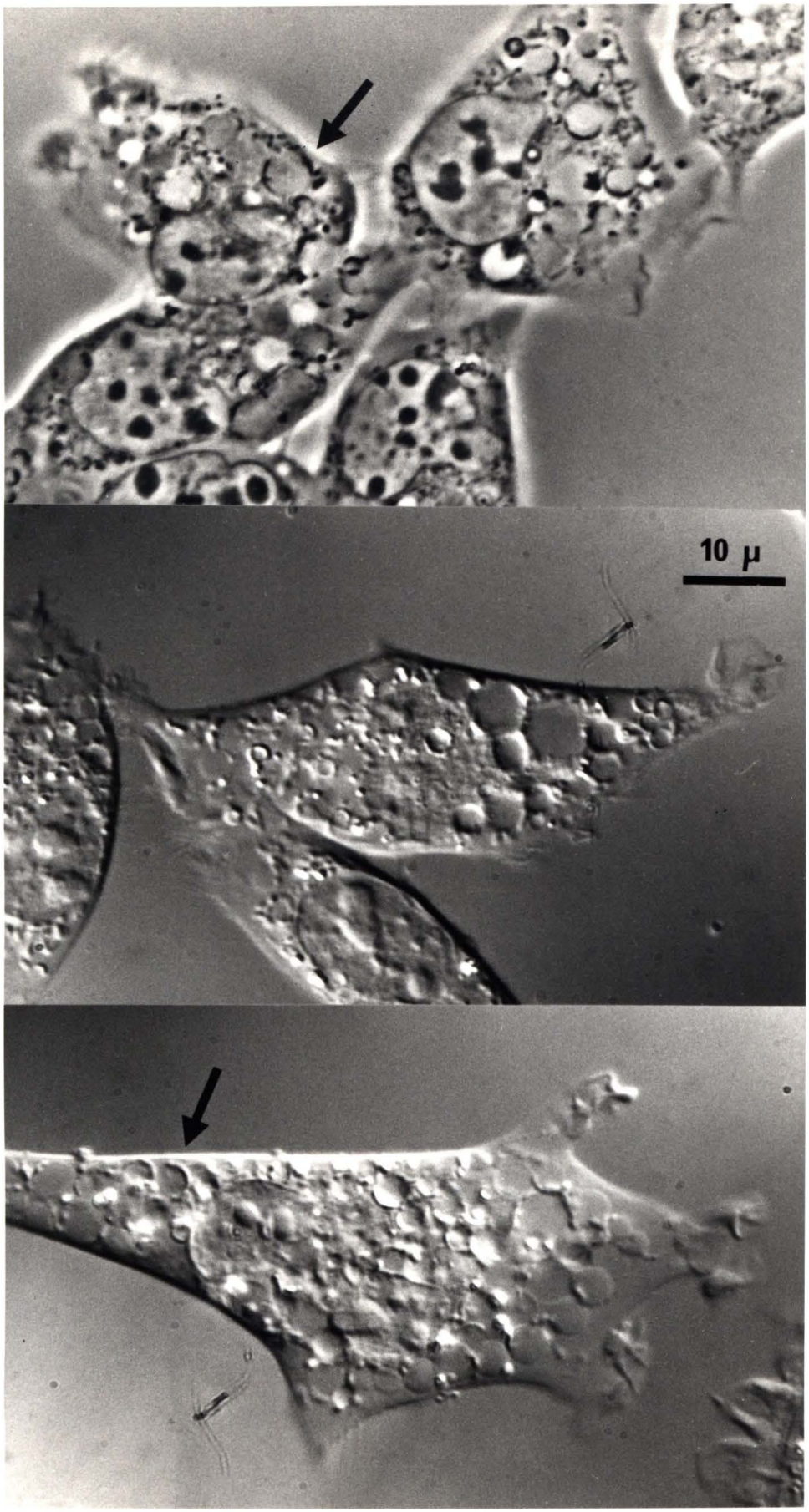
In PCC cells essentially no difference was noted between the phase dense sucrose induced vesicles and those produced by dextran (figs. 19 and 20).

Figures 16-18

Figure 16. Chinese hamster cells treated with dextran for 9 hours. Note the presence of vesicles which are grey in phase, as well as normal phase lucent endocytotic vesicles. The arrow points to a phase dense ring which partially encircles a grey phase vesicle. These phase dense rings can be seen partially surrounding nearly every grey phase vesicle. Phase contrast. X 1500.

Figure 17. Nomarski micrograph of Chinese hamster cells treated with dextran for 9 hours. Note that many of the vesicles do not appear as normal depressions, as do normal endocytotic vesicles. Some of the vesicles have a "crust" which partially encircle the vesicles, and correspond to the phase dense rings seen in fig. 16. X 1500.

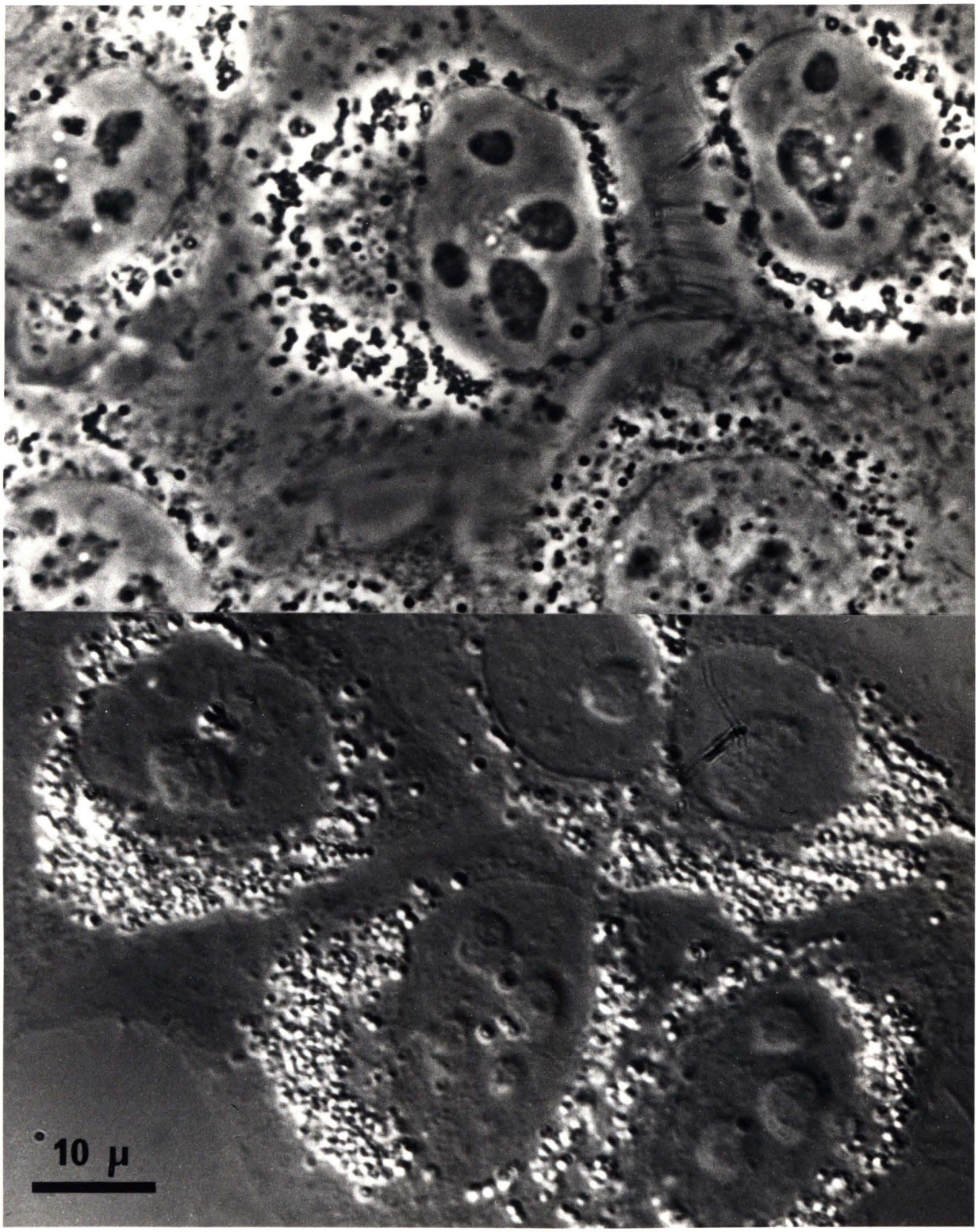
Figure 18. Nomarski micrograph of Chinese hamster cells treated with dextran for 9 hours. X 1500.


10 μ

Figures 19-20

Figure 19. Prostatic cancer cells treated with dextran for 9 hours. Note the accumulation of phase dense vesicles in the perinuclear zone. Phase contrast. X 1900.

Figure 20. Nomarski micrograph of prostatic cancer cells treated with dextran for 9 hours. Note the accumulation of vesicles, which appear as white bumps, in the perinuclear zone. X 1900.

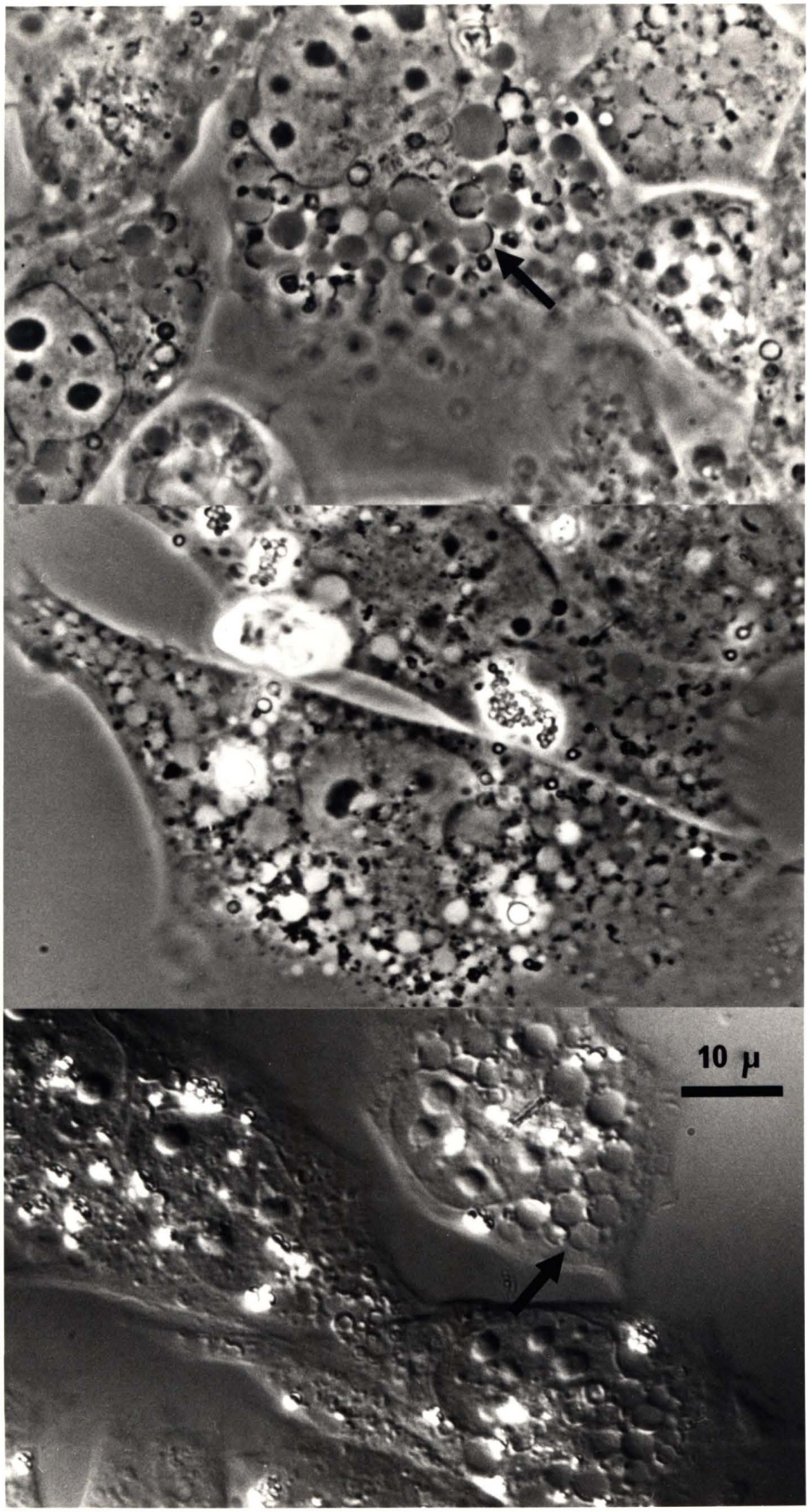

10 μ

Figures 21-23

Figure 21. Chinese hamster cells treated with PVP for 9 hours. Note the accumulation of grey phase vesicles, many of which have phase dense rings partially encircling them (arrow). Phase contrast. X 1500.

Figure 22. Chinese hamster cells treated with PVP for 9 hours. Note the presence of numerous phase dense granules in the vicinity of the endocytotic vesicles. These granules are probably lysosomes. Phase contrast. X 1500.

Figure 23. Chinese hamster cells treated with PVP for 9 hours. Arrow points to an endocytotic vesicle with a "crust" encircling it. Nomarski optics. X 1500.

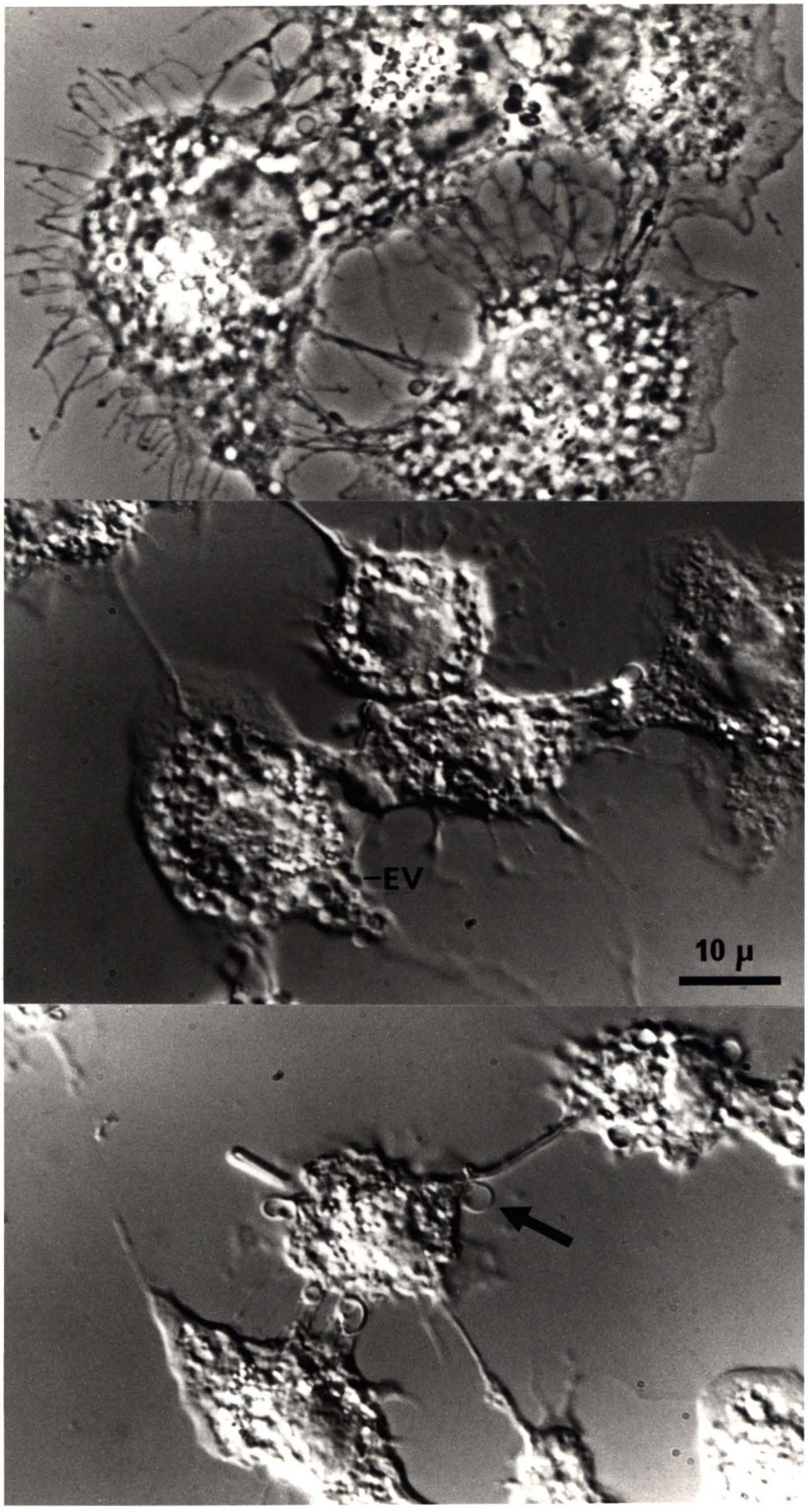

10 μ

Figures 24-26

Figure 24. Prostatic cancer cells treated with PVP for 9 hours. Note that the cytoplasm has retracted from its original well spread morphology. The dark threads radiating from each cell are membrane and cytoplasm which remained attached to the substrate and could not retract. Phase lucent endocytotic vesicles, not normally seen in PCC cells, are clearly visible. Phase contrast. X 1500.

Figure 25. Nomarski micrograph of prostatic cancer cells treated with PVP for 9 hours. Vesicles are clearly visible within the cytoplasm of these cells, which are presumably endocytotic vesicles (EV). X 1500.

Figure 26. Nomarski micrograph of prostatic cancer cells treated with PVP for 9 hours. Note the areas of peculiar membrane ruffling (arrow), which were not, however, forming endocytotic vesicles, as their appearance seems to indicate. X 1500.


EV
10 μ

Morphological Effects of PVP

PVP (Plasdone C. General Aniline and Film Corp., New York, N.Y.) when present in CTM at a concentration of 10 % weight per volume produced similar effects in CHA cells as dextran (figs. 21 through 23), except that extremely numerous phase dense granules were also noticed (fig. 22) which were not so apparent in dextran treated CHA cells.

In very marked contrast to the effect produced in CHA cells, PVP seemed to cause very deleterious effects on PCC cells. These effects, first detectable after 3 hours, reached a maximum effect between 6 to 8 hours, when the cells showed evidence of having retracted their formerly well spread cytoplasm. This effect is very dramatic, since the plasma membrane remained attached to the substrate at the same points, while the rest of the cell retracted, leaving spindle shaped, dendritic pseudopdia (figs. 24 through 26). Some cells exhibited areas of what appeared to be intense endocytosis and membrane ruffling, but long term observation of these areas revealed little movement, and no actual formation of vesicles (fig. 26).

Morphological Effects of Cytochalasin B (CB)

CHA cells were grown in a 5 ml Wild Plankton Chamber and observed and photographed with a Wild M 40 Inverted Microscope (Wild Heerbrugg Lts., Heerbrugg, Switzerland) in an incubating room maintained at 36.5°C. When cytochalasin B (Imperial Chemical Industries Ltd., Alderly Park, Cheshire, England) was added to the medium in which the cells were growing, to a final concentration of 25 μ g/ml,

Figure 27. Time-lapse series of the effect of cytochalasin B (CB) on Chinese hamster cells. The numbers in the white squares indicate the time which has elapsed since the addition of CB to the growth medium. Note that retraction of the cytoplasm from the pseudopodia begins within one minute after the addition of CB (final concentration of 25 μ g/ml). Near maximal effect is observed after only 10 minutes, at which time most of the cells have become rounded, although they are still attached to the substrate. While the plasma membrane remains attached to the substrate, the cytoplasm retracts, causing the pseudopodia to form thin dendritic processes. Phase contrast. White rectangle in the first micrograph is 40 μ long. X 400.

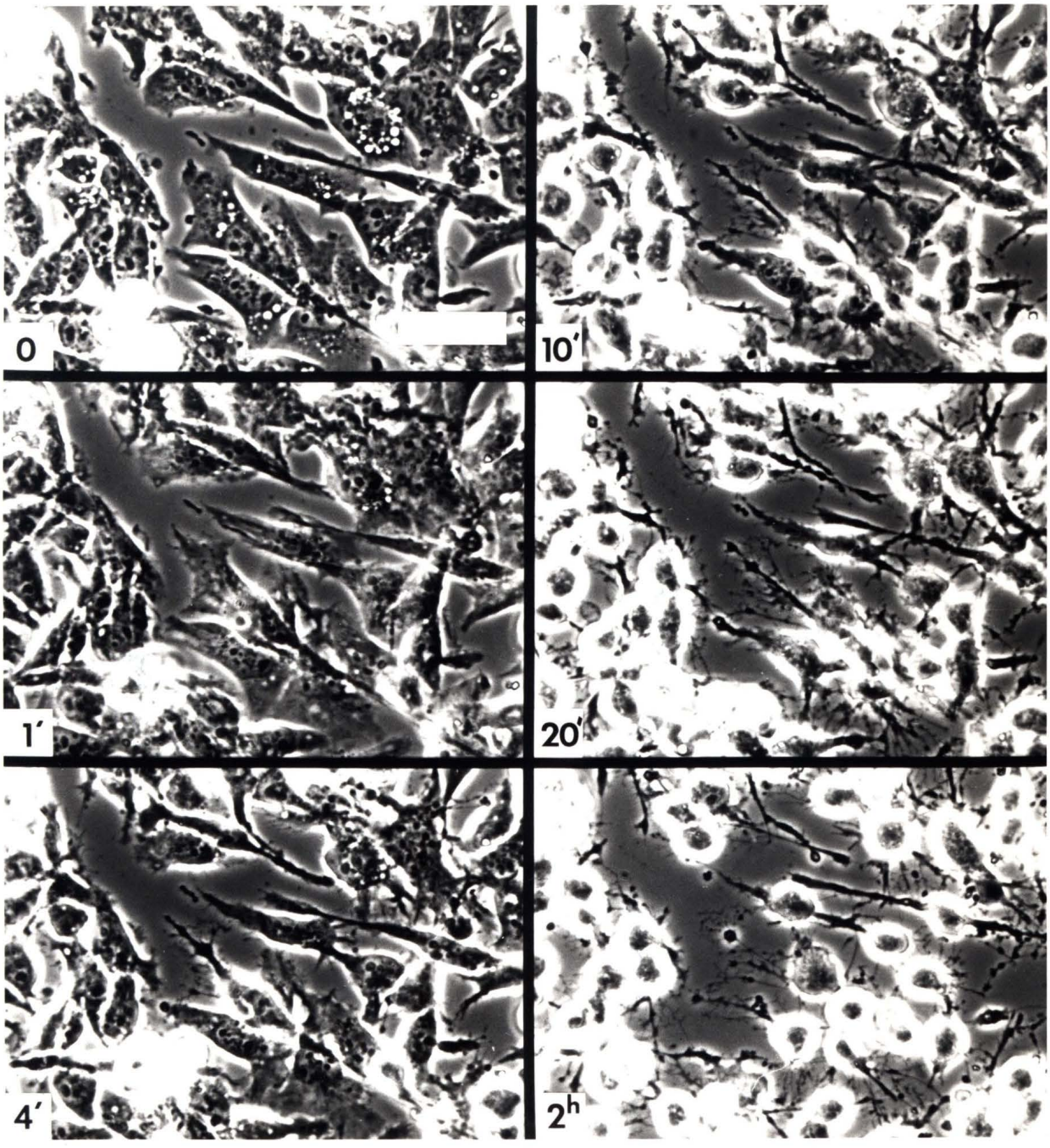
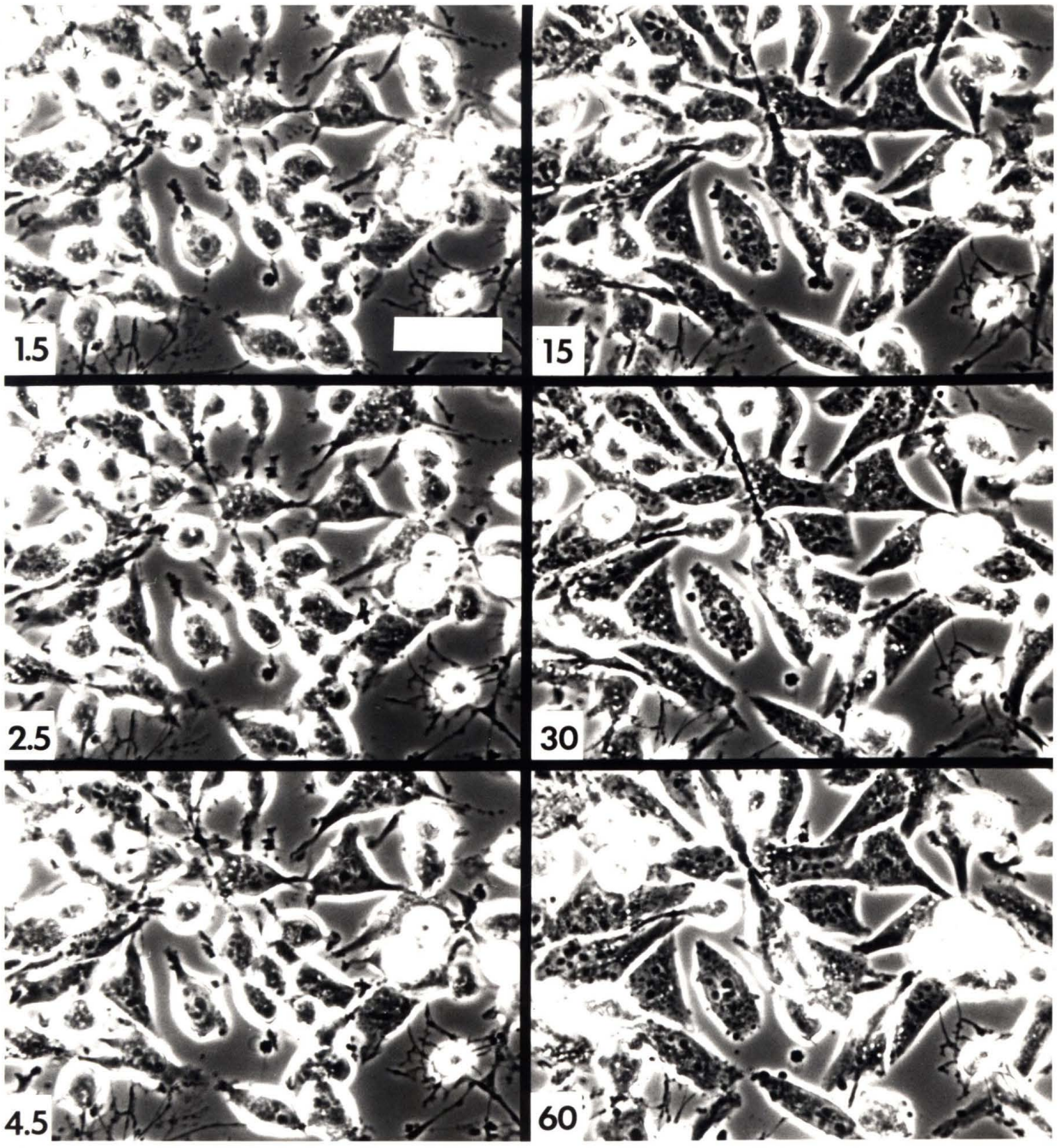

0
10′
1′
20′
4′
2h

Figure 28. Time-lapse series of the reversal of the cytochalasin B (CB) effect on Chinese hamster cells. These are cells from the same culture as shown in fig. 27, which have been incubated with 25 μ g/ml of CB for 2 hours, after which the medium was replaced with fresh, normal medium. The numbers in the white squares indicate the time in minutes which has elapsed since the replacement of the medium. Note that the cells have already begun to recover from the CB at 1.5 minutes (compare with CHA cells treated for 2 hours with CB shown in fig. 27). After 15 minutes the cells have nearly completely returned to their normal appearance. The white rectangle in the first micrograph is 40 μ long. Phase contrast. X 400.


