To the Graduate Council:

I am submitting herewith a dissertation written by Kurt Amsler entitled "Development of Na^+-Dependent Hexose Transport in a Pig Renal Epithelial Cell Line, $LLC\text{-}PK_1$." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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DEVELOPMENT OF Na^+-DEPENDENT HEXOSE TRANSPORT IN A PIG RENAL EPITHELIAL CELL LINE, LLC-PK_1

A Dissertation

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Degree

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ABSTRACT

LLC-PK_1 cells in culture develop the ability to concentrate hexose. The concentrative ability is dependent on the Na^+-electrochemical gradient, and is inhibited by phlorizin with $K_{I,0.5}$ = 0.2 μM. Development of the transport function can be accelerated by addition of the Friend cell inducer hexamethylene bisacetamide and by the phosphodiesterase inhibitors dibutyryl cAMP, theophylline, and 1-methyl-3-isobutyl xanthine. In cultures treated with any of these chemicals development of hexose concentrating capacity is inhibited by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). In all cases the development of hexose accumulating capacity is correlated with growth arrest and increased intracellular cAMP levels. However the results suggest that these changes may be necessary but are not sufficient conditions for induction of the hexose transport function.

The level of concentrative hexose uptake begins to increase only after the level of Na^+-dependent amino acid transport has decreased (step-down). The decrease is accelerated by those compounds that accelerate appearance of concentrative hexose transport and is retarded by TPA which retards appearance of the hexose transport function. Addition of TPA to stepped-down cultures produces a rapid increase in the rate of amino acid transport. This increase is dependent on protein and RNA synthesis.

The LLC-PK_1 cell line was cloned and three clones were derived. Each of these clones is capable of developing Na^+-dependent hexose

transport. One of these clones was used to analyze the development of hexose accumulating capacity in a cell population using a mathematical model developed by J. S. Cook. Modulation of only two parameters can yield the experimentally obtained results. One of these two possible mechanisms, regulation through a changing driving force for uptake, has been ruled out. It is concluded that development of concentrative hexose transport is probably due to an increase in the fraction of the cell population which is capable of concentrative hexose uptake. Expression of this transport function is an irreversible process. Further, the decision of a cell to express concentrative hexose uptake occurs just prior to or at the same time as expression of the transport function.

The number of Na^+-dependent hexose transport sites can be determined by measuring the number of Na^+-dependent phlorizin binding sites. During development of hexose accumulating capacity there is a corresponding increase in the number of Na^+-dependent phlorizin binding sites with no consistent alteration in the affinity of binding. Thus development of hexose accumulating capacity in a cell population appears to be due to the progressive expression of active Na^+-dependent hexose transporters.

The photoaffinity label for the Na^+-dependent hexose transporter, 4-azido-phlorizin, was studied to determine its usefulness in this system. The compound reversibly inhibits hexose uptake with $K_{I,0.5}$ = 1 μM when the uptake is carried out in the dark. Exposure of cells to 4-azido-phlorizin and blacklight results in an irreversible inhibition of hexose uptake. This inhibition is dependent on the presence of Na^+

in the incubation medium and can be blocked by simultaneous inclusion of phlorizin or D-glucose during exposure to light. Therefore a radioactive derivative of this compound, at a high enough specific activity, can be used to specifically and irreversibly label the Na^+-dependent hexose transporter expressed by these cells.

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CHAPTER I

GENERAL INTRODUCTION

The study of differentiation and development at the cellular level has been aided in recent years by the development of continuous cell lines which in the presence of appropriate stimuli express differentiated properties. Studies using these various model systems have provided insights into the molecular events regulating the acquisition and maintenance of the differentiated phenotype.

The investigation of epithelial cell differentiation has utilized a number of model systems (Handler et al., 1980). These include two cell lines derived from kidney, the MDCK cell line from an adult dog kidney and the LLC-PK_1 cell line derived from a juvenile pig kidney (Hull et al., 1976). With these lines morphological and functional aspects of epithelial cell differentiation have been studied in vitro.

At confluence MDCK cells form a continuous monolayer. The cells display a polarized cellular morphology with a microvillar surface, the brushborder membrane, facing the growth medium and a relatively smooth surface, the basal membrane, facing the substratum (Leighton et al., 1969; Misfeldt et al., 1976; Cereijido et al., 1978; Rindler et al., 1979; Cereijido et al., 1980). The apical membrane is separated from the basal and lateral membranes by tight junctions (Misfeldt et al., 1976; Cereijido et al., 1978; Rindler et al., 1979) which seal the monolayer, forming a permeability barrier impeding the passive diffusion of substances across the monolayer. There may be some disposition of

intracellular organelles towards the basal surface (Cereijido et al., 1978). LLC-PK_1 cells display a similar morphology at confluence (Perantoni and Berman, 1979; Mullin et al., 1980; Misfeldt and Sanders, 1981). These features are typical of kidney epithelial cells in vivo.

The morphologic polarity is mirrored by a functional polarity both in MDCK and LLC-PK_1 cells in culture and in kidney epithelial cells in vivo. In vivo, kidney epithelial cells display an asymmetric distribution of both membrane enzyme and transport components. For example, in the proximal tubular portion of the nephron Na^+-K^+-ATPase and the Na^+-independent hexose transport system are localized to the basolateral surface whereas a number of disaccharidases, alkaline phosphatase, and the Na^+-dependent hexose transport system are localized to the brushborder membrane. In both MDCK cells (Barker et al., 1978; Cereijido et al., 1980; Simmons, 1981) and LLC-PK_1 cells (Mills et al., 1979) at confluence the Na^+-K^+-ATPase has been localized by autoradiography of [^{3}H]ouabain binding sites to the basolateral membrane. In addition, lactoperoxidase-catalyzed iodination of each cell surface indicates that MDCK cell monolayers possess a different set of accessible proteins at each of the two surfaces (Richardson and Simmons, 1979).