1.5
15
2.5
30
4.5
60

retraction of the cytoplasm from the pseudopodia began within one minute (fig. 27). This continued quite rapidly until after 10 minutes nearly all the cells were rounded in appearance, but still attached to the substrate. Near maximal effect is seen after only 20 minutes.

Replacement of the CB containing medium with fresh CTM results in a rapid reversal of the effect (fig. 28), with the cells appearing nearly normal again after only 20 minutes, when nearly all the cells have appearently recovered from the treatment.

Similarly treated CHA cells, grown on coverslips and observed with high resolution optics through a Zeiss Ultraphot II, revealed the details of the effect of CB on these cells, as well as PCC cells, which were also examined by this method. Both PCC and CHA cells retracted their cytoplasm in the presence of CB, while the plasma membrane remained attached to the substrate, with the result that thin dendritic processes were formed (figs. 29 through 32). In spite of the gross morphological alterations brought about by this drug, the previously formed endocytotic vesicles normally seen in CHA cells remained unaltered. Membrane ruffling was completely inhibited in both cell lines, and the normal formation of endocytotic vesicles by CHA cells was also inhibited.

Interestingly, some of the CHA cells seemed much more resistant to the effects of CB than other cells, in that the cytoplasm did not retract as much. This may, however, only be due to differences in attachment of the cells to the substrate.

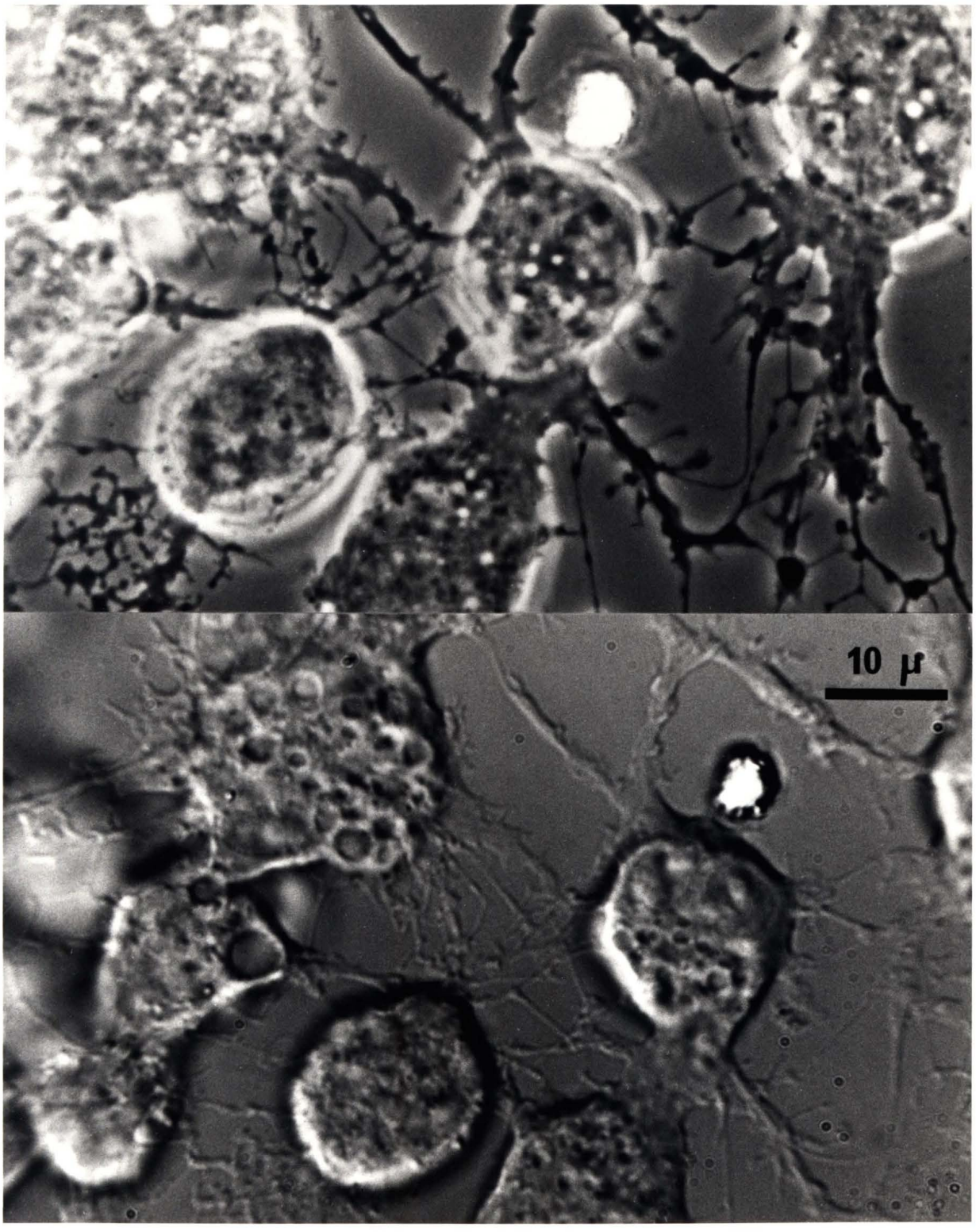
Morphological Effects of Insulin

An apparent increase in membrane ruffling was noted when CHA cells were incubated with 5.0 I.U./ml of insulin (crystalline bovine pancreas insulin, 26.4 I.U. per mg., Sigma). However, no endocytotic activity was observed in most of these areas of membrane ruffling (figs. 33-34).

Figures 29-30

Figure 29. Cytochalasin B treated Chinese hamster cells. Note that the cytoplasm has retracted causing the pseudopodia to become thin dendritic processes. Phase lucent endocytotic vesicles can be seen in the rounded up portion of the cells. The plasma membrane has remained attached to the substrate. Phase contrast. X 1900.

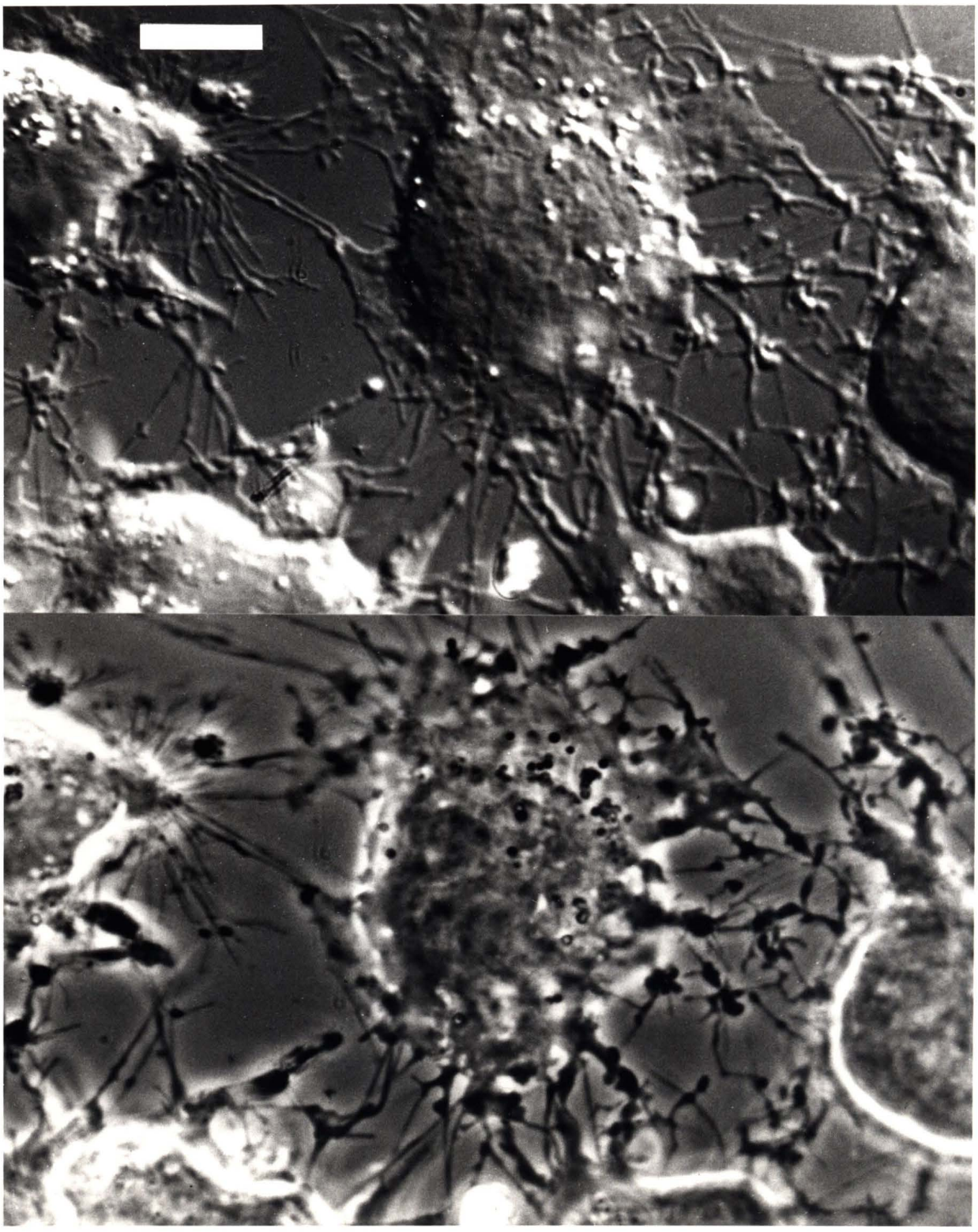
Figure 30. Nomarski micrograph of cytochalasin B treated Chinese hamster cells. The phase lucent endocytotic vesicles are more clearly visible in this micrograph than in the phase contrast micrograph of nearly the identical field shown in fig. 29. X 1900.


10 μ

Figures 31-32

Figure 31. Cytochalasin B treated prostatic cancer cells. The cells have rounded up in much the same manner as cytochalasin B treated Chinese hamster cells (figs. 29-30). Phase contrast. X 1900.

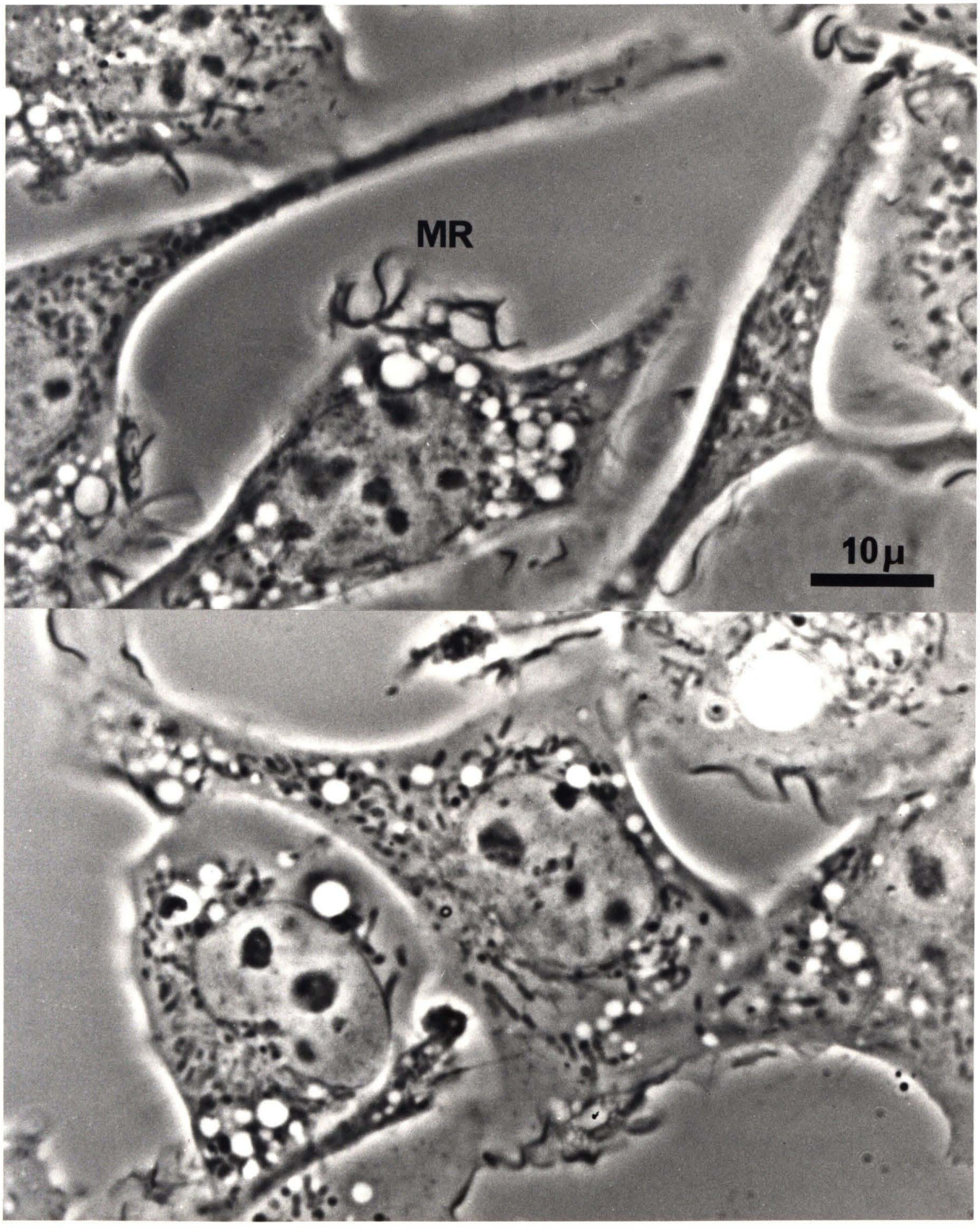
Figure 32. Nomarski micrograph of cytochalasin B treated prostatic cancer cells. Same field as in fig. 31. White rectangle is 10 μ long. X 1900.



Figures 33-34

Figure 33. Effect of insulin on Chinese hamster cells. Incubation of Chinese hamster cells with 5.0 I.U./ml of insulin for one hour resulted in an apparent increase in membrane ruffling in these cells. A typical area is seen beneath the letters MR. Endocytotic activity was absent in most of these areas of membrane ruffling. Phase contrast. X 1900.

Figure 34. Effect of insulin on Chinese hamster cells. Areas of membrane ruffling induced by insulin can be seen on these cells. Phase contrast. X 1900.


MR
10 µ

Endocytotic Uptake of Radioisotopically Labelled Compounds

General Characterisation of the System

To establish that the radioactivity remaining in the sample vials after incubation with sucrose-^{3}H, followed by washing with saline, was attributable to a metabolic process, and not merely to adsorption of the sucrose-^{3}H to either the cells or the walls of the vial itself, two preliminary experiments were performed. The first (fig. 35) shows the rate of incorporation of sucrose-^{3}H by CHA cells at 22° and 37°C. The second experiment (fig. 36) demonstrates the inhibitory effect of 10^{-2} M iodoacetate on the uptake of sucrose-^{3}H. Both experiments clearly established both that the uptake of sucrose-^{3}H was indeed a metabolically dependent process, which for reasons discussed earlier must occur by endocytosis.

Several experiments were preformed using the inhibitor of endocytosis, cytochalasin B (CB) on both CHA and PCC cells. The results are shown in figs. 37 through 39. Clearly the uptake of sucrose-^{3}H was inhibited by CB, with the effect being very pronounced with a concentration as low as 5 μ g/ml, the rate of uptake being only 52% that of the control, untreated cells. Interestingly, the PCC cells did not respond as well to CB as did the CHA cells, as even 20 μ g/ml of CB allowed the uptake of sucrose-^{3}H to occur at 77% of the rate of the control, untreated cells. With CHA cells, the rate of uptake of sucrose-^{3}H was reduced to 36% of the control

Figures 35 and 36

Figure 35. The effect of temperature on the uptake of sucrose-^{3}H by Chinese hamster cells. Ordinate indicates the counts per minute of radioactivity remaining in the sample vials after washing with saline. Abscissa indicates the number of minutes that the sample vials were incubated prior to washing with saline. Concentration of sucrose-^{3}H in the medium was 3.92×10^{-7} M 0.666 µCi/ml. Mean cell count was 173,000 ± 20,000 cells per vial. Each point on the graph represents the mean of three samples. The lines drawn represent the least squares linear regression lines for 37° and 22°C. ▲= 37 C, ●= 22 C.

Slopes of the linear regression lines:

22° C = 0.78 ± 0.13 CPM/minute
37° C = 1.50 ± 0.30 CPM/minute

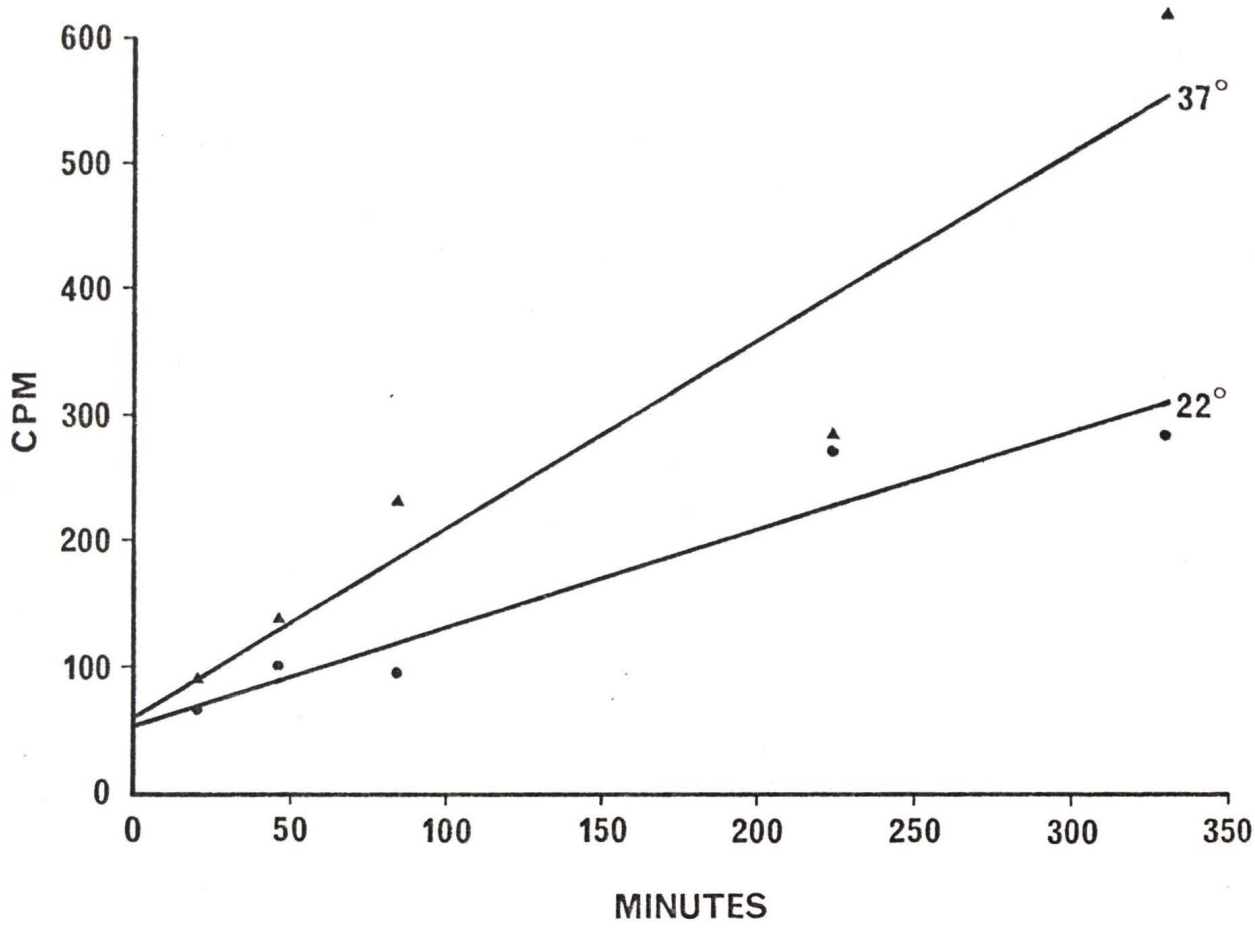
Rates are significantly different ($P < 0.05$)

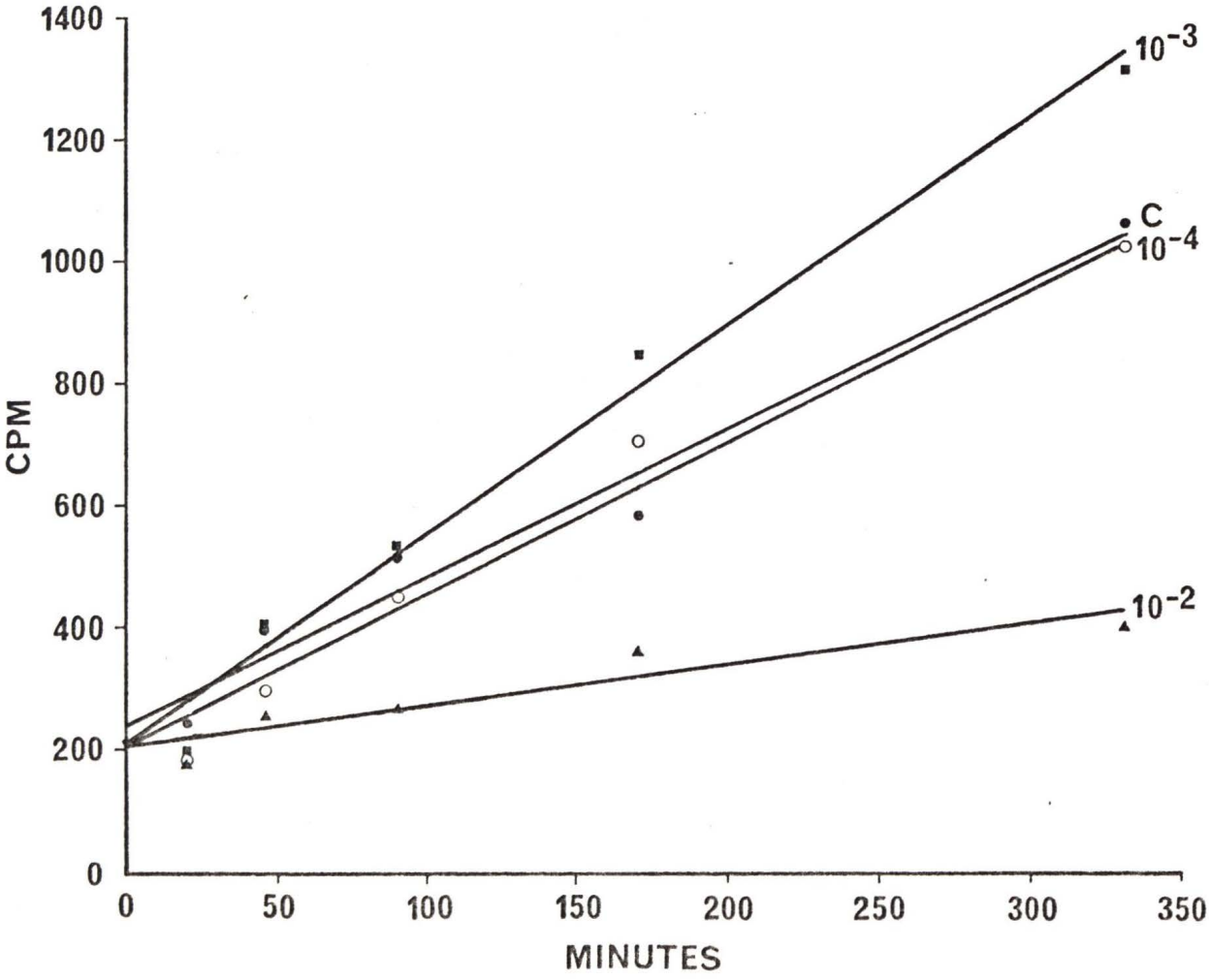
Figure 36. The effect of iodoacetate on the uptake of sucrose-^{3}H by Chinese hamster cells. Numbers at the end of each regression line indicate the concentration of iodoacetate in the medium in moles per litre. C = control rate of uptake in the absence of iodoacetate. Each point on the graph represents the mean of 3-4 samples. Sucrose-^{3}H concentration = 3.92×10^{-7} M 0.666 µCi/ml. Mean cell count = 486,000 ± 32,000 cells per vial. ● = Control samples, o = 10^{-4} M iodoacetate, ■ = 10^{-3} M, ▲ = 10^{-2} M.

Slopes of the linear regression lines:

C = 2.43 ± 0.26 CPM/minute
10^{-4} = 2.61 ± 0.18 CPM/minute
10^{-3} = 3.47 ± 0.25 CPM/minute
10^{-2} = 0.68 ± 0.12 CPM/minute

10^{-3} rate is significantly higher than control ($P < 0.05$).


600
500
400
300
200
100
0
CPM
37°
22°
0
50
100
150
200
250
300
350
MINUTES


1400
1200
1000
800
600
400
200
0
CPM
10-3
C
10-4
10-2
0
50
100
150
200
250
300
350
MINUTES

Figures 37 and 38

Figure 37. Inhibition of the uptake of sucrose-^{3}H by Chinese hamster with cytochalasin B (CB). Sucrose-^{3}H concentration = 3.92 X 10^{-7} M, 0.666 µCi/ml. Mean count = 943,000 ± 68,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. ● = control samples, ▲ = 5 µg/ml CB, ■ = 10 µg/ml CB, o = 20 µg/ml CB

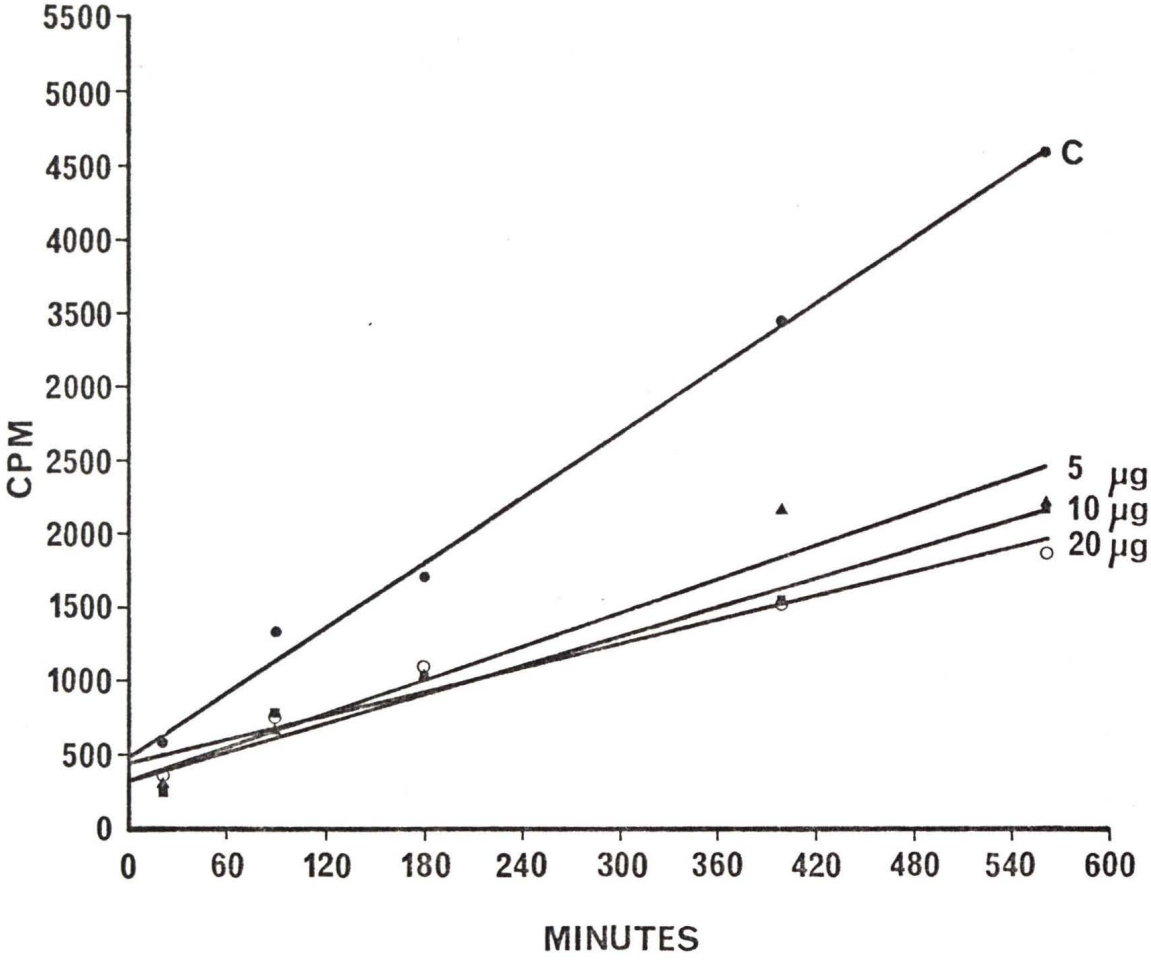
Slopes of the linear regression lines:

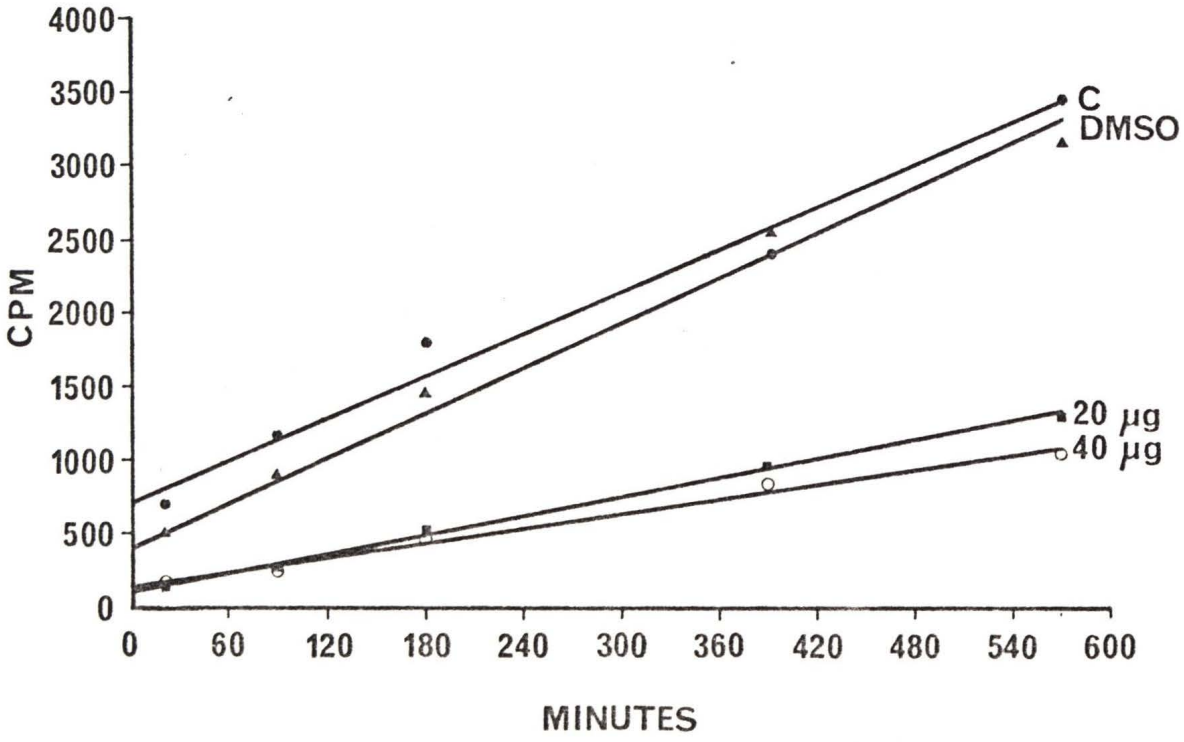
C (control) = 7.33 ± 0.28 CPM/minute
5 µg/ml CB = 3.78 ± 0.53 CPM/minute
10 µg/ml CB = 3.26 ± 0.34 CPM/minute
20 µg/ml CB = 2.67 ± 0.28 CPM/minute

Figure 38. Inhibition of the uptake of sucrose-^{3}H by Chinese hamster with cytochalasin B (CB). Sucrose-^{3}H concentration = 3.92 X 10^{-7} M, 0.666 µCi/ml. Mean cell count = 1,215,000 ± 110,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. The line labelled DMSO represents the rate of sucrose-^{3}H uptake in the presence of 0.4% by volume of dimethylsulfoxide (DMSO). This is the same concentration of DMSO added to the vials containing CB, since CB was introduced in a DMSO solution, ▲ = DMSO, ■ = 20 µg/ml, CB, o = 40 µg/ml CB. ● = C (control).

Slopes of the linear regression lines:

C (control) = 4.74 ± 0.38 CPM/minute
DMSO = 5.09 ± 0.36 CPM/minute
20 µg/ml CB = 2.12 ± 0.07 CPM/minute
40 µg/ml CB = 1.65 ± 0.11 CPM/minute


5500
5000
4500
4000
3500
2000
2500
2000
1500
1000
500
0
CPM
0
60
120
180
240
300
360
420
480
540
600
MINUTES
C
5 µg
10 µg
20 µg


4000
3500
3000
2500
2000
1500
1000
500
0
CPM
0
60
120
180
240
300
360
420
480
540
600
MINUTES
C
DMSO
20 µg
40 µg

Figures 39 and 40

Figure 39. Inhibition of the uptake of sucrose-^{3}H by prostatic cells in the presence of cytochalasin B (CB). Sucrose-^{3}H concentration = 4.61 X 10^{-7} M, 0.784 µCi/ml. Mean cell count = 1,190,000 ± 172,000 cells per vial. Each point represents the mean of 3-5 samples. ● = control samples, ▲= 5 µg/ml CB, ■= 10 µg/ml CB, o = 20 µg/ml CB.

Slopes of the regression lines:

C (control) = 4.46 ± 0.33 CPM/minute
5 µg/ml CB = 2.95 ± 0.55 CPM/minute
10 µg/ml CB = 3.35 ± 0.13 CPM/minute
20 µg/ml CB = 3.38 ± 0.11 CPM/minute

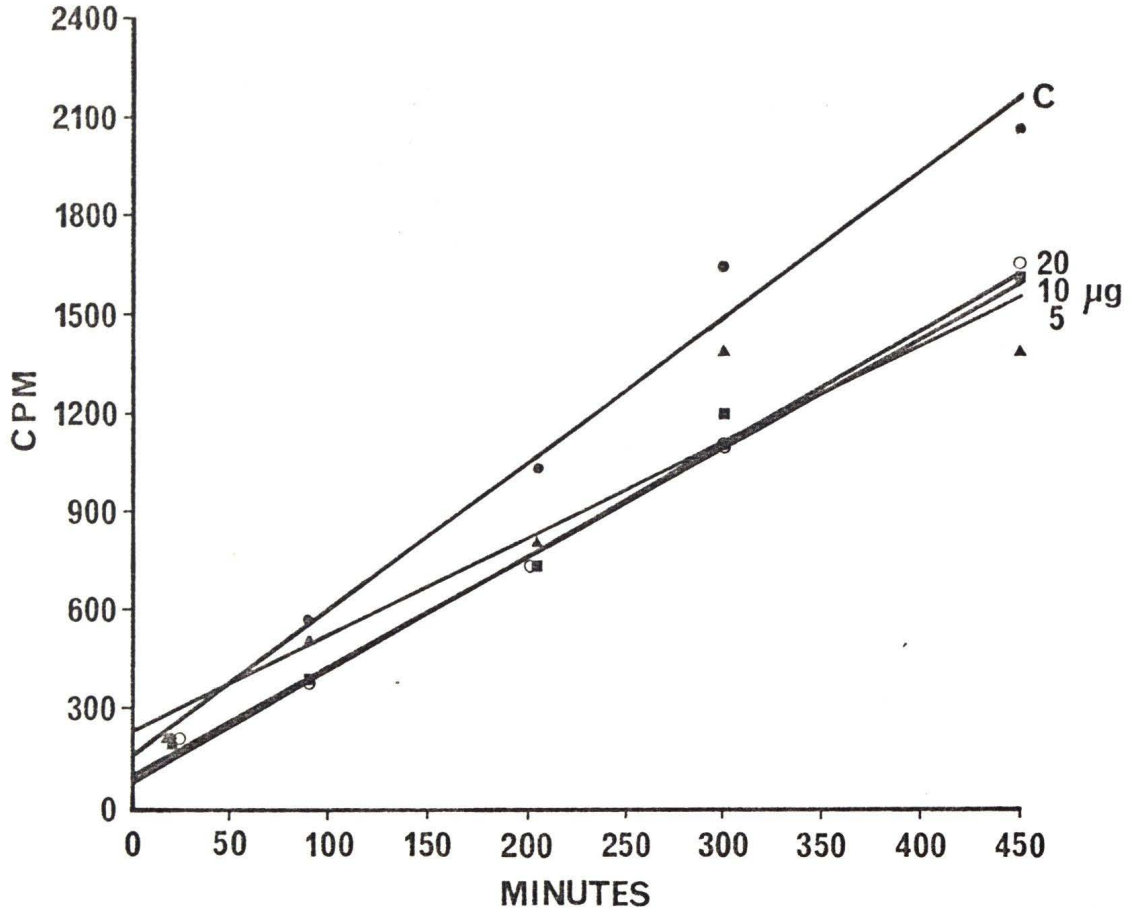
5 µg/ml CB rate is significantly lower than control ($P < 0.05$).

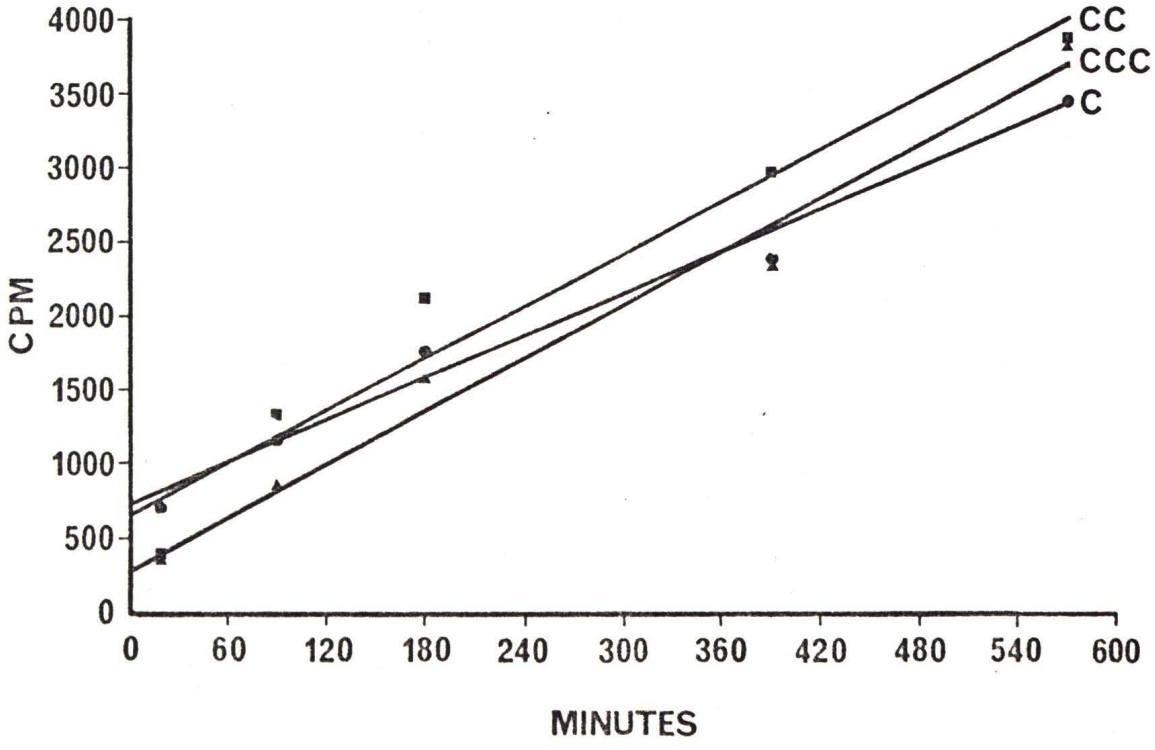
Figure 40. The effect of the uptake of 3.92 X 10^{-7} M sucrose-^{3}H (0.666 µ Ci/ml) in the presence of 0.08 M cold sucrose (CC) and followed by a 5 minute incubation with 0.08 M cold sucrose in the absence of labelled sucrose (CCC), as compared to the uptake of labelled sucrose alone, without further treatment (C). Experimental conditions were the same as in fig. 38. Each point on the graph represents the mean of 3-5 samples. ● = control samples (C), ■= CC samples, ▲ = CCC.

Slopes of the regression lines:

C = 4.74 ± 0.38 CPM/minute
CC = 5.88 ± 0.76 CPM/minute
CCC = 5.98 ± 0.50 CPM/minute

Rates are not significantly different.


2400
2100
1800
1500
1200
900
600
300
0
CPM
0
50
100
150
200
250
300
350
400
450
MINUTES
C
20
10 µg
5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3500
3000
2500
2000
1500
1000
500
0
CPM
0
60
120
180
240
300
360
420
480
540
600
MINUTES
CC
CCC
C

rate in the presence of 20 µg/ml. Strangely, with CHA cells the rate of uptake of sucrose-^{3}H was increasingly inhibited by increasing concentrations of CB, whereas with PCC cells maximum inhibition occurred at 5 µg/ml, with greater concentrations having no further significant effect.

While the previously described experiments demonstrated that the uptake of sucrose-^{3}H was clearly a metabolic process, dependent on endocytosis, it seemed desirable to further characterize the kinetics of the process. To this end the experiment shown in fig. 40 was performed. This experiment was actually another part of the experiment shown in fig. 38. The regression line labelled C represents the samples incubated only with labelled sucrose, while the line labelled CC represents the samples incubated with labelled sucrose and 0.08 M cold sucrose. The line labelled CCC represents the samples which were incubated with both labelled sucrose and 0.08 M cold sucrose, followed by a cold chase with 0.08 M cold sucrose for 5 minutes at 36.5°C. Since no significant change was observed between the rate of sucrose-^{3}H uptake in the samples containing sucrose-^{3}H alone and those containing sucrose-^{3}H and cold sucrose, it is convincingly demonstrated that sucrose in a concentration of 0.08 M does not increase the rate of sucrose-^{3}H uptake at a concentration of 3.92×10^{-7} M, and is therefore probably not itself an inducer of endocytosis, as was considered by Munro (58) to be a possible

explanation for the sucrose induced vesiculation observed with CHA cells both by Munro and myself.

In addition, this experiment demonstrated that sucrose-^{3}H can enter cells only passively, as a result of its presence in solution in the medium, for if there were an active transport system capable of selectively transporting sucrose across the plasma membrane, independently of endocytosis, then the rate of sucrose-^{3}H uptake in the presence of 0.08 M cold sucrose would be very much less than the rate observed with sucrose-^{3}H alone, such was not, however, the case. Any possible binding of sucrose-^{3}H to the membrane of the endocytotic vesicles was also ruled out by this experiment, since any such binding would have resulted in a decreased rate of endocytosis in the presence of cold sucrose, as the cold sucrose would be competing for binding sites with the sucrose-^{3}H. Futhermore, since sample vials treated with a 0.08 M sucrose cold chase, following incubation with sucrose-^{3}H in the presence of 0.08 M cold sucrose, also did not show any decrease in the rate of sucrose-^{3}H uptake, it can be concluded with certainty that the sucrose-^{3}H remaining in the sample vials following washing with saline, represents sucrose-^{3}H which has been more or less permanently taken up by the cells, and which is not merely loosely bound in some way to the plasma membrane of the cells or to the walls of the scintillation vials themselves. This experiment clearly established

the usefulness of sucrose-^{3}H as a quantitative marker of endocytosis.

Effect of the Presence of Serum and PVP on Endocytosis

Both serum (19) (81) and PVP (80) have been shown to be inducers of endocytosis on the basis of morphological observations in other cell systems. This is clearly not the case with CHA cells, as the results shown in fig. 41 indicate. Neither serum at a concentration of 10% volume per volume, nor PVP at a concentration of 10% weight per volume, either with or without serum, had any significant effect on the rate of endocytosis. Serum also had no significant effect on endocytosis in the experiment shown in fig. 43.

Effect of Dextran on Endocytosis

The effect of dextran on the uptake of sucrose-^{3}H was investigated, with the results shown in figs. 42 and 43. 10% weight per volume of dextran (T 70, Pharmacia) increased the rate (not statistically significantly, however) of endocytosis by 48% (fig. 42) whereas lower concentrations had no significant effect. 7.5% dextran (fig. 43) actually decreased the rate of endocytosis somewhat, both in the presence of serum (88% of control; not however statistically significant) and in the absence of serum (68% of control; statistically significant, $P < 0.02$).

Figures 41 and 42

Figure 41. The effect of serum and PVP on the uptake on sucrose-^{3}H. C = normal CTM, PS = 10% PVP in normal CTM, PNS = 10% PVP in serum free CTM, CNS = serum free CTM, CNSS = serum free CTM plus 0.08 M cold sucrose. 3.92×10^{-7} M sucrose-^{3}H (0.666 µCi/ml). Mean cell count = 1,860,000 ± 197,000 cells per vial. Each point on the graph represents 3-5 samples. ● = C samples, ▲ = CNS samples, ■ = CNSS samples, o = PS samples, Δ = PNS samples.

Slopes of the regression lines:

C = 4.95 ± 0.46 CPM/minute
CNS = 4.66 ± 1.03 CPM/minute
CNSS = 3.89 ± 0.44 CPM/minute
PS = 3.95 ± 0.44 CPM/minute
PNS = 4.24 ± 0.48 CPM/minute

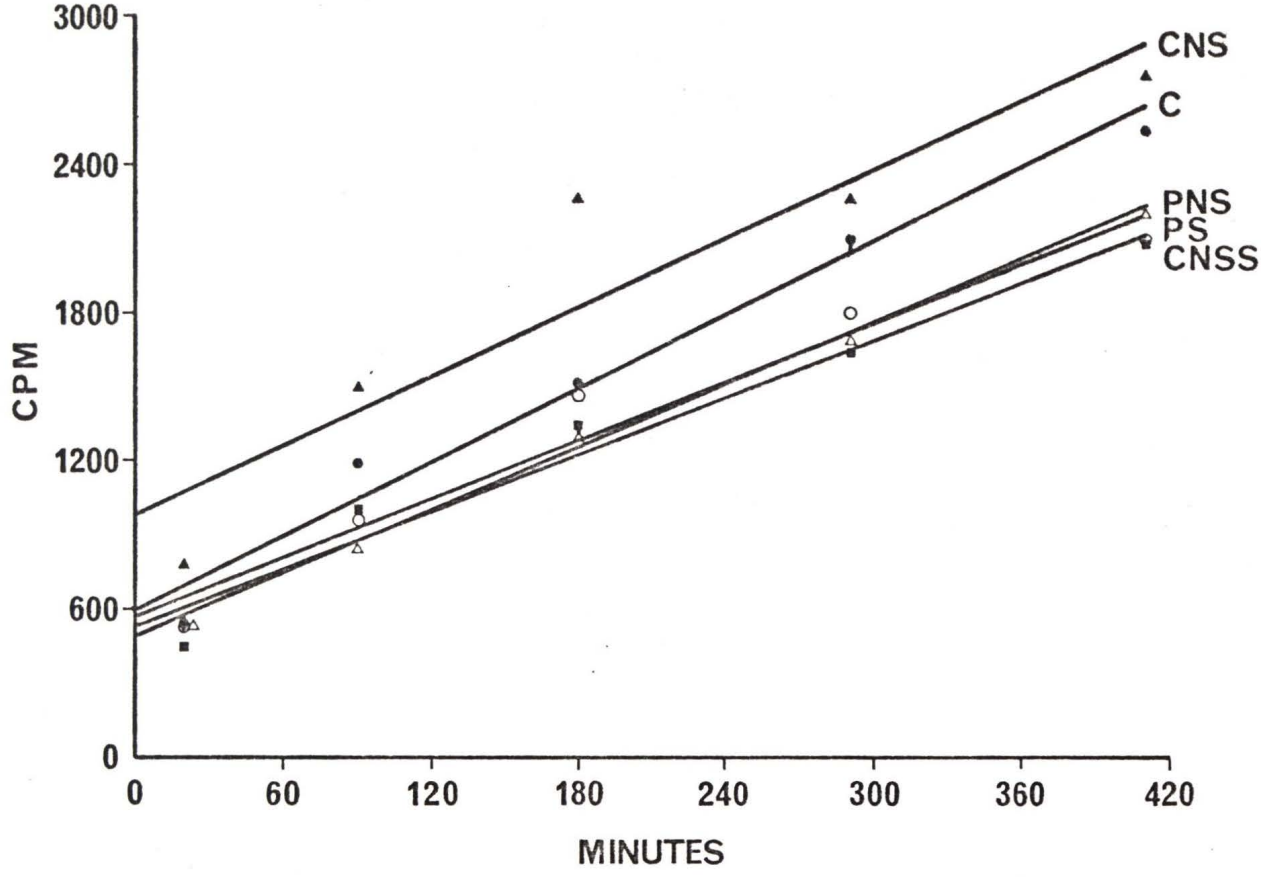
Rates are not significantly different.

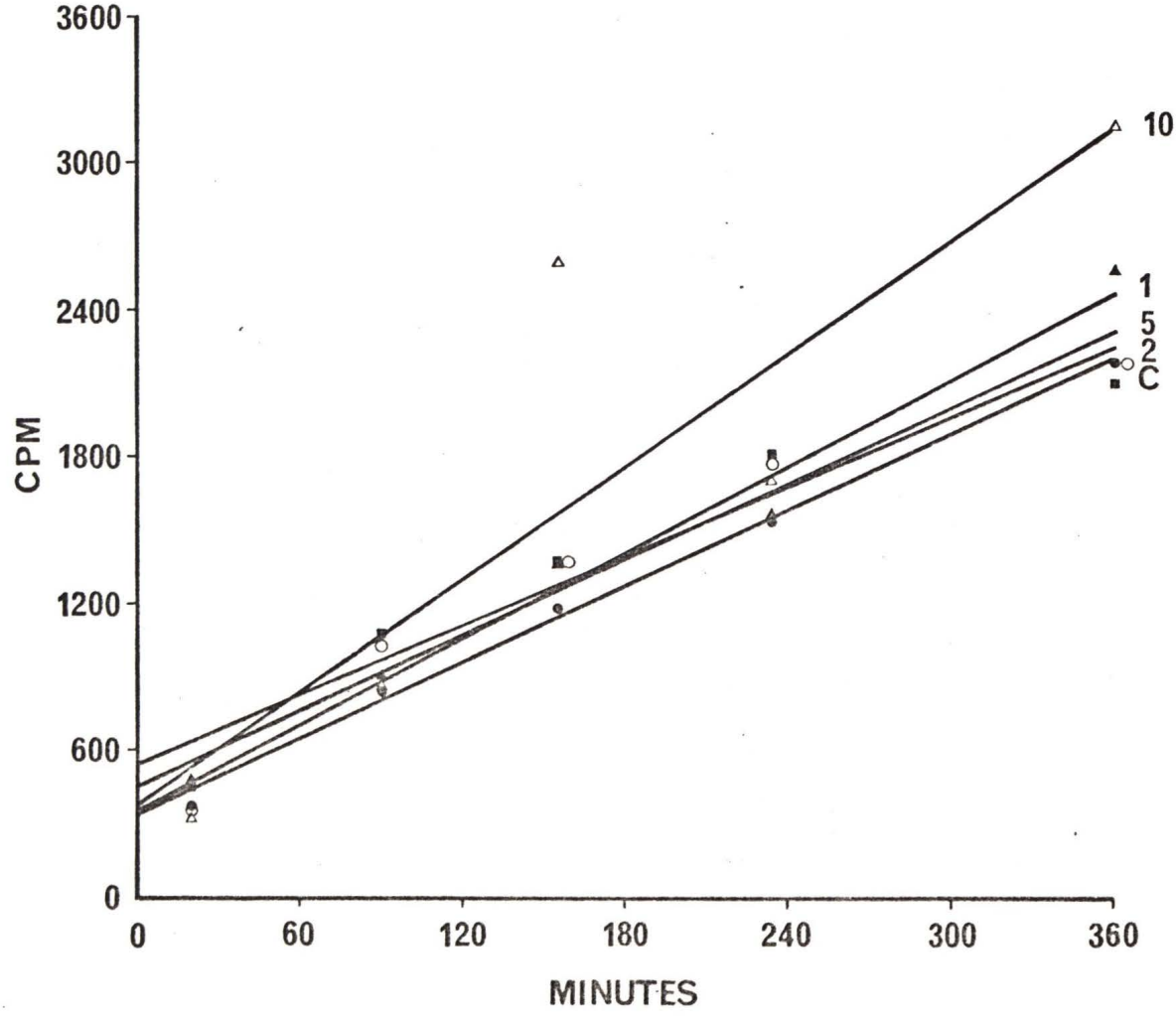
Figure 42. The effect of dextran on the uptake of sucrose-^{3}H. Figures after each regression line indicate the concentration of dextran, weight per volume, in CTM. 3.92×10^{-7} M sucrose-^{3}H (0.666 µCi/ml). Mean cell count = 1,985,000 ± 102,000 cells per vial. Each point of the graph represents 3-5 samples. ● = C samples (no cold dextran), ▲ = 1% dextran samples, ■ = 2% dextran samples, o = 5% dextran samples, Δ = 10% dextran samples.

Slopes of the regression lines:

C = 5.20 ± 0.19 CPM/minute
1% = 5.89 ± 0.47 CPM/minute
2% = 4.72 ± 0.68 CPM/minute
5% = 5.15 ± 0.67 CPM/minute
10% = 7.69 ± 2.54 CPM/minute

Rates are not significantly different.


3000
2400
1800
1200
600
0
CPM
0
60
120
180
240
300
360
420
MINUTES
CNS
C
PNS
PS
CNSS


3600
3000
2400
1800
1200
600
0
CPM
0
60
120
180
240
300
360
MINUTES
10
1
5
2
C

Figures 43 and 44

Figure 43. The effect of dextran on the uptake of sucrose-^{3}H both in the presence of and in the absence of serum. C = CTM, CNS = CTM without serum, 7.5 = 7.5% dextran in CTM, 7.5 NS = 7.5% dextran in CTM without serum. 3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 1,720,000 ± 130,000 cells per vial. Each point on the graph represents 3-5 samples. ● = C samples, ■ = CNS samples, ▲ = 7.5 samples, o = 7.5 NS samples.

Slopes of the regression lines:

C = 7.12 ± 0.35 CPM/minute
CNS = 5.32 ± 0.71 CPM/minute
7.5 = 6.23 ± 0.26 CPM/minute
7.5 NS = 4.86 ± 0.57 CPM/minute

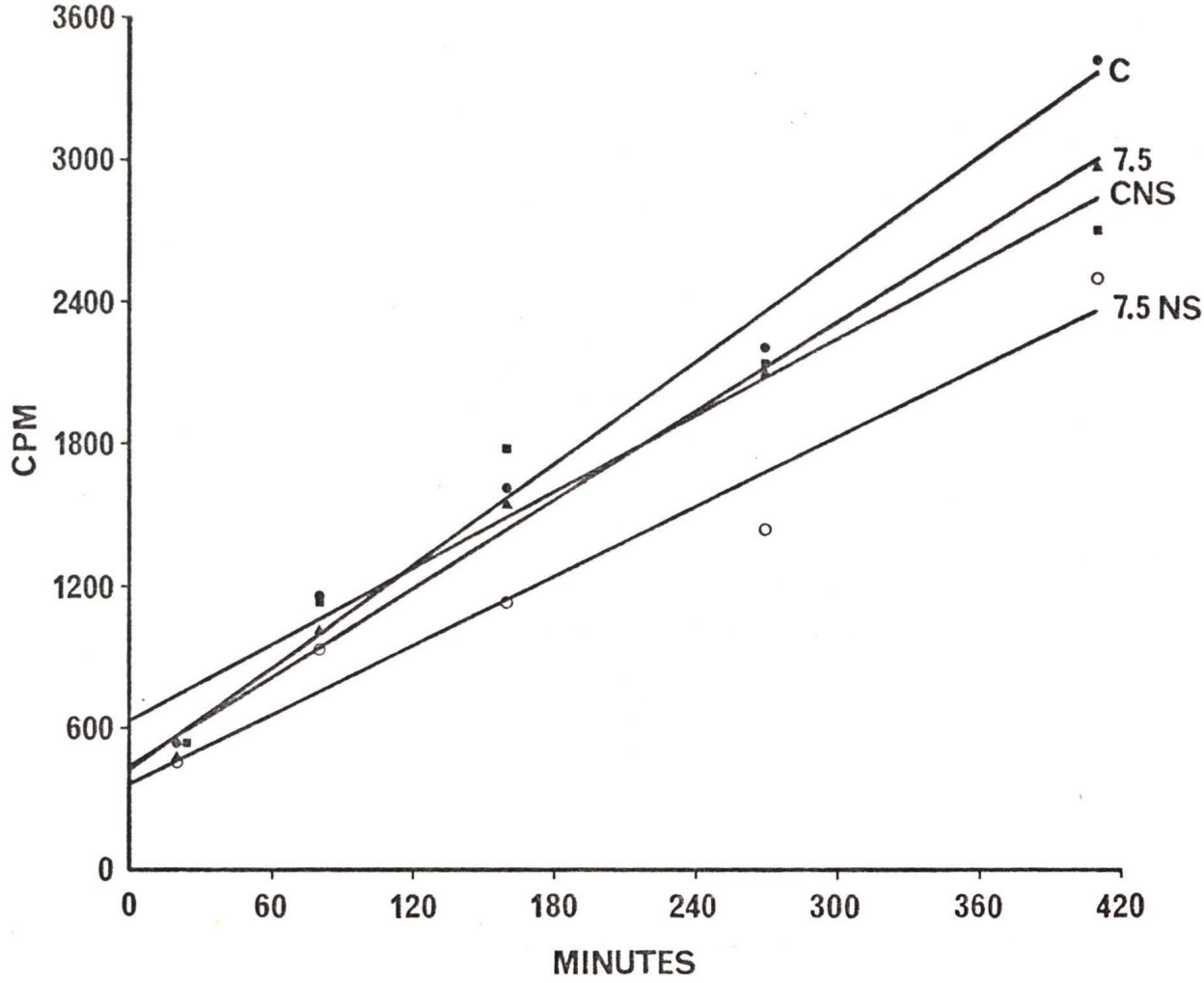
C, CNS, and 7.5 rates are not significantly different. 7.5 NS significantly less than C ($P < 0.02$).

Figure 44. The uptake of dextran-^{3}H in the presence of cold dextran. Numbers after each line indicate the percent in weight per volume of cold dextran in the CTM. Dextran-^{3}H concentration = 0.762×10^{-6} g/ml, 0.309 µCi/ml. Mean cell count = 1,912,500 ± 117,700 cells per vial. Each point of the graph represents 3-5 samples. ● = 0% dextran samples, ▲ = 1% samples, ■ = 2% samples, o = 5% samples, △ 10% samples.

Slopes of the regression lines:

0% = 17.5 ± 2.49 CPM/hour
1% = 18.6 ± 1.43 CPM/hour
2% = 18.6 ± 2.25 CPM/hour
5% = 23.0 ± 2.87 CPM/hour
10% = 37.6 ± 6.27 CPM/hour

0%, 1%, 2% and 5% rates not significantly different. 10% is significantly greater than 0% ($P < 0.005$).


3600
3000
2400
1800
1200
600
0
CPM
0
60
120
180
240
300
360
420
MINUTES
C
7.5
CNS
7.5 NS

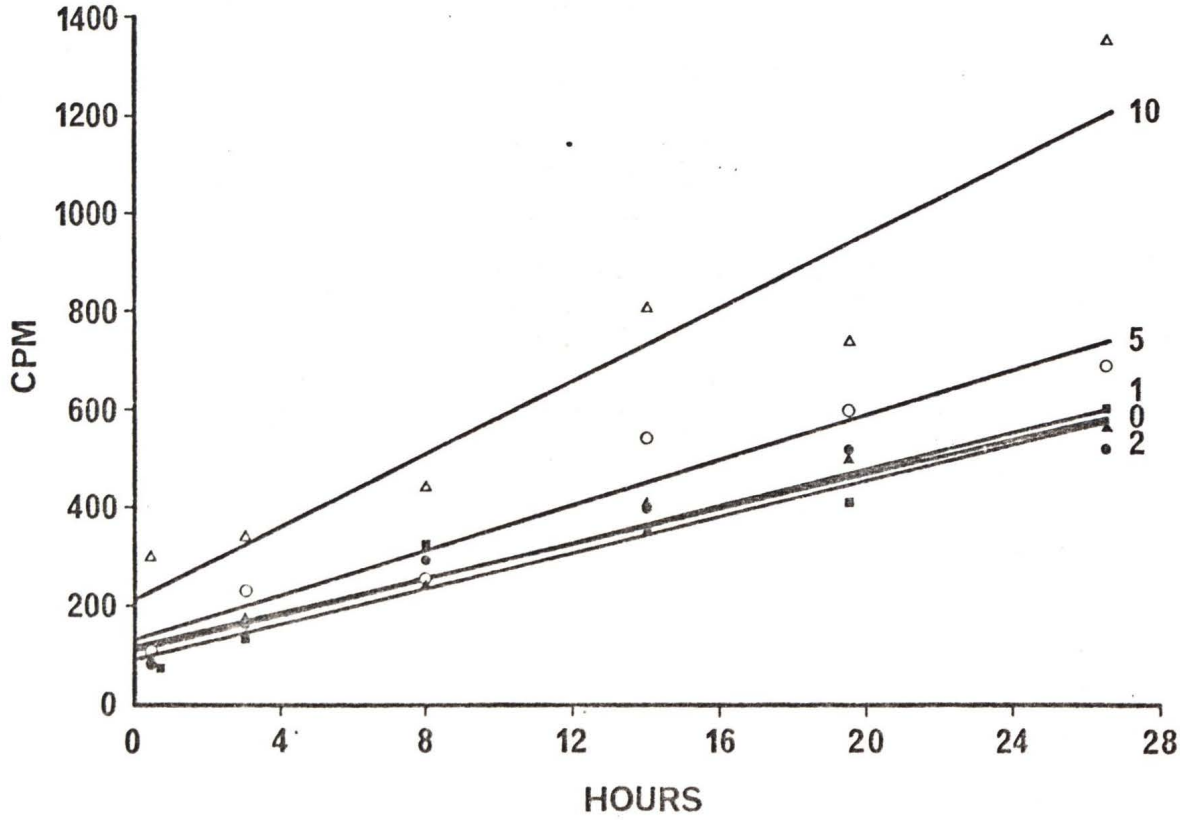

1400
1200
1000
800
600
400
200
0
CPM
0
4
8
12
16
20
24
28
HOURS
10
5
1
0
2

Figure 44 shows the results of an experiment in which the uptake of dextran-^{3}H was measured in the presence of cold dextran (T 70, Pharmacia). Some increase in the rate of endocytosis of dextran-^{3}H in the presence of 5% weight per volume of cold dextran (131% of control, 0% cold dextran rate; not however statistically significant) and 10% cold dextran (215% of control; significant, $P < 0.005$) can be seen. However, the fact that the addition of cold dextran does not decrease the rate of dextran-^{3}H uptake, clearly indicates that dextran does not bind to the plasma membrane of these cells, otherwise the rate of dextran-^{3}H uptake would be less in the presence of cold dextran, due to competition for binding sites.

Uptake of PVP-^{14}C

In order to quantitate the uptake of PVP-^{14}C, which can enter cells only by endocytosis, the experiment shown in figs. 45 and 46 was performed. Very sporadic results were obtained, both with dialysed and undialysed PVP-^{14}C, and both in the presence of and in the absence of serum. These results suggested an unusual phenomenon.

To determine whether this effect was due to an abnormally and sporadically altered rate of endocytosis in the presence of this concentration of PVP (.021 and 0.51 mg/ml, as opposed to 100 mg/ml in the previous experiment shown in fig. 41, which did not alter the rate of endocytosis) or was perhaps due to adsorption to the glass walls of the vials, the double labelling experiment shown in fig. 47 was performed.

Figures 45 and 46

Figure 45. Uptake of PVP-^{14}C. UD = undialysed PVP-^{14}C in CTM, UD NS = undialysed PVP-^{14}C in CTM without serum. Concentration of K 17.8 PVP-^{14}C = 0.021 mg/ml, 0.20 µCi/ml. Mean cell count = 1,177,000 ± 52,500 cells per vial. Each point on the graph represents 3-5 samples. ▲...▲ = UD, ●---● = UD NS.

Slopes of regression lines:

UD = 1.69 ± 1.87 CPM/minute
UD NS = 3.27 ± 1.85 CPM/minute

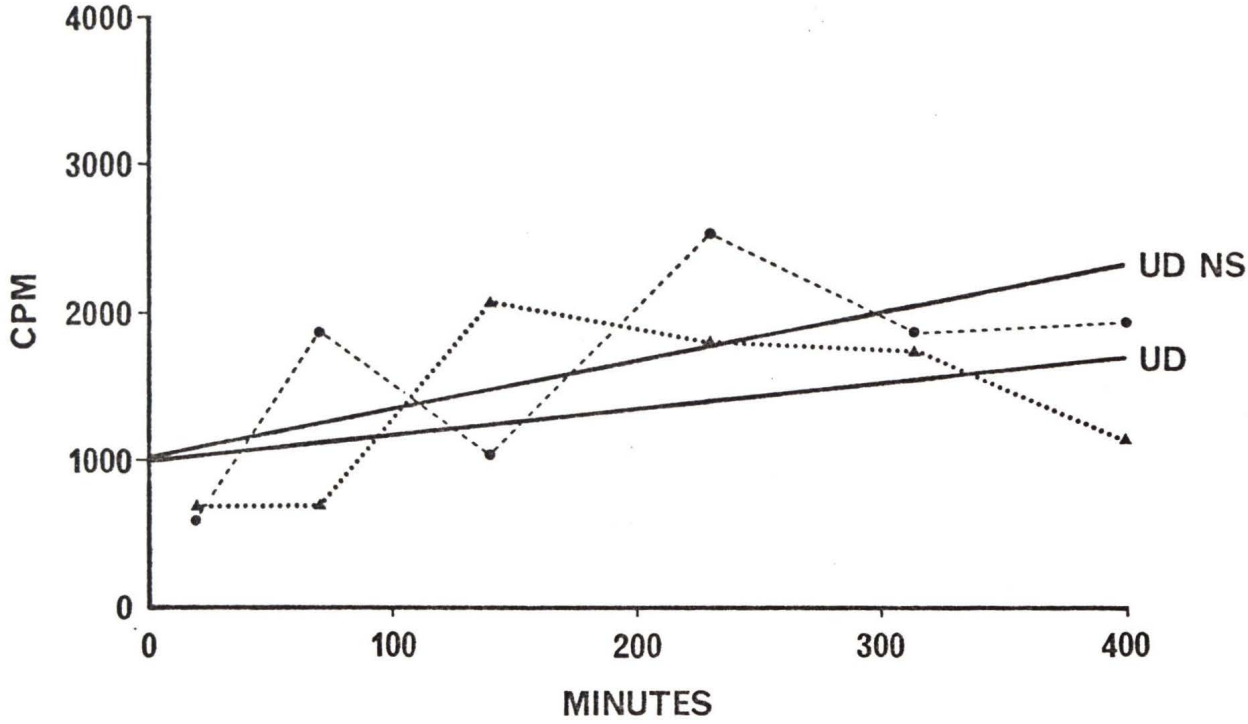
Regression lines are not significantly different.

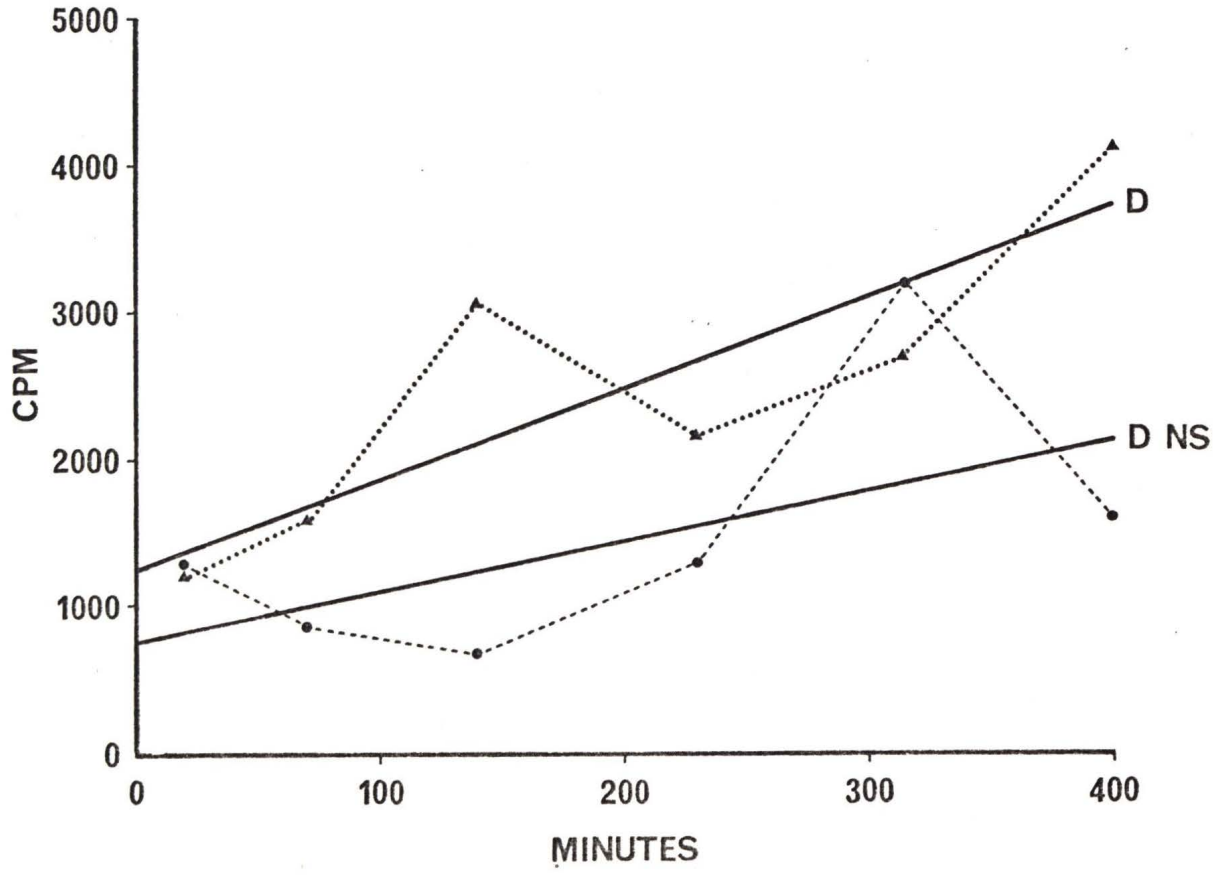
Figure 46. Uptake of dialysed PVP-^{14}C. D = dialysed PVP-^{14}C in CTM, D NS = dialysed PVP-^{14}C in CTM without serum. Concentration of dialysed K 17.8 PVP-^{14}C = 0.051 mg/ml 0.48 µCi/ml. Mean cell count = 1,177,000 ± 52,500 cel per vial. Each point of the graph represents 3-5 samples. ▲...▲ = D, ●---● = D NS.

Slopes of regression lines:

D = 6.12 ± 1.96 CPM/minute
D NS = 3.44 ± 2.64 CPM/minute

Regression lines are not significantly different.


4000
3000
2000
1000
0
CPM
0
100
200
300
400
MINUTES
UD NS
UD


5000
4000
3000
2000
1000
0
CPM
0
100
200
300
400
MINUTES
D
D NS

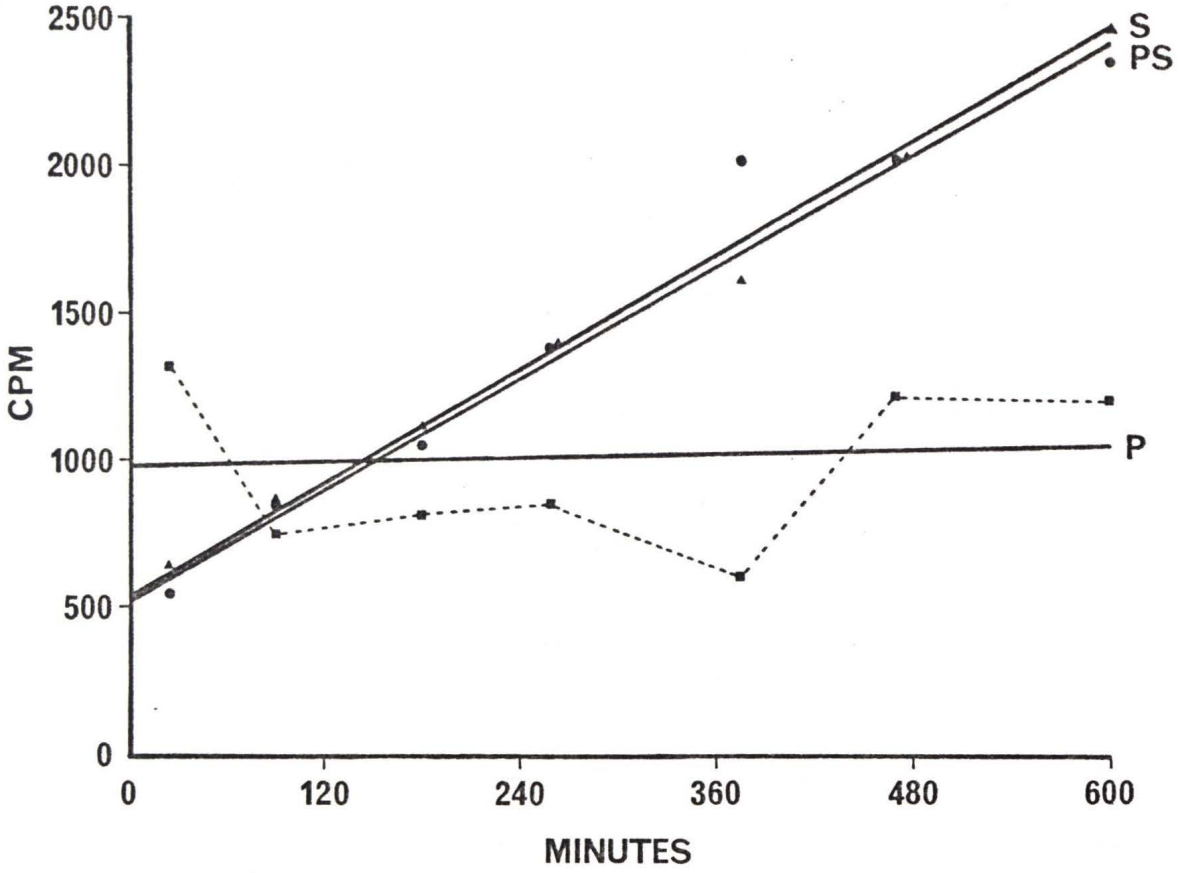
Figures 47 and 48

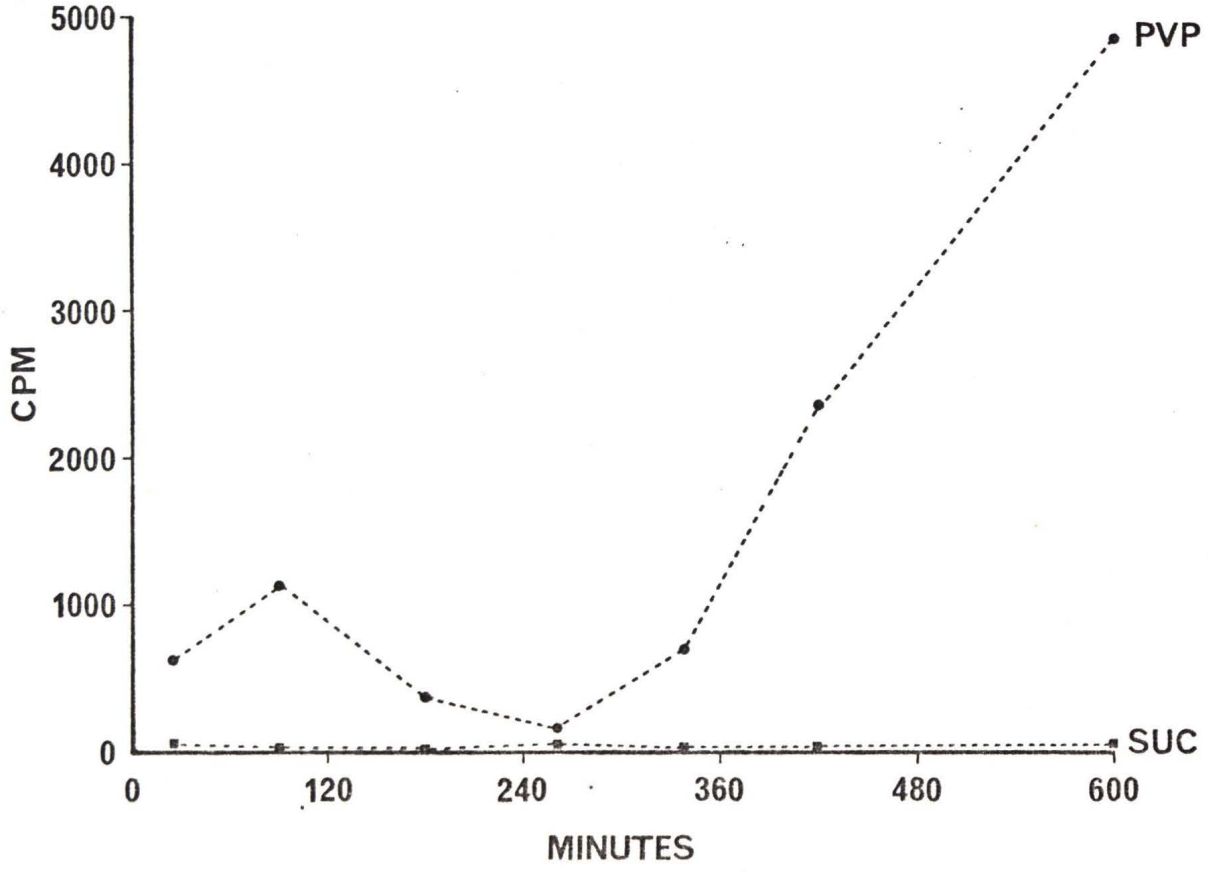
Figure 47. Uptake of sucrose-^{3}H and PVP-^{14}C. S = sucrose-^{3}H in CTM, counted in full tritium channel, PS = sucrose-^{3}H in CTM in the presence of PVP-^{14}C, counted in short tritium channel, with corrections made for difference in counting efficiency in this channel, and with PVP-^{14}C crosstalk substracted. P = PVP-^{14}C counted in short ^{14}C channel, and with sucrose-^{3}H crosstalk substracted. Sucrose-^{3}H concentration = 2.34×10^{-7} M, 0.40 µCi/ml. PVP-^{14}C concentration (undialysed K 17.8) = 0.072 mg/ml, 0.72 µCi/ml. Mean cell count = 2,010,000 ± 210,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. ● = S samples, ▲ = PS samples, ■ = P samples.

Slopes of regression lines:

S = 3.22 ± 0.28 CPM/minute
PS = 3.12 ± 0.12 CPM/minute
P = 0.24 ± 0.60 CPM/minute

Figure 48. Adsorption of sucrose-^{3}H and PVP-^{14}C to scintillation vials without cells. Isotope concentrations same as for the above experiment. Each point of the graph represents the mean of 4 samples. Note that the sucrose-^{3}H levels remain very near the background level (25-30 CPM), while considerable PVP-^{14}C remains in the vials.


2500
2000
1500
1000
500
0
CPM
0
120
240
360
480
600
MINUTES
S
PS
P


5000
4000
3000
2000
1000
0
CPM
0
120
240
360
480
600
MINUTES
PVP
SUC

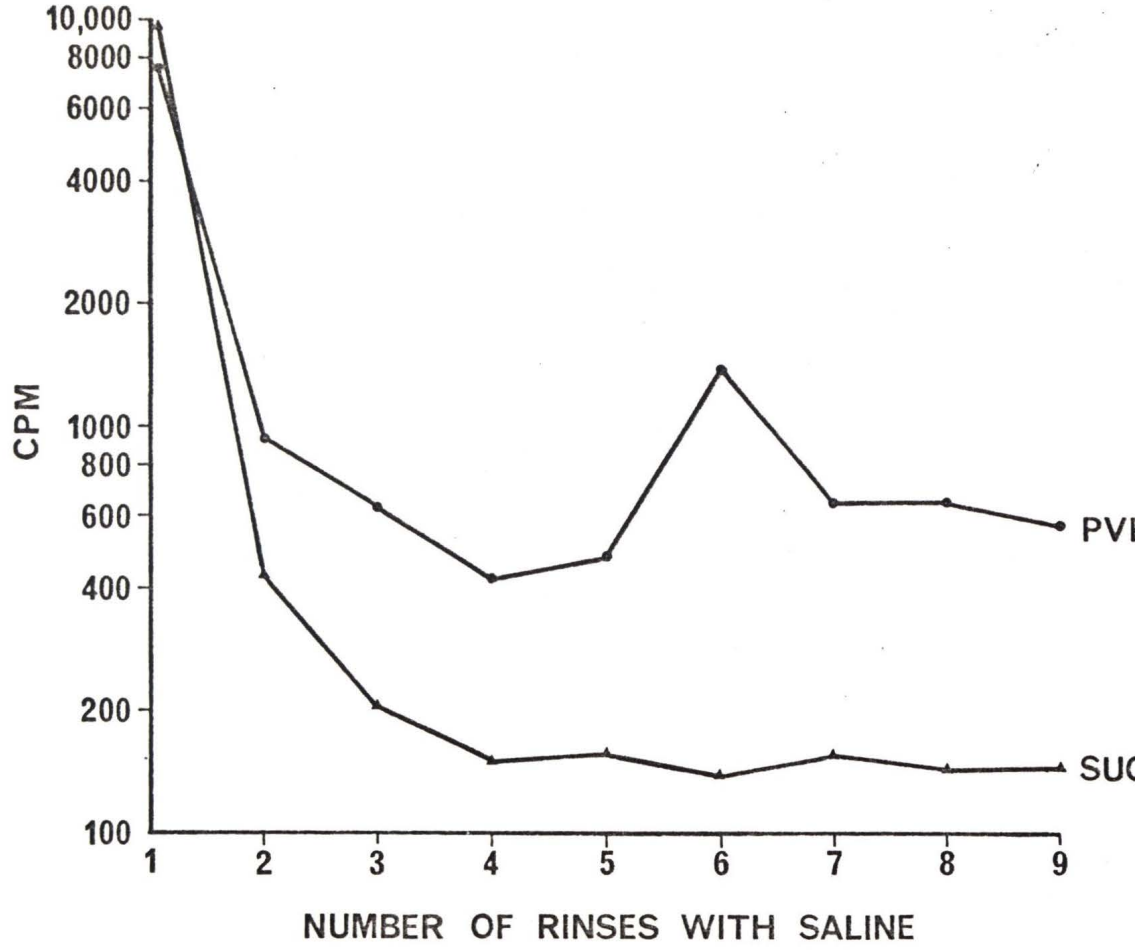
The results of this experiment clearly demonstrate that the PVP-^{14}C had no effect on the rate of uptake of sucrose-^{3}H when both were present in the same sample vials, however the results for the uptake of PVP-^{14}C were again sporadic, as no steady increase in the radioactivity remaining in the vials as a function of time occurred, as was observed with sucrose-^{3}H. This clearly demonstrated that the sporadic behaviour of PVP-^{14}C was not due to endocytosis. In the same experiment, when PVP-^{14}C and sucrose-^{3}H were incubated in clean scintillation vials without cells, the results shown in fig. 48 were obtained. Clearly PVP-^{14}C was adhering to the walls of the scintillation vials, whereas sucrose-^{3}H was not.

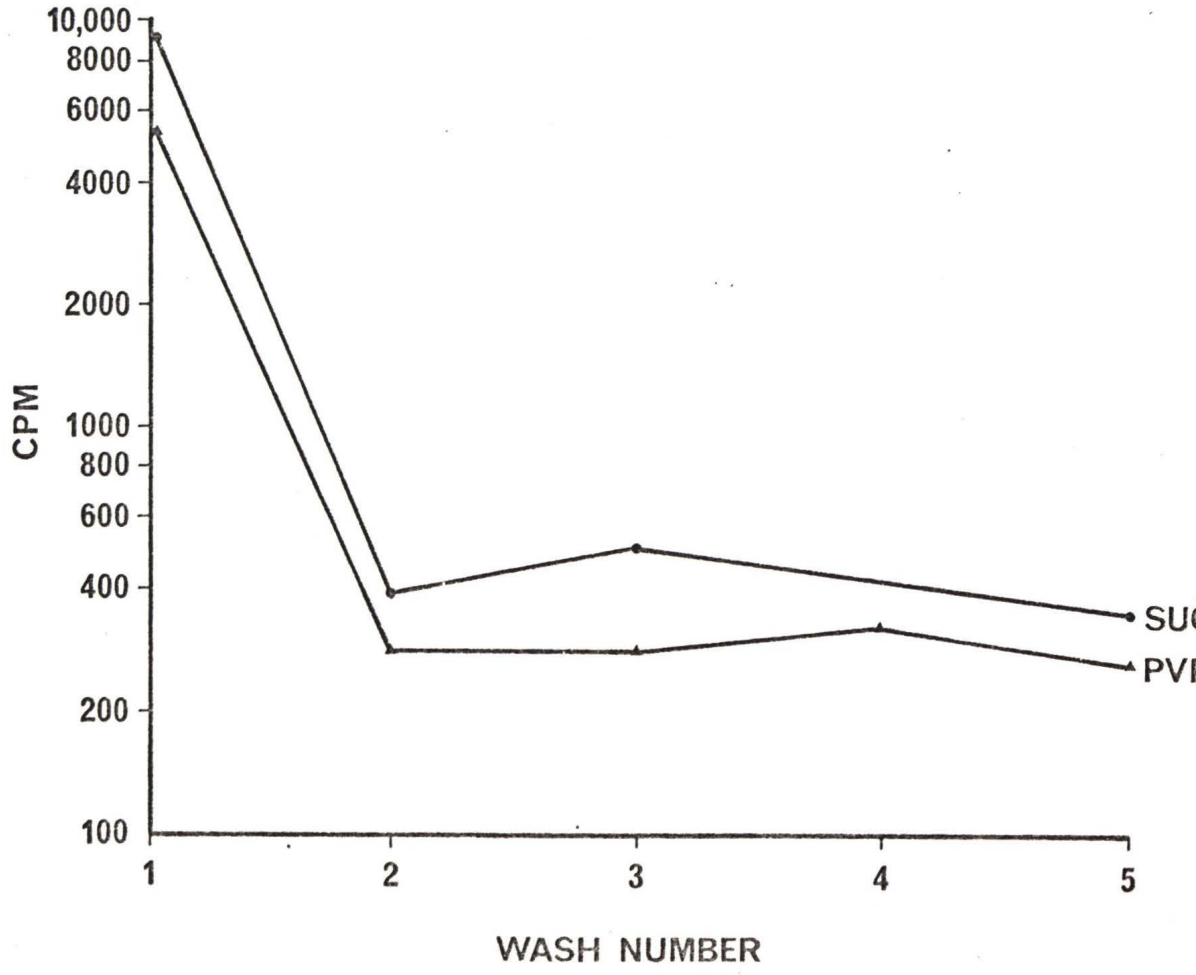
To determine whether the phenomenon observed with PVP-^{14}C could be explained by an inadequate washing of the sample vials with saline, following the incubation with PVP-^{14}C, the experiment shown in fig. 49 was performed. In this experiment the sample vials, all of which were confluent with CHA cells at the bottom, were incubated with either PVP-^{14}C or sucrose-^{3}H for 20 minutes at 22°C, after which they were washed with either 1, 2, 3, ... or 9 changes of saline. The results, plotted as counts per minute remaining as a function of the number of rinses with saline, and corrected for differences in counting efficiencies between ^{3}H and ^{14}C, as well as differences in the amount of sucrose-^{3}H and PVP-^{14}C added, clearly indicates that PVP-^{14}C has a greater affinity for the sample vials, which persists even after 9 rinses with saline.

Figures 49 and 50

Figure 49. Relative affinity of PVP-^{14}C and sucrose-^{3}H for sample vials containing cells. Note that the ordinate is a logarithmic scale. Each point on the graph represents the mean of 5 samples. CPM has been corrected for differences in counting efficiencies of ^{14}C and ^{3}H and for differences in the amount of activity added per vial. All sample vials were confluent with cells. PVP-^{14}C concentration = 0.111 mg/ml, 1.04 µCi/ml, sucrose-^{3}H concentration = 6.54×10^{-7} M, 1.11 µCi/ml plus cold sucrose to make 0.111 mg/ml sucrose.

Figure 50. Adsorptivity of PVP-^{14}C to cells compared to sucrose-^{14}C. Details of experiment given in Materials and Methods section. Ordinate is a logarithmic scale.


10,000
8000
6000
4000
2000
1000
800
600
400
200
100
CPM
1
2
3
4
5
6
7
8
9
PVP
SUC
NUMBER OF RINSES WITH SALINE


10,000
8000
6000
4000
2000
1000
800
600
400
200
100
CPM
1
2
3
4
5
SUC
PVP
WASH NUMBER

These experiments demonstrated that PVP-^{14}C adheres to the sample vials rather tenaciously, both with and without cells being present. These experiments did not, however, establish whether PVP could bind in any way to cell membranes. Since it was of interest to determine whether or not any such binding occurred, the experiment shown in fig. 50 was performed. This experiment involved scraping the cells off the glass after incubation with PVP-^{14}C or sucrose-^{14}C, so that any binding of PVP-^{14}C to the glass itself would not interfere with the detection of any possible binding of PVP-^{14}C to the cells. As is readily apparent, PVP-^{14}C levels are actually less than with sucrose-^{14}C, and since sucrose has already been shown not to bind to the plasma membrane of these cells, we can safely conclude that PVP also has no affinity for the plasma membrane of these cells.

Effect of Conditioned Medium on Endocytosis

To verify the effect observed by Munro (58) with CHA cells that endocytosis is stimulated by the mere replacement of the medium with fresh growth medium, the experiment shown in fig. 51 was performed. The results clearly indicate that when the growth medium (CTM) in which the CHA cells had been maintained since the innoculation of the culture (hereafter referred to as conditioned medium (CM)) was left in the sample vials, the rate of endocytosis was nearly 3 times the rate observed with fresh CTM. This effect

was just the opposite of what would be expected on the basis of the work by Munro, and warranted further investigation.

When the experiment with CM was repeated (fig. 52) similar results were obtained. In this experiment the CM was removed from the vials, pooled, its pH read, justed from 7.0 to the normal value of 7.2, and then replaced in the sample vials, in order to eliminate pH, or disturbance of the cells when the medium is changed, as possible causes of this effect. Some of the CM was millipored, in order to remove dead cells, and other debris from the medium, which might have conceivably been the causative agents of this effect, this however proved not to be the case. CTM which had been "conditioned" by PCC cells, instead of CHA cells, has some effect, but not nearly as great an effect as observed when the medium was "conditioned" by CHA cells.

It was at first suspected that something was present in CM, which was not present in fresh CTM, that caused this increase in endocytosis, therefore, an experiment in which CM was diluted with CTM was performed (fig. 53). The results of this experiment ruled out the possibility that a hormone, for example, or other highly specific regulator was secreted by CHA cells into the medium, which might be responsible for the CM effect. The possibility still remained that a metabolic product of the CHA cells was responsible for the CM effect, and that it must be present in

Figures 51 and 52

Figure 51. The effect of conditioned medium (CM) on the uptake of sucrose-^{3}H. C = normal fresh CTM, CM = conditioned medium. 3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 890,000 ± 95,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. ▲ = CM samples, ● = C samples.

Slopes of the regression lines:

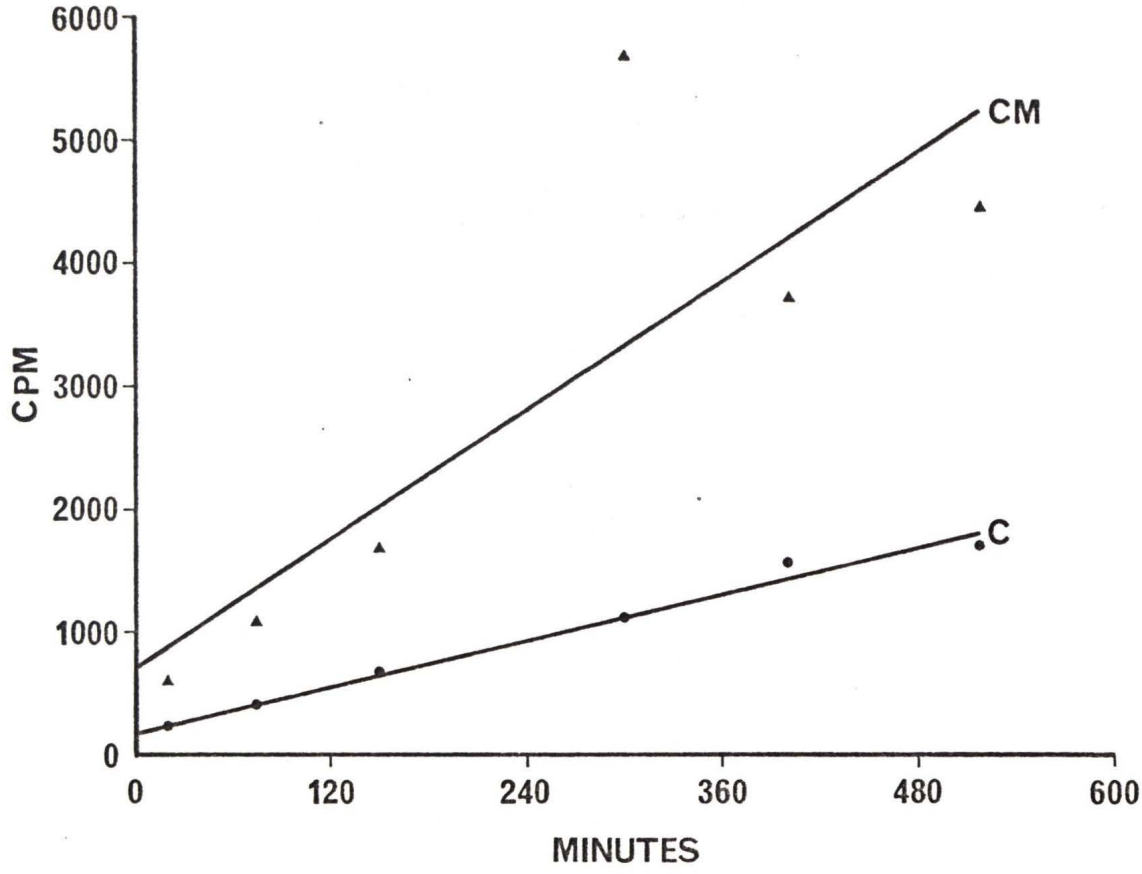
C = 3.13 ± 0.21 CPM/minute
CM = 8.65 ± 2.97 CPM/minute

Figure 52. The effect of CM on the uptake of sucrose-^{3}H. CM = conditioned medium which was removed from the sample vials, pooled, and its pH readjusted to 7.2 with NaOH. CMM = same as CM except it was, in addition, passed through a 0.22 µ Millipore filter (Millipore Filter Corp., Bedford, Massachusetts). PCM = CTM conditioned by PCC cells rather than CHA cells. Its pH was also readjusted to 7.2. 3.92×10^{-7} M sucrose-^{3}H (0.666 µCi/ml). In this experiment, the medium was removed from all sample vials, replaced with 2 ml of sterile saline, which was then immediately removed by vacuum suction and replaced with the test medium. Mean cell count = 912,000 ± 65,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. C = normal fresh CTM. ● = C samples, ■ = CM samples, ▲ = CMM samples, o = PCM samples.

Slopes of regression lines:

C = 3.49 ± 0.18 CPM/minute
CM = 13.04 ± 1.53 CPM/minute
CMM = 10.99 ± 0.48 CPM/minute
PCM = 5.14 ± 0.25 CPM/minute

PCM rate is significantly higher than C ($P < 0.01$).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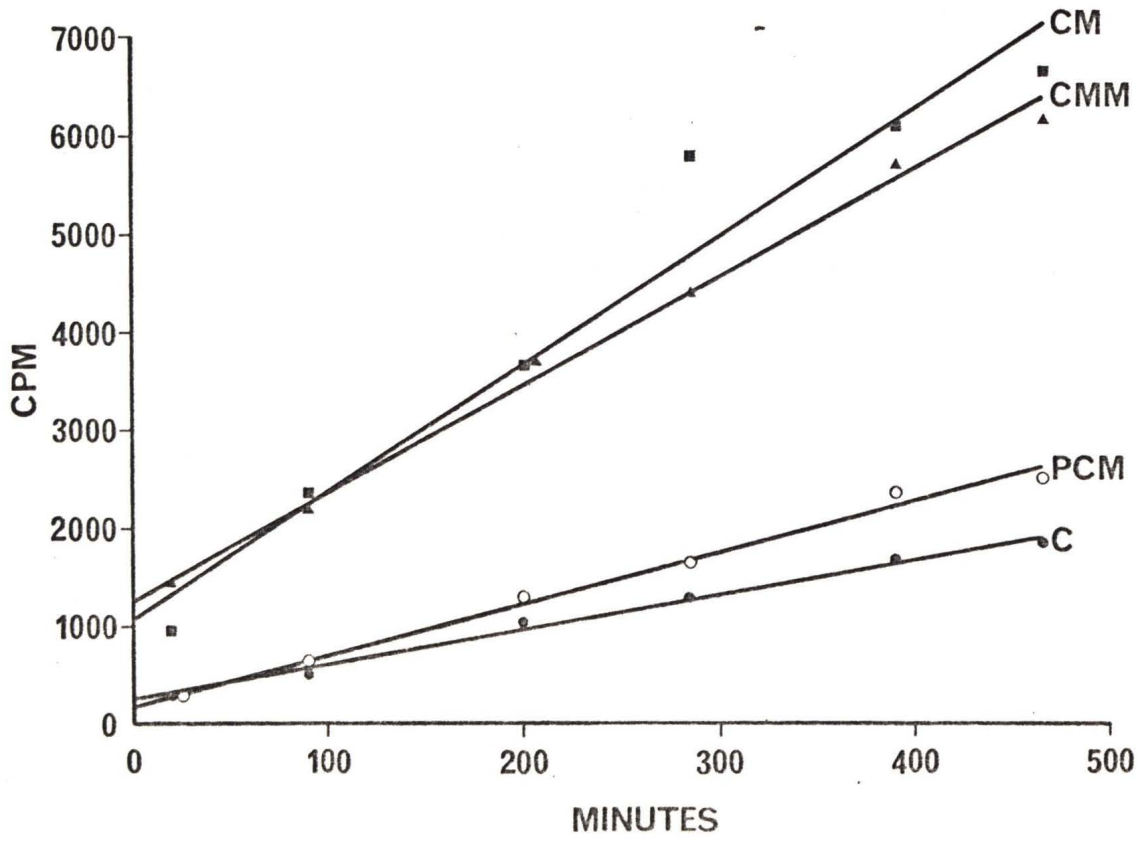
5000
4000
3000
2000
1000
0
CPM
CM
C
0
120
240
360
480
600
MINUTES


7000
6000
5000
4000
3000
2000
1000
0
CPM
CM
CMM
PCM
C
0
100
200
300
400
500
MINUTES

a relatively high concentration in order to exert its effect. To investigate this possibility, the experiment shown in fig. 54 was performed. In this experiment CM, less highly diluted with CTM than in the experiment shown in fig. 53, also lacked the effect observed with undiluted CM. This suggested that perhaps some constituent of CTM was metabolised and removed from the medium, and its absence was responsible for the effect observed with CM.

To test the above hypothesis, glutamine, all the vitamins normally present in CTM, and glucose were added back to CM in the hopes that one of these would obliterate the CM effect. The results (fig. 55) clearly indicate that when glucose was added to CM in the concentration normally present in CTM, the increased rate of endocytosis normally observed with CM was not observed, but rather is reduced to the rate observed with normal CTM. This immediately suggested that glucose was in some way an inhibitor of endocytosis, which is not what one would expect, since it has already been demonstrated that the inhibition of glycolysis by iodoacetate resulted in the inhibition of endocytosis, demonstrating that glucose utilization is necessary for normal levels of endocytosis.

The uptake of sucrose-^{3}H was investigated using glucose free medium (GFM) with increasing concentrations of glucose added.

Figures 53 and 54

Figure 53. The effect of the dilution of CM with CTM. The dilutions are 1/4 CM :3/4 CTM (■), 1/16 CM : 15/16 CTM (o), 1/64 CM : 63/64 CTM (△) CM (▲) is undiluted, C (●) is fresh CTM. 3.92 X 10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 880,000 ± 51,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. Regression lines shown for CM and C only.

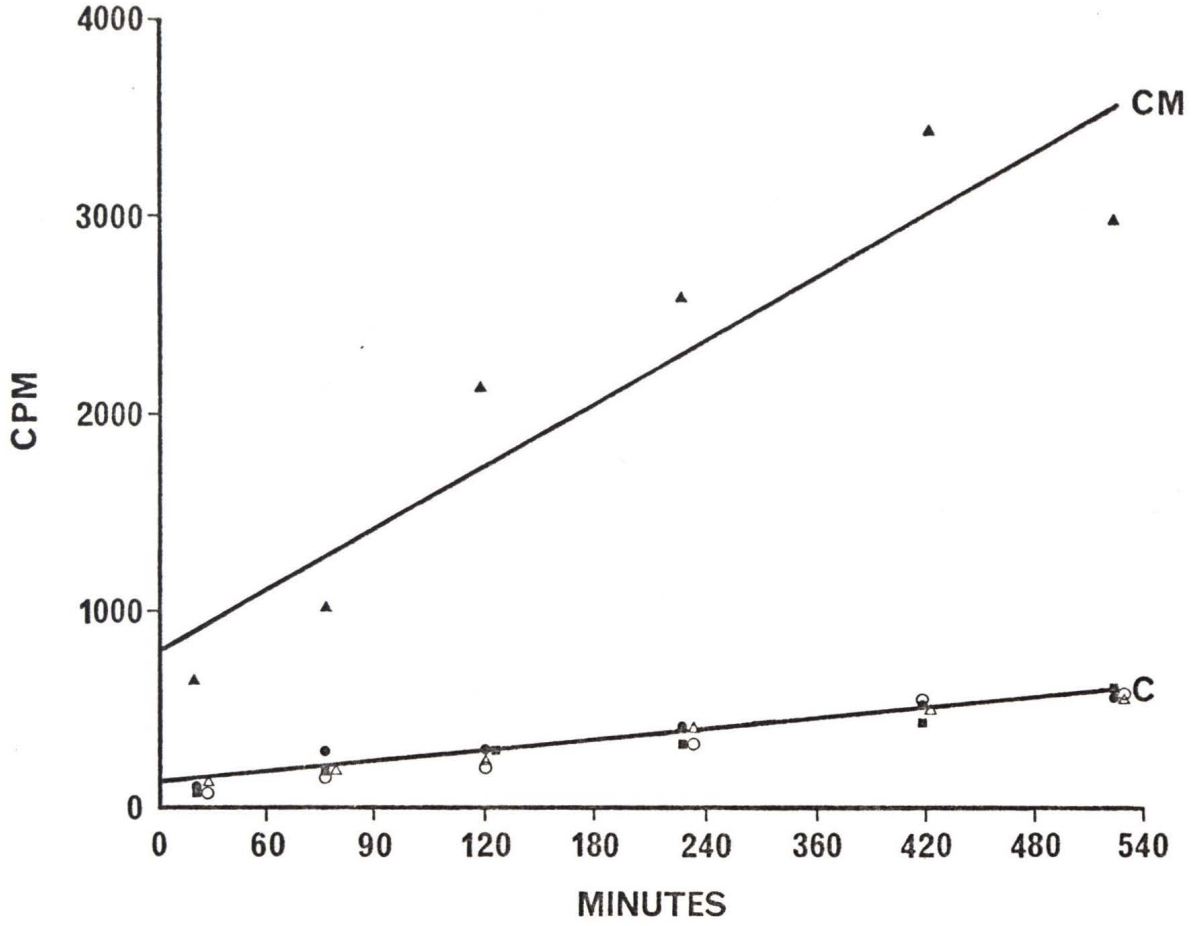
Slopes of the regression lines:

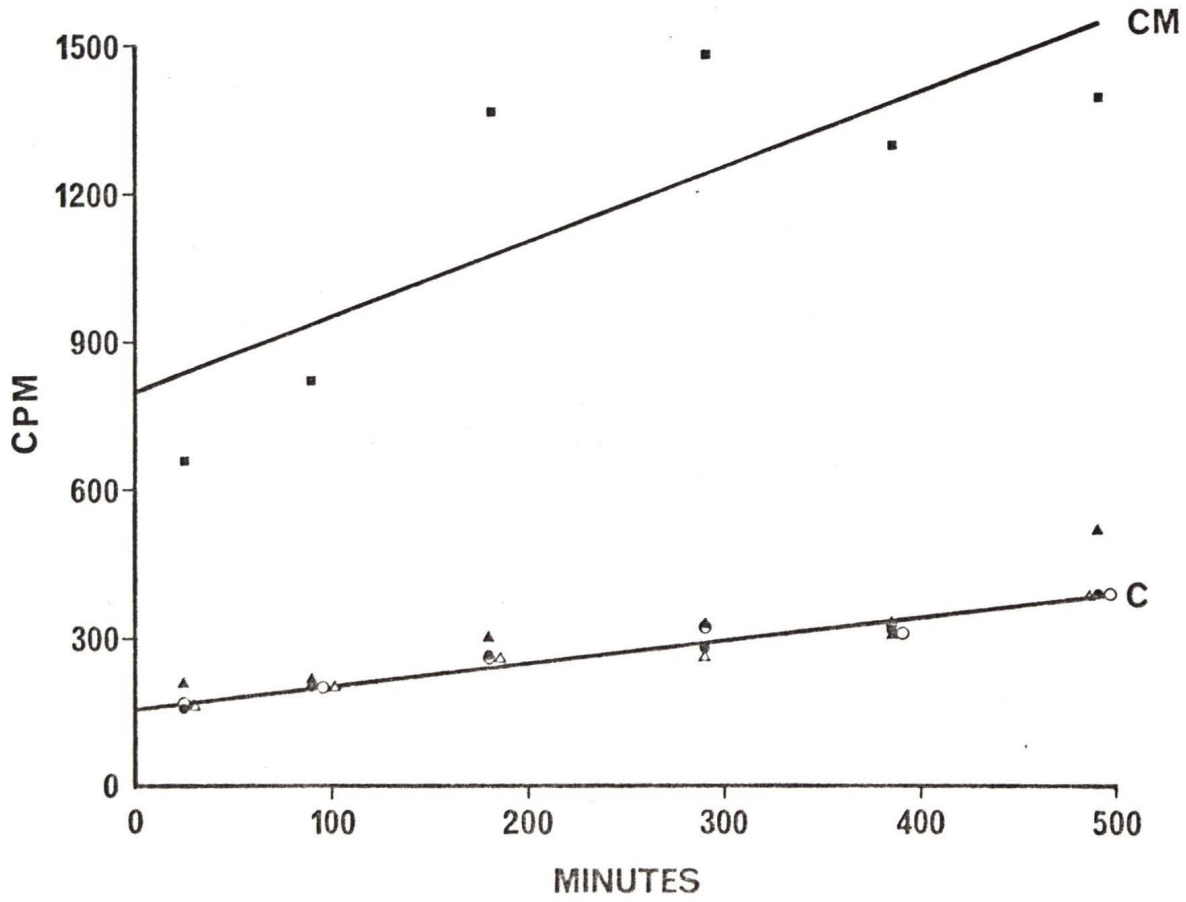
CM = 5.24 ± 1.08 CPM/minute
C = 0.85 ± 0.12 CPM/minute
1/4 CM : 3/4 CTM = 0.93 ± 0.08 CPM/minute
1/16 CM : 15/16 CTM = 1.06 ± 0.09 CPM/minute
1/64 CM : 63/64 CTM = 0.92 ± 0.08 CPM/minute

Figure 54. The effect of the dilution of CM with CTM. The dilutions are: 3/4 CM : 1/4 CTM (▲), 1/2 CM : 1/2 CTM (o), 1/4 CM : 3/4 CTM (△). CM (■) is undiluted, C (●) is fresh CTM. 3.92 X 10^{-7} M sucrose-^{3}H (0.666 µCi/ml). Mean cell count = 587,000 ± 112,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. Regression lines shown for CM and C only.

Slopes of the regression lines:

CM = 1.53 ± 0.59 CPM/minute
C = 0.46 ± 0.05 CPM/minute
3/4 CM : 1/4 CTM = 0.58 ± 0.13 CPM/minute
1/2 CM : 1/2 CTM = 0.63 ± 0.16 CPM/minute
1/4 CM : 3/4 CTM = 0.45 ± 0.05 CPM/minute


4000
3000
2000
1000
0
CPM
CM
C
0
60
90
120
180
240
360
420
480
540
MINUTES


1500
1200
900
600
300
0
CPM
CM
C
0
100
200
300
400
500
MINUTES

Figures 55 and 56

Figure 55. The effect on endocytosis of the addition of glutamine, vitamins, and glucose to CM. GA (△) = glutamine, V (■) = vitamins, GLU (o) = glucose, all added to CM in the same concentration as they would normally be present in CTM. CM (▲), C (●) = normal CTM. 3.92×10^{-7} M sucrose-^{3}H (0.666 µCi/ml). Mean cell count = 510,000 ± 81,000 cells per vial. Each point on the graph represents the mean of 3-5 samples.

Slopes of the regression lines:

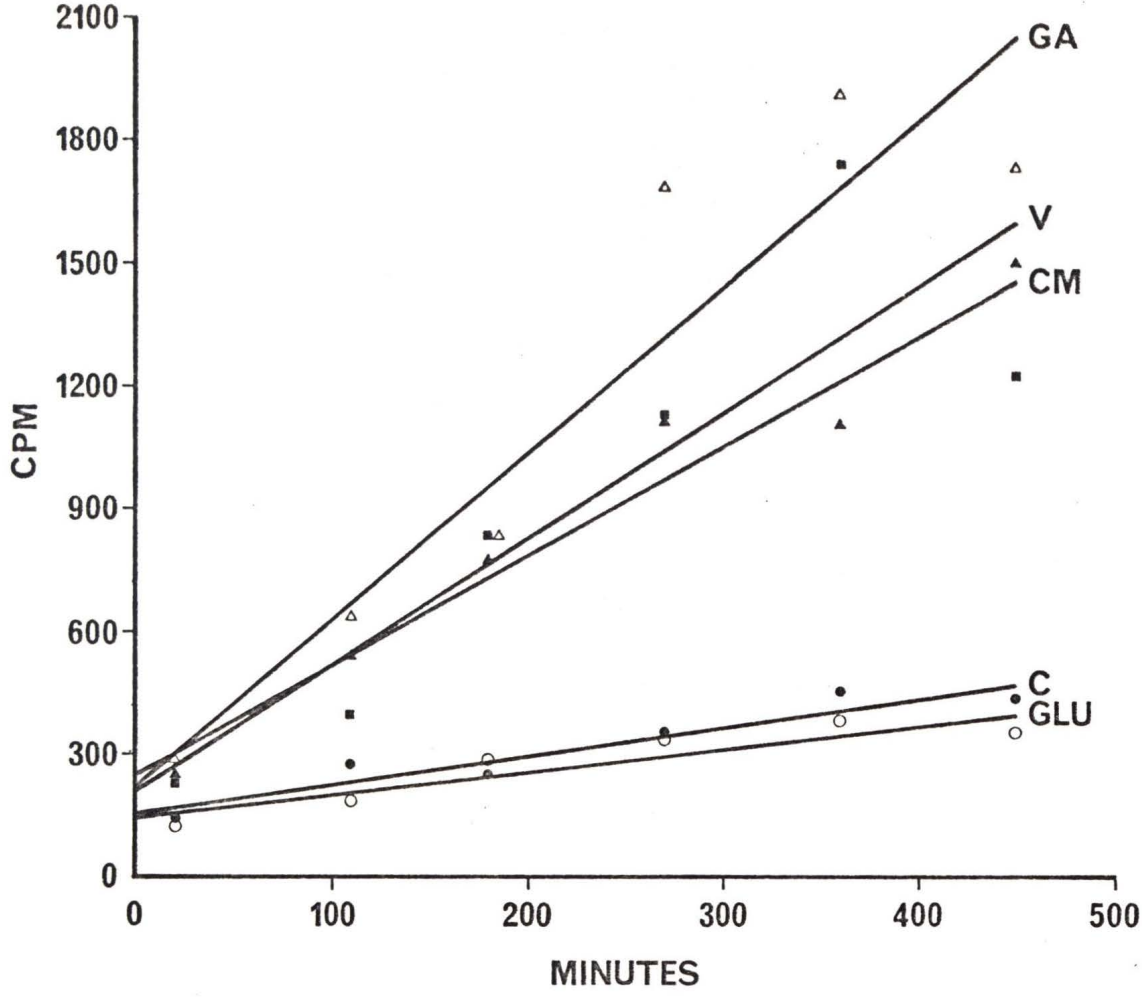
C = 0.70 ± 0.11 CPM/minute
CM = 2.65 ± 0.27 CPM/minute
GLU = 0.60 ± 0.13 CPM/minute
V = 3.07 ± 0.83 CPM/minute
GA = 4.06 ± 0.75 CPM/minute

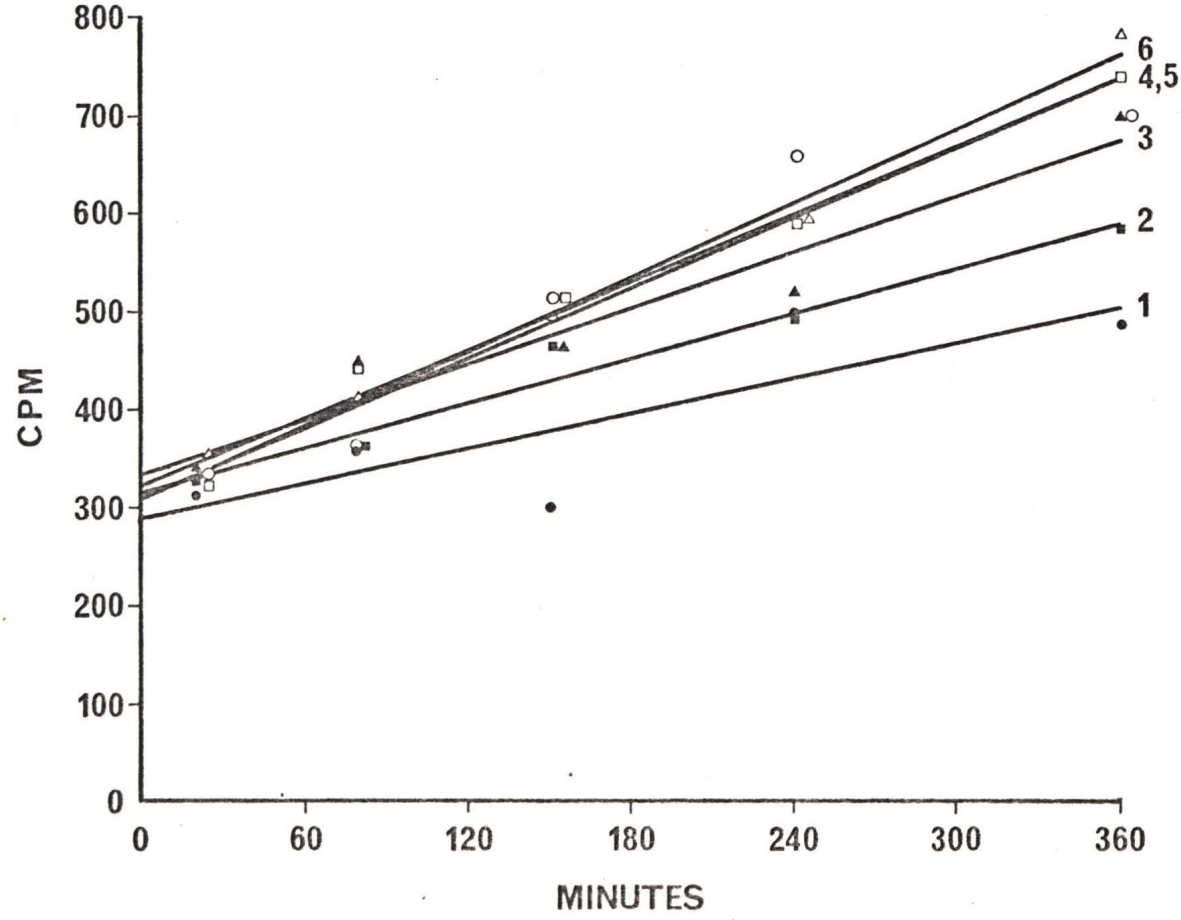
GA rate is significantly greater than CM rate ($P < 0.05$).

Figure 56. The effect of increasing concentrations of glucose on endocytosis. Glucose free medium prepared as described in Materials and Methods section. Concentrations of glucose added to GFM: 1 (●) = 1.0 mg/ml, 2 (■) = 0.2 mg/ml, 3 (▲) = 0.04 mg/ml, 4 (o) = 0.008 mg/ml, 5 (□) = 0.0016 mg/ml, 6 (△) = no glucose. 3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 1,067,000 ± 131,000 cells per vial. Each point on the graph represents the mean of 3-5 samples.

Slopes of the regression lines:

1 = 0.59 ± 0.23 CPM/minute
2 = 0.76 ± 0.08 CPM/minute
3 = 0.95 ± 0.13 CPM/minute
4 = 1.19 ± 0.19 CPM/minute
5 = 1.15 ± 0.08 CPM/minute
6 = 1.27 ± 0.06 CPM/minute


2100
1800
1500
1200
900
600
300
0
CPM
0
100
200
300
400
500
MINUTES
GA
V
CM
C
GLU


800
700
600
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400
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200
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0
CPM
0
60
120
180
240
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360
MINUTES
6
4,5
3
2
1

The results, shown in fig. 56, clearly demonstrate the effect of increasing concentrations of glucose on endocytosis. When this effect is expressed as % maximum rate of endocytosis (that observed with GFM) as a function of the $\log_{10}$ of the concentration of glucose added to GFM, the results shown in fig. 58 are observed, which suggest a logarithmic response of endocytosis to glucose.

In the experiment shown in fig. 56, the maximum rate of endocytosis, the rate obtained with GFM without glucose, was only about 2 times the rate of GFM with 1 mg/ml of glucose added, which is the same as CTM, whereas in previous experiments the rate with CM was 3 to 5 times the rate with CTM. To investigate this effect further, the experiment shown in fig. 58 was performed. In this experiment an extremely high rate of endocytosis (unreproducible in subsequent experiments) was observed with CM, whereas the rate with GFM was only 2 times the rate with CTM. Clearly additional factors, in addition to the elimination of glucose from the medium, must be responsible for the maximum effect observed with CM.

In order to investigate more rapidly the effect of other compounds on the rate of endocytosis observed with CM, the experimental procedure was altered. Rather than assaying the incorporation of sucrose-^{3}H at regular intervals of time, samples were taken only at the beginning of the incubation, and after 8 hours of incubation. This procedure made possible the assay of

Figures 57 and 58

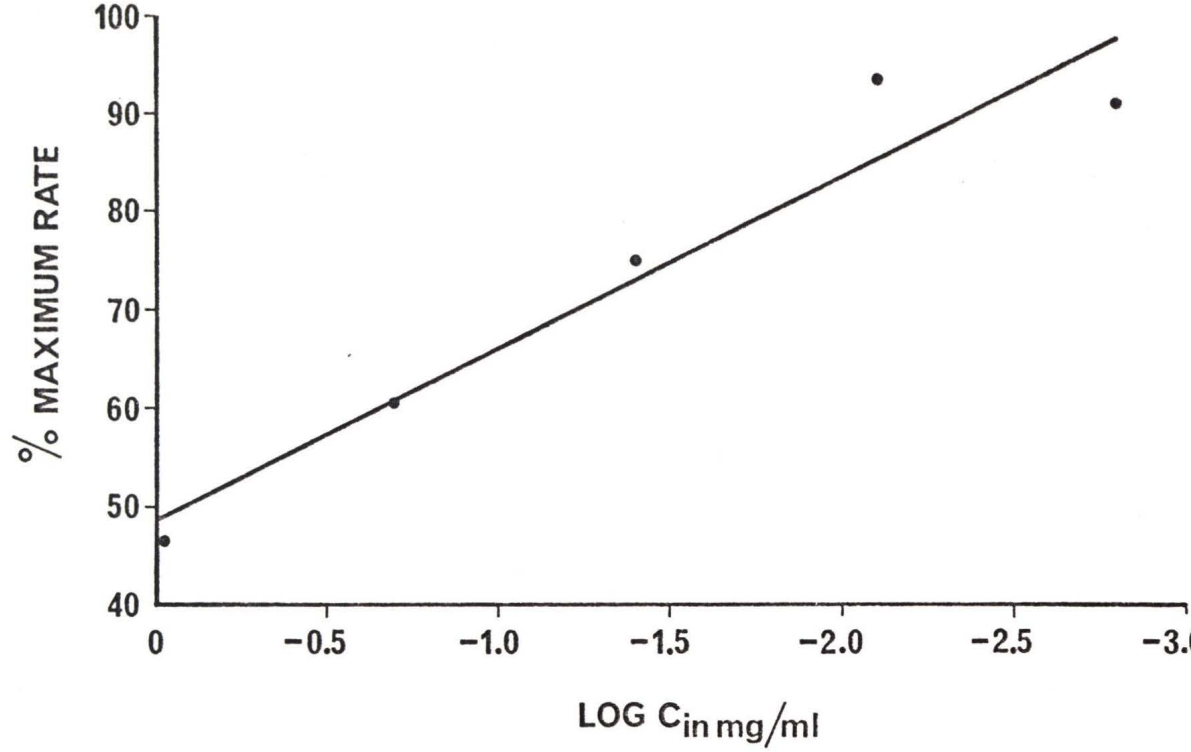
Figure 57. The effect of increasing concentrations of glucose on endocytosis. Data same as for fig. 56. Results expressed as % of maximum rate as observed with GFM as a function of the $\log_{10}$ of the concentration in mg/ml of glucose added to GFM.

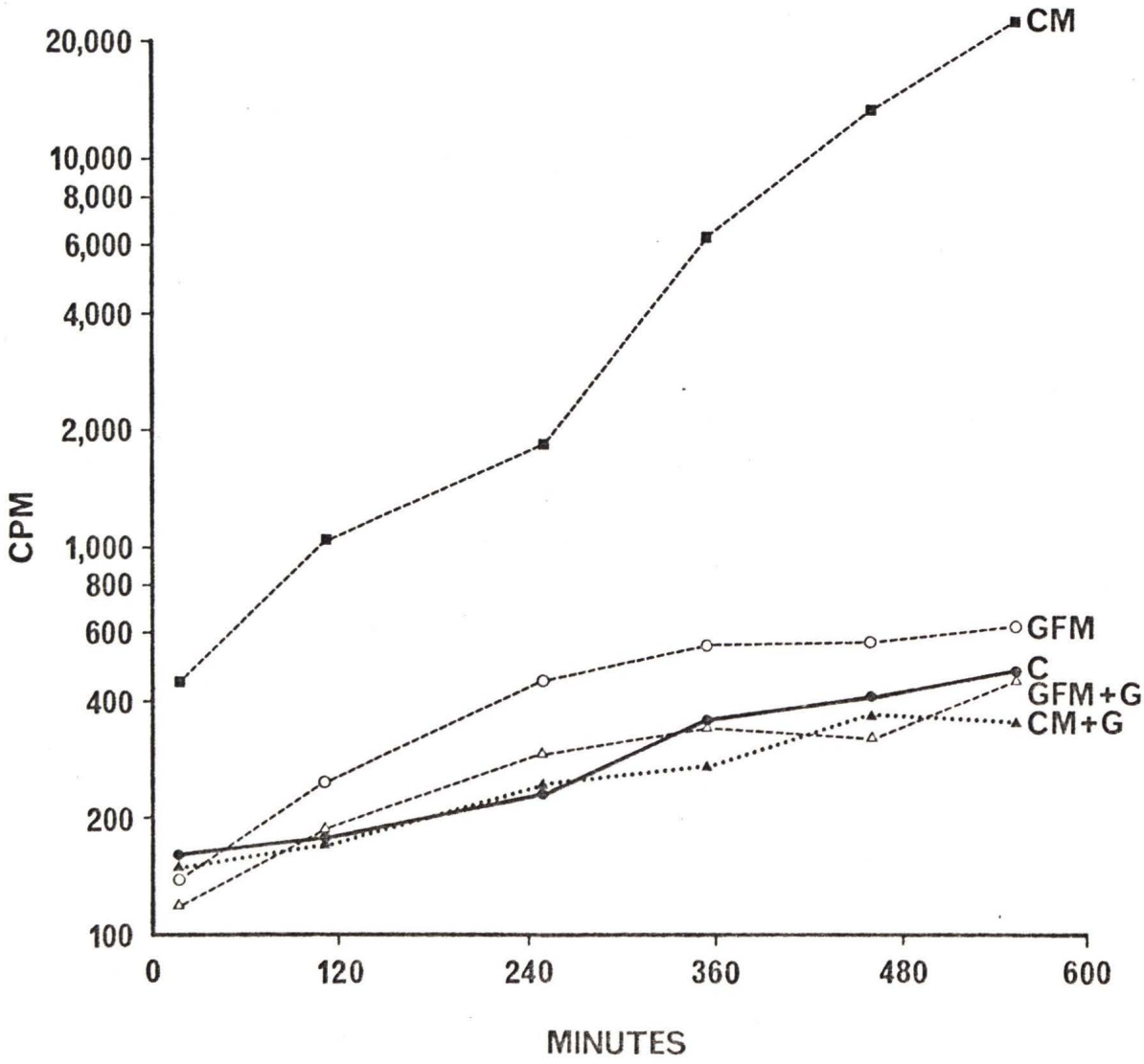
Figure 58. Comparison between the rate of endocytosis with CM, GFM, CTM, GFM plus glucose and CM plus glucose. CM = (■---■), GFM = (o----o), C, which is CTM = (●——●), GFM + G = GFM plus 1 mg/ml of glucose = (Δ----Δ), CM + G = CM plus 1 mg/ml of glucose = (▲----▲). 3.92×10^{-7} M sucrose-^{3}H, (0.666 uCi/ml). Mean cell count = 1,115,000 ± 129,000 cells per vial. Each point on the graph represents the mean of 3-5 samples.

Slopes of regression lines (not shown):

C = 0.61 ± 0.07 CPM/minute
CM =38.9 ± 9.01 CPM/minute
GFM = 0.91 ± 0.13 CPM/minute
GFM + G = 0.55 ± 0.08 CPM/minute
CM + G = 0.48 ± 0.05 CPM/minute

GFM rate is significantly greater than C rate ($P < 0.05$).


100
90
80
70
60
50
40
% MAXIMUM RATE
0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
LOG C in mg/ml


20,000
10,000
8,000
6,000
4,000
2,000
1,000
800
600
400
200
100
CPM
CM
GFM
C
GFM+G
CM+G
0
120
240
360
480
600
MINUTES

more variables, as fewer sample vials were required per variable. The results of the first such experiment are shown in histogram form in fig. 59. In each bar in the histograms, the counts per minute remaining at times 0 are shown by the bottom line, while the counts per minute remaining after 8 hours incubation are shown by the top line. The standard error for the top line is indicated by a vertical line. Interestingly, both glucose and mannose, an epimer of glucose capable of being utilized in glycolysis (47) reduced the rate of endocytosis to below the rate observed with CTM. Fructose also had a significant effect, as well as sodium pyruvate (fig. 60), but the amino acids and ATP had little effect. Strangely, glucose-1-phosphate had much less effect than glucose.

Possible Hormone Effects of Endocytosis

Using the above described simplified method of assaying changes in the rate of endocytosis, the effects of several hormones were investigated.

The first of such experiments, in which the effect of the hormonal second messenger, 3',5' cyclic adenosine monophosphate (cAMP) was investigated, is shown in fig. 60. Neither cAMP nor the dibutyryl derivative (di-cAMP), which is more permeable to cell membranes and resists attack by the enzyme phosphodiesterase (65), had any significant effect on the rate of endocytosis, either with CM or CTM. Theophylline at a concentration of 10^{-3} M was

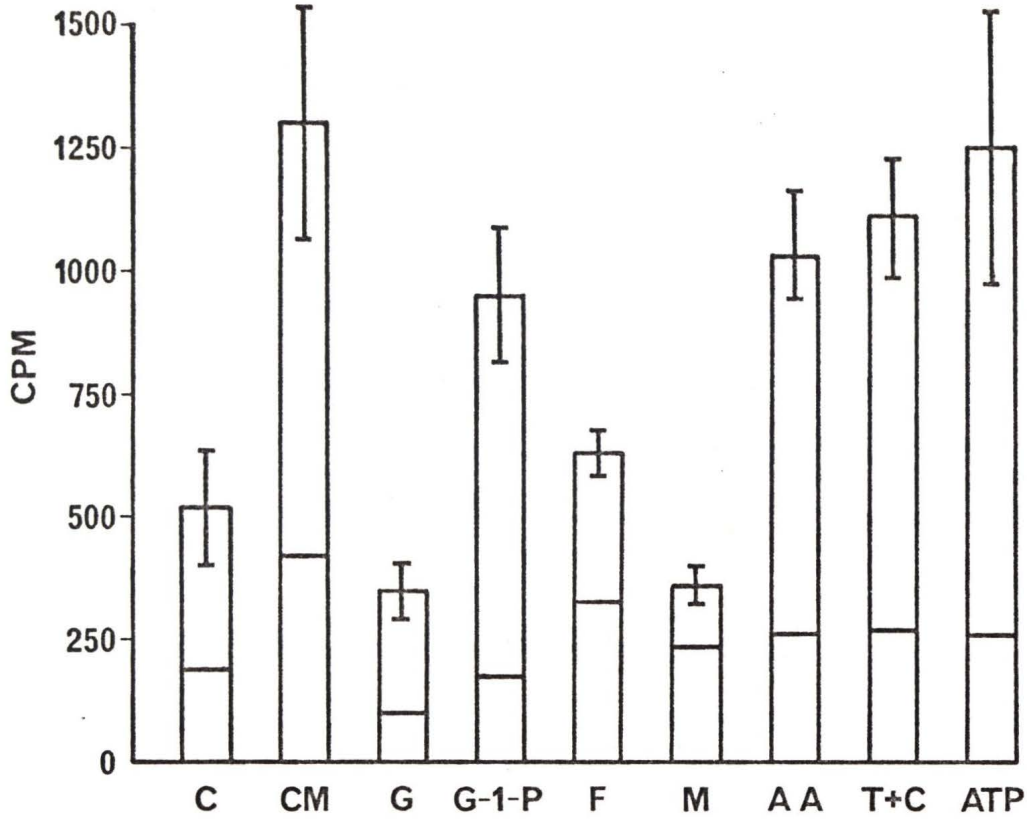
Figures 59 and 60

Figure 59. Assay of the effect of various compounds on the rate of endocytosis by Chinese hamster cells with conditioned medium (CM). The bottom line of each bar represents uptake of sucrose-^{3}H at time 0 (mean of 4 samples), and the top line of each bar represents the uptake of sucrose-^{3}H at the end of 8 hours incubation at 36.5° C (mean of 6 samples). Vertical lines indicate the standard error of the mean . 8 hours incubation.

C = normal CTM
CM = conditioned medium
G = CM plus 1 mg/ml D-glucose
G-1-P = CM plus 1 mg/ml D-glucose-1-phosphate
F = CM plus 1 mg/ml D-fructose
M = CM plus 1 mg/ml of D-mannose
AA = CM plus all the amino acids normally present in CTM added at that concentration, except tyrosine and cysteine
T + C = CM plus tyrosine and cysteine added to CM in the concentration normally present in CTM
ATP = CM plus 1 mg/ml of adenosine triphosphate
3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 1,022,000 + 105,000 cells per vial.

Figure 60. The effect of cyclic AMP and dibutyryl cyclic AMP on endocytosis in Chinese hamster cells.

C = normal CTM
CM = conditioned medium
G = CM + 1 mg/ml D-glucose
P = CM + 1 mg/ml sodium pyruvate (Sigma)
T = CM + 10^{-3} M theophylline (Sigma)
cAMP = CM + 10^{-4} M adenosine3' : 5'-cyclic monophosphoric acid (Sigma), (cAMP) + theophylline
dAMP = CM + N^{6}, O^{2} -dibutyryl adenosine 3' : 5'-cyclic monophosphoric acid (Sigma) 10^{-4} M + theophylline
T_C= theophylline + CTM
$cAMP_C$ = cAMP + CTM + theophylline
$dAMP_C$ = di-cAMP + CTM + theophylline
8 hour incubation. 3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 1,250,000 + 194,000 cells per vial.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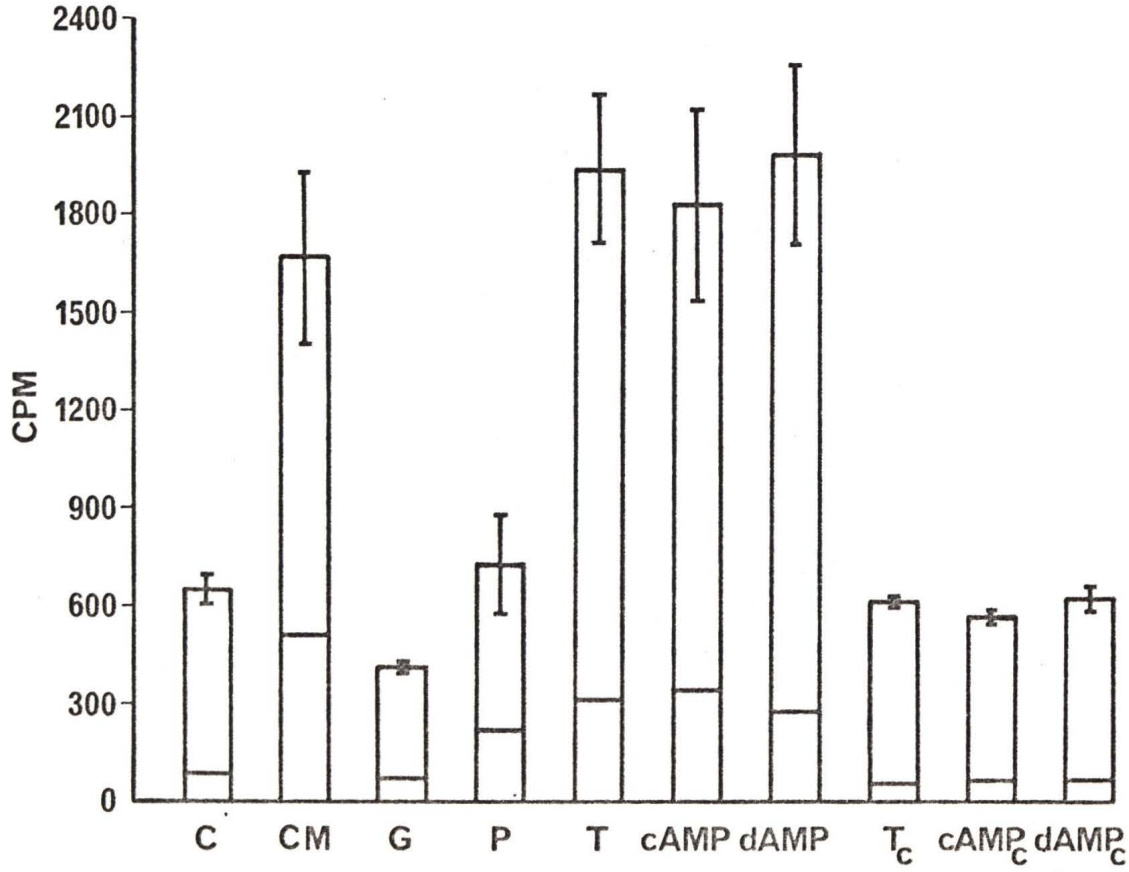
1250
1000
750
500
250
0
C
CM
G
G-1-P
F
M
A A
T+C
ATP


CPM
2400
2100
1800
1500
1200
900
600
300
0
C
CM
G
P
T
cAMP
dAMP
T_c
$cAMP_c$
$dAMP_c$

added with both cAMP and di-cAMP, which, because of its inhibitory action on phosphodiesterase (66), would prolong and enhance any cAMP or di-cAMP effect. Theophylline alone had no significant effect on the rate of endocytosis with either CM or CTM.

The second experiment, shown in fig. 61, demonstrated that insulin, neither in the presence of, nor in the absence of di-cAMP, had any significant effect on the rate of endocytosis with either CM or CTM.

The hormones, testosterone and 17-β-estradiol, also had no effect with either CM or CTM (fig. 62). No synergistic effects with di-cAMP or insulin were observed either.

Consistency of the Rate of Endocytosis

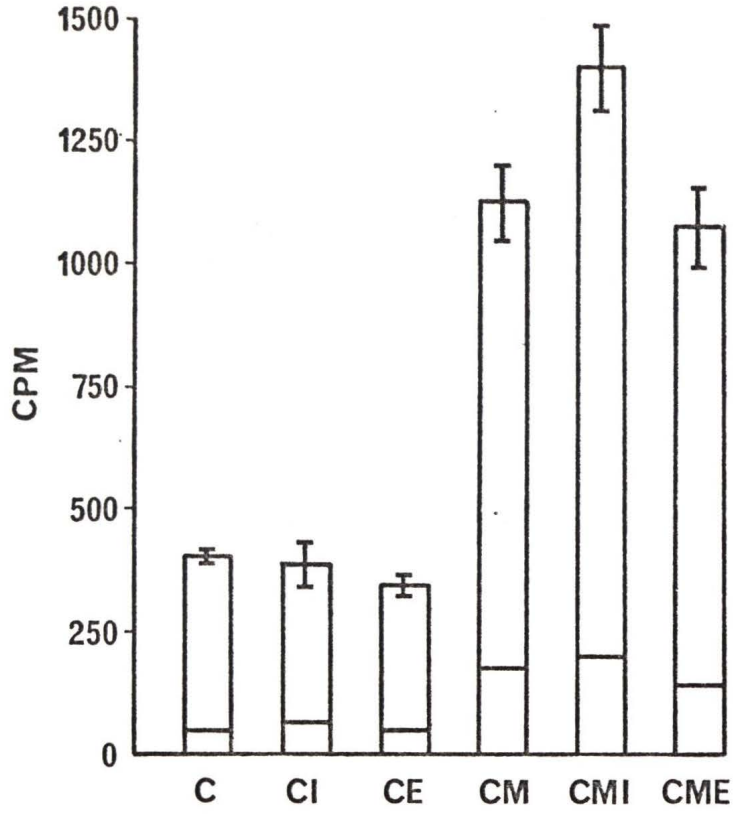
A decline in the rate of endocytosis with CTM was noted over the months of experimentation. In attempt to determine the cause of this effect, scintillation vials were thoroughly cleaned with chromic acid, after which the rate of endocytosis was determined with cells grown in these vials (fig. 63). When the rate of endocytosis with cells in these vials is compared with the rate observed in a previous experiment in which the vials were not cleaned with chromic acid, no significant difference is seen (fig. 63, experiments 7 and 8). This indicates that any possible residual contaminants which might have accumulated in the vials during the course of these experiments did not effect the rate of endocytosis.

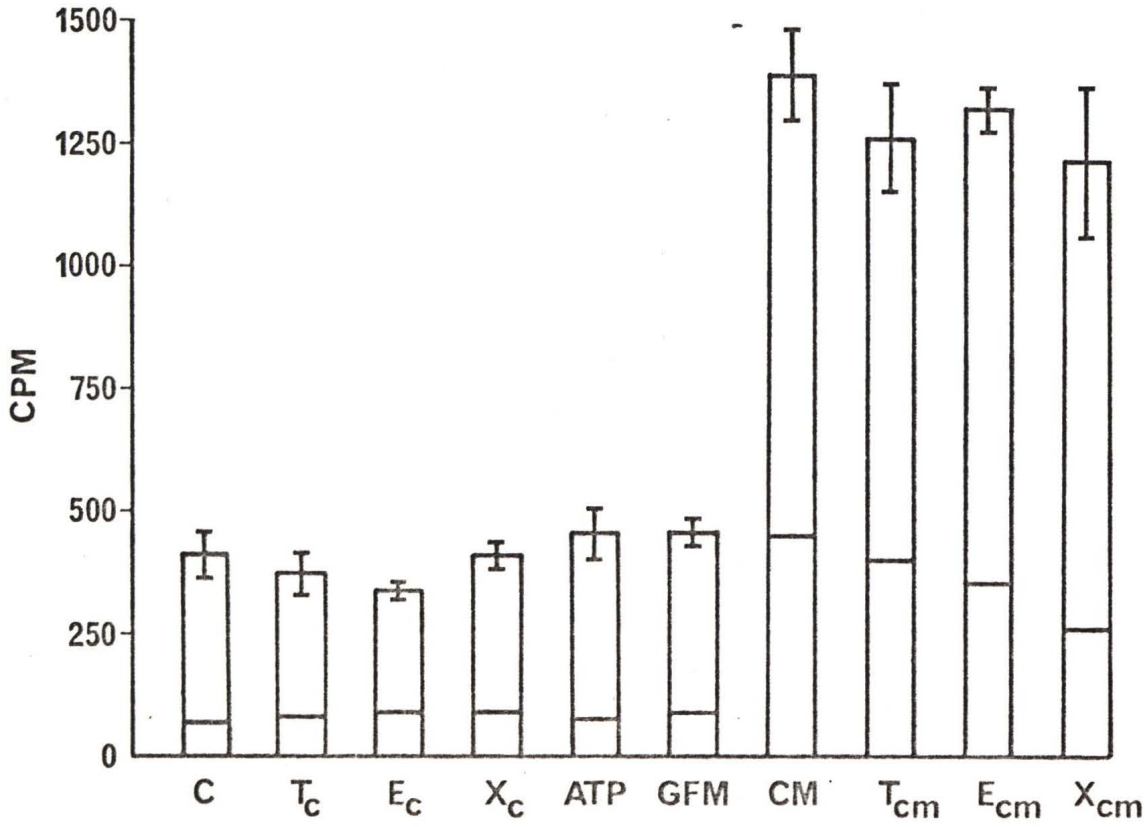
Figure 61. The effect of insulin on endocytosis by Chinese hamster cells. Insulin (bovine pancreas crystalline insulin, 26.4 I.U. per milligram, Sigma), used at 5.0 I.U. per ml. Theophylline and di-cAMP used at the same concentrations as in fig. 60. 3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 716,000 + 136,000 cells per vial. 8 hour incubation.

C = CTM
CI = CTM + insulin
CE = CTM + insulin + theophylline + di-cAMP
CM = CM
CMI = CM + insulin
CME = CM + insulin + di-cAMP

Figure 62. The effect of testosterone and 17-β-estradiol on endocytosis by Chinese hamster cells. Testosterone (Nutritional Biochemicals Corp., Cleveland, Ohio) used at 10^{-6} M, 17-β-estradiol (Sigma) used at 10^{-6} M. 3.92×10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). 8 hour incubation. Mean cell count = 687,000 + 94,000.

C = CTM
T_c = testosterone + CTM
E_c = 17-β-estradiol + CTM
X_c = testosterone + estradiol + di-cAMP + theophylline
ATP = 2 mg/ml ATP in CTM
GFM = GFM
CM = CM
T_{cm} = testosterone + CM
E_{cm} = estradiol + CM
X_{cm} = testosterone + estradiol + di-cAMP + theophylline


1500
1250
1000
750
500
250
0
CPM
C
CI
CE
CM
CMI
CME


1500
1250
1000
750
500
250
0
CPM
C
T_c
E_c
X_c
ATP
GFM
CM
T_{cm}
E_{cm}
X_{cm}

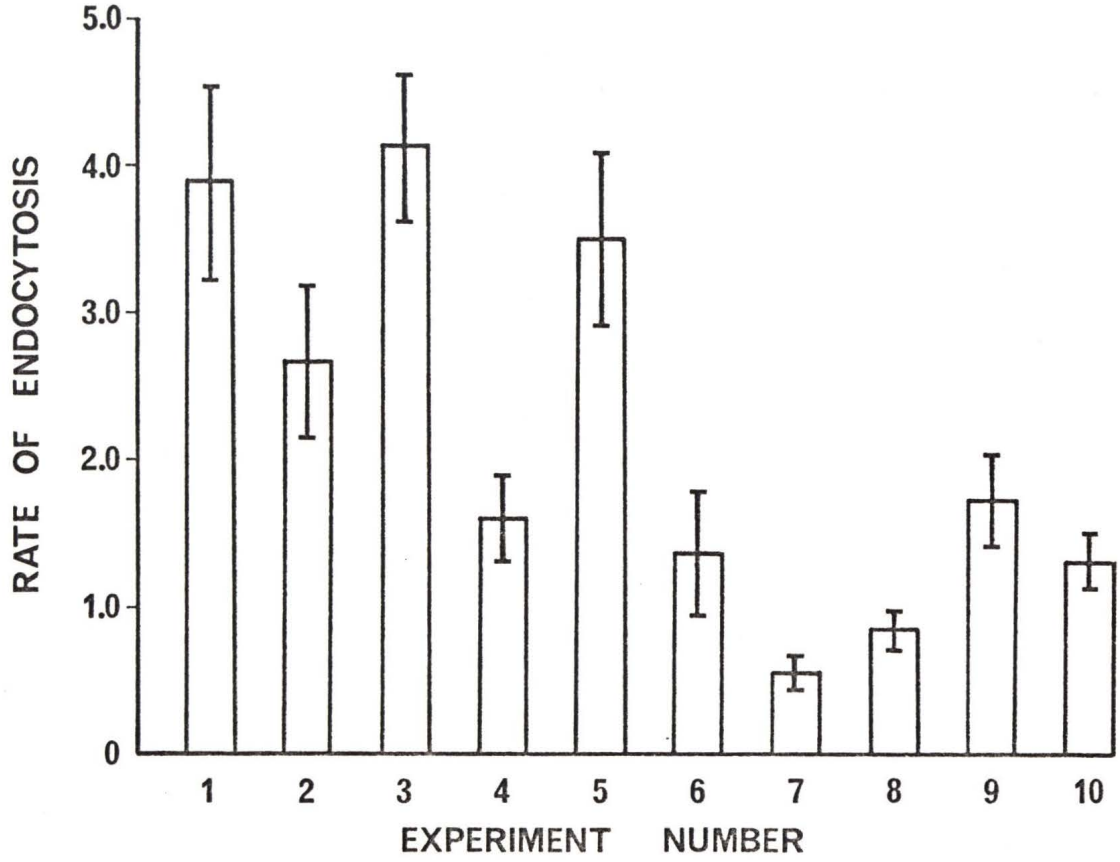
In order to investigate whether the CHA cell line had in some manner transformed to a less actively endocytosing state, CHA cells which had been frozen according to the method of Porterfield and Ashwood-Smith (63) after receipt of the cell line, and stored in liquid nitrogen, were thawed and subcultured. The rate of endocytosis with these cells is also shown in fig. 63. As can be seen, the cells which had been thawed (referred to as "resurrected cells") did not show the same relatively high rate observed in the earlier experiments. Nor did these cells exhibit the usual increased rate of endocytosis with CM (not shown). When this experiment was repeated (fig. 63), after two weeks with the same resurrected cells, which had by then undergone two additional passages, the rate of endocytosis was still considerably less than that observed in the earlier experiments. In this experiment some indication of an enhanced rate of endocytosis with CM was observed (not shown). Of considerable importance is the fact that the original rate of endocytosis observed in earlier experiments could not be reproduced, and remains unexplained.

Prostatic Cancer Cells (PCC) and the Conditioned Medium Effect

Two experiments were performed in order to determine whether the conditioned medium effect on endocytosis was a general phenomenon or only specific for CHA cells. The results are shown

Figure 63. Comparison of the rates of endocytosis by Chinese hamster cells in different experiments. Rate of endocytosis expressed in CPM/minute as determined by the slopes of the regression lines, per 10^6 cells. All rates of endocytosis shown are with CTM, with identical concentrations of sucrose-^{3}H. Vertical lines equal total standard error for the rate of endocytosis and mean cell count per vial.

Experiment No.	Figure in which it appears.
1	fig. 38
2	fig. 41
3	fig. 43
4	fig. 47
5	fig. 51
6	fig. 55
7	fig. 58
8	chromic acid cleaned scintillation vials
9	first resurrected cell experiment
10	second resurrected cell experiment


RATE OF ENDOCYTOSIS
5.0
4.0
3.0
2.0
1.0
0
1
2
3
4
5
6
7
8
9
10
EXPERIMENT NUMBER

Figures 64 and 65

Figure 64. The effect of CM and glycerine on endocytosis by PCC cells. CM was conditioned by the PCC cells used in this experiment, Millipored, and adjusted to pH 7.2. 1% and 5% glycerine by volume. Mean cell count = 1,170,000 ± 152,000 cells per vial. 3.92 X 10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Each point on the graph represents the mean of 3-5 samples. C = (●), CM = (■), 1% glycerine = (△), 5% glycerine = (o).

Slopes of the regression lines:

C (control) = 2.41 ± 0.19 CPM/minute
CM = 2.09 ± 0.48 CPM/minute
1% glycerine = 1.78 ± 0.45 CPM/minute
5% glycerine = 1.58 ± 0.35 CPM/minute

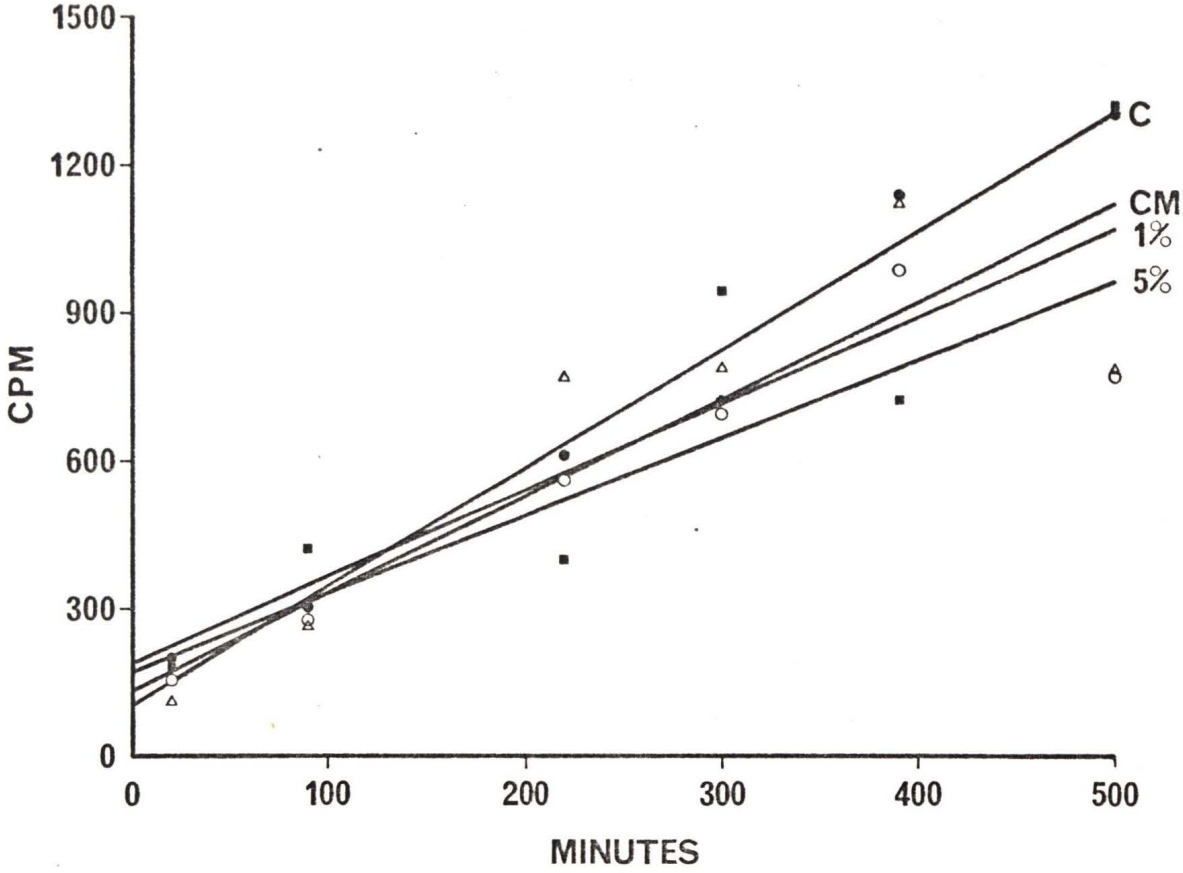
Rates are not significantly different.

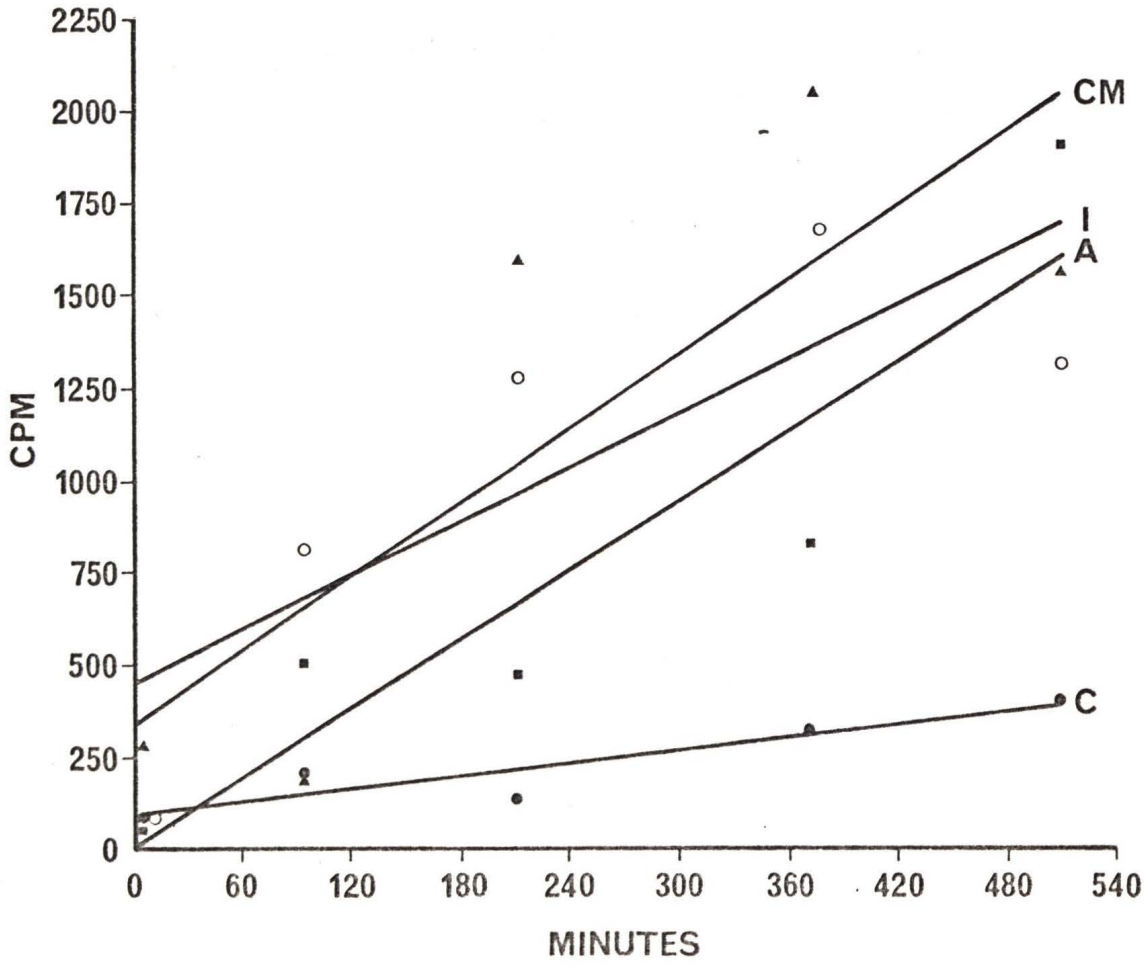
Figure 65. The effect of CM, di-cAMP and insulin on endocytosis by PCC cells. Di-cAMP and insulin used in the same concentrations as in previous experiments. 3.92 X 10^{-7} M sucrose-^{3}H, (0.666 µCi/ml). Mean cell count = 842,000 ± 138,000 cells per vial. Each point on the graph represents the mean of 3-5 samples. C (●) = CTM, CM (▲) = CM, I (o) = insulin in CM, A (■) = di-cAMP + theophylline in CM.

Slopes of the regression lines:

C = 0.58 ± 0.14 CPM/minute
CM = 3.34 ± 1.40 CPM/minute
I = 2.43 ± 0.98 CPM/minute
A = 3.13 ± 0.77 CPM/minute

CM, I, and A rates are not significantly different.


1500
1200
900
600
300
0
CPM
0
100
200
300
400
500
MINUTES
C
CM
1%
5%


2250
2000
1750
1500
1250
1000
750
500
250
0
CPM
0
60
120
180
240
300
360
420
480
540
MINUTES
CM
I
A
C

in figs. 64 and 65. Clearly the conditioned medium effect, although observed in one experiment with PCC cells, is not so reproducible as with CHA cells. Attempts to alter the rate of endocytosis with glycerin, di-cAMP and insulin were unsuccessful.

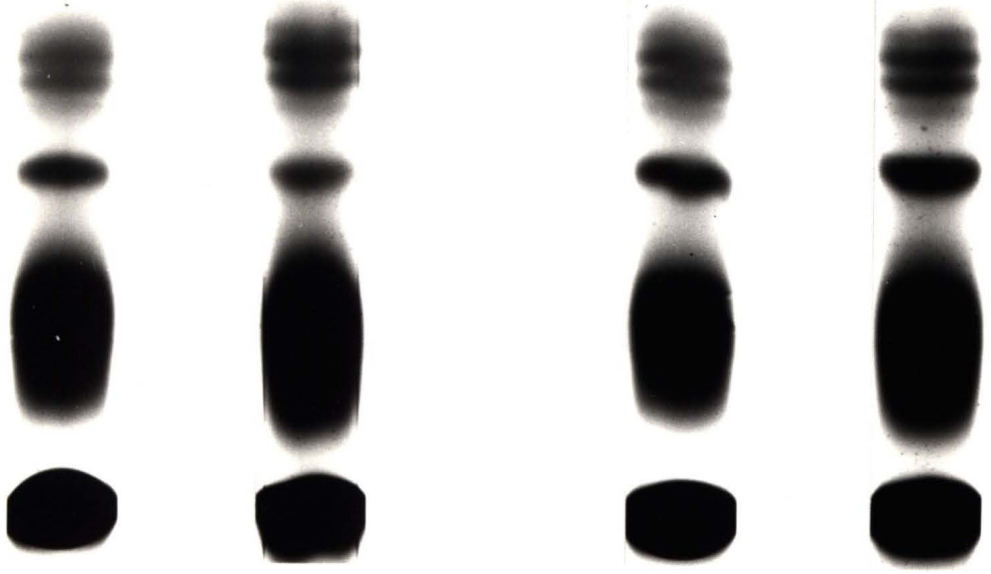
Electrophoresis

Polyacrylamide disc gel electrophoresis was performed on two samples each of CTM and CM conditioned by CHA cells. The results of three runs, two of which are shown in fig. 66, revealed no difference in the zone patterns. It therefore seems unlikely that there is any alteration of the protein component of CM compared to CTM. If a protein alteration with CM had been demonstrated, it could possibly explain the greater rate of endocytosis with CM as compared to GFM.

Oxygen Consumption Studies

The results shown in fig. 67 indicate that CB had no substantial effect on respiration by CHA cells, and hence is not a respiratory inhibitor with these cells. In order to determine whether there was any correlation between the CM effect and respiration, the experiment shown in fig. 68 was performed. The results clearly indicate that the rate of respiration is significantly less when the cells are in CM than in either GFM or CTM. Thus the increased rate of endocytosis observed with CM is not due to an overall increase in metabolism by these cells.

Figure 66. Electrophoretic zone patterns of CM and CTM proteins. 0.04 M tris-glycine pH 8.3 buffer, 250 volts, 25 milliamperes. Direction of migration from top to bottom. Numbers indicate different runs. Zones were more distinct in the original gels.


1
3
1
3
CM
CTM

Figures 67 and 68

Figure 67. Effect of cytochalasin B (CB) on respiration by Chinese hamster cells. Ordinate = change in % saturation of O_2. Abscissa = time in minutes. Slopes indicated are the mean of two experiments. C = CTM + 0.25% DMSO, CB = CTM + 25 µg/ml CB + 0.25% DMSO. 1.8 X 10^{-6} cells/ml. Eosin viability 46% after scraping cells off of stock culture flasks, and 28% after O_2 consumption determination, due to homogenizing effect of magnetic stirrer.

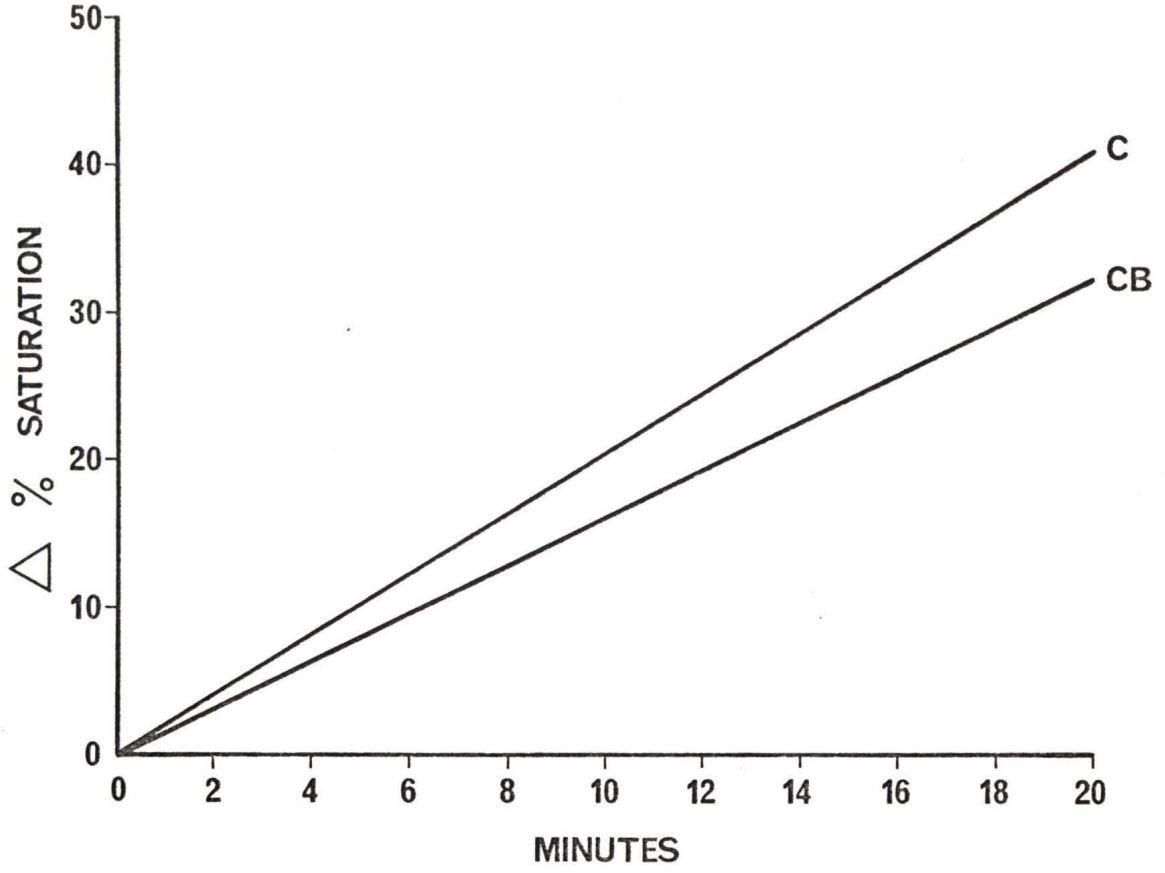
C = 2.05 ± 0.06 %/minute
CB = 1.61 ± 0.04 %/minute

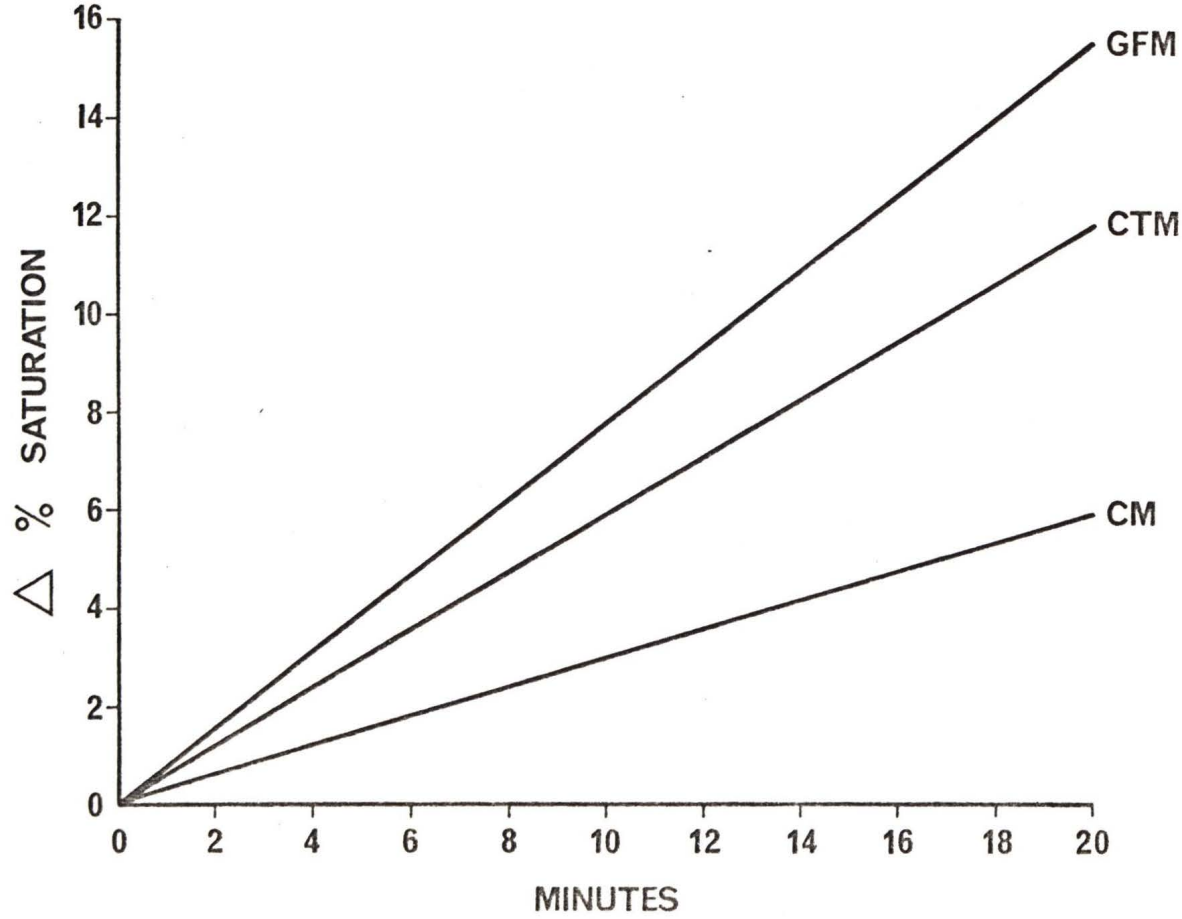
Rates are significantly different ($P < 0.001$).

Figure 68. Respiration by Chinese hamster in glucose free medium (GFM), conditioned medium (CM) and normal medium (CTM). Change in % saturation of O_2 as a function of time. Slopes indicated are the mean of three experiments. 5 X 10^{-5} cells/ml.

CTM = 0.59 ± 0.14 %/minute
GFM = 0.78 ± 0.17 %/minute
CM = 0.34 ± 0.03 %/minute

CTM and GFM rates are not significantly different. CM rate is significantly lower than CTM rate ($P < 0.05$).


50
40
30
20
10
0
△ % SATURATION
C
CB
0
2
4
6
8
10
12
14
16
18
20
MINUTES


16
14
12
10
8
6
4
2
0
△ % SATURATION
GFM
CTM
CM
0
2
4
6
8
10
12
14
16
18
20
MINUTES

DISCUSSION

Insulin, cyclic AMP, polyvinylpyrrolidone (PVP), serum, and ATP, all of which were inducers of endocytosis in various other cell systems, had no effect on endocytosis in Chinese hamster cells. Therefore generalities concerning the induction of endocytosis should be viewed with caution, particularly since the mechanism is unknown by which inducers of endocytosis cause the necessary movement of microfilaments that result in vesicle formation. It was suspected that the intracellular levels of cyclic AMP might be a factor in the regulation of microfilament activity, in this case endocytosis, as is the case in pigment granule translocation in frog melanocytes (56), exocytosis in the toad urinary bladder (54), and endocytosis in thyroid slices (11). However, the work presented here with hamster cells does not support this.

While hamster cells have been shown by autoradiography to take up 17-β-estradiol-^{3}H, which then is found concentrated on or in the nucleus (1), 17-β-estradiol has no effect on endocytosis in hamster cells, at least within 8 hours incubation, and therefore does not facilitate its own uptake. Since the hamster cells were derived from an ovary cell, it would not have been surprising had the hamster cells responded to 17-β-estradiol with an altered rate of endocytosis, since estrogens are known to exert direct

influences on ovaries (79). Testosterone, which might have been expected to have had the opposite effect of 17-β-estradiol, also had no influence on the rate of endocytosis. The negative results obtained with cyclic AMP, 17-β-estradiol, testosterone, and insulin indicate that hormones probably do not directly influence endocytosis in Chinese hamster cells.

The results obtained with conditioned medium (CM) do, however, suggest the existence of regulatory systems which control the rate of endocytosis. When hamster cells were incubated with CM, the rate of endocytosis was increased, usually 3 to 5 times the rate observed with normal medium (CTM), an effect which could be noticeably reversed with concentrations of D-glucose as low as 1.6×10^{-6} g/ml (8.9×10^{-6} M). D-mannose, D-fructose and sodium pyruvate (the latter compounds were not assayed for their effect at lower concentrations) resulted in the reversal of the conditioned medium effect similar to that observed with D-glucose. These results suggest the existence of a complex metabolic feedback system, since it seems unlikely that these compounds, all of which can be readily utilized by the cell through the normal glycolytic pathway, would be themselves inhibitors of endocytosis in the usual sense. Rather, it seems more likely that some product of the normal metabolism of these sugars and pyruvate results in a specific regulation of the rate of formation of endocytotic vesicles, in much the same manner as end-product (feedback) inhibition in intermediary metabolism. Such a system could

conceivably serve the function of transporting metabolisable materials into the cell only when the intracellular levels of energy sources became sufficiently low to warrant active uptake of these materials.

Clearly hamster cells are respiring at a lower level in conditioned medium (CM) than in either normal medium or glucose free medium (GFM), while endocytotic activity is greater in conditioned medium (CM) that in either CTM or GFM. Mere removal of glucose from the medium (GFM) is not sufficient to produce the relatively high rate of endocytosis observed with CM, but the addition of glucose to CM is sufficient to obliterate this effect, which further complicates the matter.

These results all suggest that some normal product of glycolysis, or perhaps the Kreb's cycle, since pyruvate also had some effect, has a regulatory effect on endocytosis. ATP is a possible candidate for this role, despite the fact that it had no effect when added to CM. ATP may not be able to enter the cell, either directly across the cell membrane, or indirectly across the plasma membrane which has invaginated to form endocytotic vesicles, since the plasma membrane is known to possess ATPase enzymes which would hydrolyze ATP to ADP + P_i.

In any case, further elucidation of the CM effect will require extensive biochemical and ultrastructural studies to determine what intracellular changes occur when hamster cells are incubated in CM as compared to CTM or CM plus glucose.

Nevertheless the importance of this effect should not be overlooked, since this is the first reported incident in which cells were observed to endocytose at a greater rate when their environmental milieu became deficient in essential nutrients. This observation suggests that cells may endocytose in response to their nutritional requirements.

Both the morphological observations and the studies of sucrose-^{3}H uptake, with and without cold sucrose present, support the observations of Munro (58) and Wagner *et al.* (82) that sucrose is taken up by endocytosis, and if present in a sufficient concentration to exert an osmotic effect, can cause endocytotic vesicles to accumulate. The accumulation of these sucrose laden vesicles is not due to an induction or stimulation of endocytosis, as indicated both by this work and the results obtained by Wagner *et al.* The rate of endocytosis by Chang liver cells was shown by Wagner *et al.* to decline after 3 hours exposure to 0.08 M sucrose, an effect not observed with hamster cells. They attribute this decline to a lack of available plasma membrane, since the plasma membrane utilized to form endocytotic vesicles in the presence of 0.08 M sucrose cannot be reutilized, due to the persistence of these vesicles. The same effect might have been observed with hamster cells if the experiment had been continued for a longer period of time, and the lack of inhibition of endocytosis by 0.08 M sucrose

after 3 hours with hamster cells may merely reflect differences in the rate of endocytosis in the two cell systems or in rates of membrane synthesis.

Cytochalasin B (CB) inhibited endocytosis in both hamster cells (to 36% of the control level with 20 µg/ml of CB) and prostatic cancer cells PCC (to 77% of the control level with 20 µg/ml of CB). Since there was no indication that higher concentrations of cytochalasin B would have inhibited endocytosis further, to any significant extent, the possibility exists that there are microfilaments which are relatively insensitive to this drug. The observations by Goldman (34) on the effect of cytochalasin B on the microfilaments of BHK-21 cells demonstrated the existence of cytochalasin B resistant microfilaments in these cells. The possibility that there are different classes of microfilaments in hamster cells and prostatic cancer cells, with different sensitivities to cytochalasin B, warrants investigation by electron microscopy.

In spite of the fact that endocytosis was not inhibited completely by cytochalasin B in either the hamster of prostatic cancer cell lines, profound morphological changes occurred with both cell lines. Most important with respect to this study is the fact that no membrane ruffling occurred in the presence of cytochalasin B. However, the formation of endocytotic vesicles was never observed to occur anywhere except in areas of membrane

ruffling, as observed with phase contrast and Nomarski optics. Endocytosis seems to continue, albeit at a reduce rate, in the absence of areas of membrane ruffling. Therefore it must be concluded that the membrane ruffling, thought to be necessary for endocytosis, may in fact not be necessary. This is supported by the failure to detect areas of classical membrane ruffling with prostatic cells, which also actively endocytosed sucrose-^{3}H. Endocytosis in the absence of membrane ruffling must result in the formation of endocytotic vesicles which are too small to be seen with the light microscope (less that 0.4 μ in diameter). The formation of such vesicles may involve cytochalasin B insensitive microfilaments.

The differences observed between the morphological effects of endocytosed sucrose and endocytosed dextran on hamster cells can be explained on the basis of the much higher molecular weight of dextran, and therefore the lower osmotic effect which would be expected. The similarities in the effects produced by dextran and sucrose can be explained on the basis that sucrose is a disaccharide, while dextran is a polysaccharide, and hence would have similar chemical properties. Since dextran was present at a concentration of 10% weight per volume in the medium, low molecular weight impurities, which may be present in commercial preparations of dextran could explain the osmotic effects observed, which resemble those produced with sucrose with hamster cells. The increase in phase density of dextran containing vesicles in hamster cells can be explained by the accumulation of dextran within these vesicles. The phase dense, semicircular rings, observed

on these vesicles, as well as on PVP laden vesicles, are a mystery. These rings may be areas where phase dense granules (probably lysosomes) are attempting to fuse with the vesicles to discharge their enzymes into the vesicles. The difference in both size and phase density between sucrose and dextran induced vesicles in hamster cells, as compared to the much smaller vesicles observed with prostatic cancer cells, strongly suggests a difference in both the size of endocytotic vesicles formed in these two cell systems, as well as their relative tendency to fuse with one another to form larger vesicles. Since the formation of endocytotic vesicles was not observed with the light microscope with prostatic cancer cells, endocytosis in these cells presumably occurs by the formation of submicroscopic (less than 0.4 μ diameter) vesicles.

The results obtained by the endocytosis of 10 % PVP by prostatic cancer cells are particularly striking, and quite in contrast to the results obtained by the endocytosis of PVP by Chinese hamster cells, which are nearly indistinguishable from the effects produced by 10 % dextran in hamster cells. Clearly PVP has deliterious effects on PCC cells which may be a direct result of damage to the lysosomal-vacular system of these cells. PVP, despite its alleged lack of toxicity (33) and widespread use as a plasma expander (33), may in fact result in the deterioration of secondary lysosomes in prostatic cancer cells, and perhaps other cell types as well, with a concomitant release of lysosomal enzymes into

the cytoplasm, which could produce the observed effects. Such a hypothesis seems reasonable, as the cytotoxicity of numerous compounds and particles, for example asbestos, silica, and carrageenan, a sulfated polygalactose, which when endocytosed are found in secondary lysosomes, have been shown to exert damaging, often carcinogenic effects on other cell and animal systems (2). Why the effect observed with PVP on prostatic cancer cells is not observed with hamster cells, is open to speculation.

The cryoprotective action of polymers such as dextran and polyvinylpyrrolidone (PVP) appear unrelated either to the endocytotic uptake of these compounds, or to binding to the plasma membrane. These compounds have been shown not to bind to the plasma membrane of hamster cells. Serum, which is known to increase the survival of cells exposed to freezing and thawing regimes, has no effect on endocytosis, and hence the uptake of cryoprotective polymers (5). Cytochalasin B, which inhibits endocytosis, also has no effect on cell survival after freezing and thawing (5). Thus it appears that the cryoprotective action of these polymers does not take place either as a result of the polymer being taken into the cell, or directly onto its surface by binding to the plasma membrane. Thus the cryoprotective action of these polymers must occur as a result of changes in the physical-chemical properties of the medium in which the cells are frozen and thawed.

In conclusion, this study has provided evidence that factors which influence endocytosis in other cell systems have no effect on hamster cells, whereas evidence has been presented with suggests a

control of endocytosis in these hamster cells in response to metabolic requirements, rather than external inducing agents.

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