In cultured monolayers epithelial cells carry out net transepithelial transport of a number of substances similar to the transport in the animal. When grown on a solid substratum, confluent MDCK monolayers contain many "domes" (Leighton et al., 1969), which are fluid-filled pockets between the monolayer and the substratum generated by transepithelial (net apical to basolateral) salt and water transport. The

fluid accumulates under the monolayer, raising it off of the substratum. When the pressure generated by the fluid becomes too great the dome bursts and the cell sheet settles on the substratum (Rabito et al., 1980). If cells are grown on a permeable substratum, such as a Millipore or Nucleopore filter, this dome formation does not occur since the transported salt and water is not trapped underneath the monolayer. Net apical to basolateral Na^+ flux has been measured in confluent MDCK monolayers (Misfeldt et al., 1976; Cereijido et al., 1978; Cereijido et al., 1980; Simmons, 1981). Ion fluxes have not been directly measured in LLC-PK_1 cells, however indirect measurements indicate that transepithelial ion and water transport also occur in this cell line. Confluent cultures of LLC-PK_1 cells, like MDCK cells, display domes when grown on a solid substratum. In addition, LLC-PK_1 cells possess a transepithelial potential difference, a manifestation of net transepithelial ion transport (Misfeldt and Sanders, 1981). Further, under appropriate conditions a short circuit current, I_{sc}, is generated. This is due to a net Na^+ flux (Misfeldt and Sanders, 1981). In addition to transepithelial ion and water transport confluent LLC-PK_1 cultures display net transepithelial D-glucose transport, the flux being oriented in an apical to basolateral direction (Misfeldt and Sanders, 1981). The presence of transepithelial D-glucose transport in cultured cells is of particular interest since this transport function is normally only observed in the terminally differentiated kidney proximal tubule, intestine, and choroid plexus.

In vivo, net transepithelial hexose transport is achieved by the asymmetric orientation of the two distinct hexose transport systems. A Na^+-dependent hexose transport mechanism is located at the apical membrane and, utilizing the energy of the Na^+-electrochemical gradient oriented into the cell, couples the transport of D-glucose to the transport of Na^+. This results in a net uptake of hexose into the cell against a concentration gradient. At the basolateral membrane surface hexose exits from the cell down its concentration gradient by Na^+-independent facilitated diffusion. The presence of net transepithelial hexose transport in LLC-PK_1 cells suggests that a similar asymmetric orientation of transport functions exists in these cells. Misfeldt and Sanders (1981) found that the I_{sc} was only generated when hexose was added to the apical bathing solution. Addition of hexose to the basal bathing solution did not generate an I_{sc}. These results are interpreted as indicating that there is cotransport of Na^+ and D-glucose into the cell only from the apical bathing solution. The Na^+ and D-glucose then exit through the basolateral membrane producing net Na^+-dependent D-glucose transport and net D-glucose-dependent Na^+ transport in an apical to basolateral direction. The Na^+ is transported out of the cell by the Na^+-K^+-ATPase and the D-glucose is transported out by a facilitated diffusive hexose transporter.

Direct evidence for the existence of a Na^+-dependent hexose transport mechanism in LLC-PK_1 cells is provided by the demonstration of concentrative hexose uptake in confluent monolayers (Mullin et al., 1980; Rabito and Ausiello, 1980). The level of concentrative hexose uptake is

measured by determining the accumulation of α-methyl-D-glucopyranoside (α-meG) in the cells against a concentration gradient. This hexose is a nonmetabolizable D-glucose analog that is highly specific for the Na^+-dependent hexose transport function. Since the cell can only concentrate hexose if this transport mechanism is present, the accumulation of α-meG can be used as a relative measure of the expression of this transport function in the cell population. Rabito (1980) has found that concentrative hexose uptake and Na^+-dependent phlorizin binding, a marker for the Na^+-dependent hexose transporter, are located at the apical surface of $LLC\text{-}PK_1$ cell monolayers grown on collagen-coated Nucleopore filters. This is in agreement with the localization of the Na^+-dependent hexose transport function in the kidney.

Concentrative hexose uptake in $LLC\text{-}PK_1$ cells is Na^+-dependent (Mullin et al., 1980; Rabito and Ausiello, 1980), and inhibited by a number of competing hexoses (Mullin et al., 1980; Rabito and Ausiello, 1980), sulfhydryl reagents (Rabito and Ausiello, 1980), and low concentrations of phlorizin (Mullin et al., 1980; Rabito and Ausiello, 1980). These characteristics are very similar to those of the Na^+-dependent hexose transport system of the renal proximal tubular segment.

The transepithelial D-glucose transport by $LLC\text{-}PK_1$ cell monolayers is also dependent on the presence of Na^+ and is inhibited when phlorizin is added to the apical but not the basal bathing solution (Misfeldt and Sanders, 1981). In addition ouabain, a potent inhibitor of Na^+-K^+-ATPase, reduces the transepithelial hexose transport when added to the basal but not the apical bathing solution (Misfeldt and Sanders, 1981).

In the presence of D-glucose the cell monolayer generated an I_{sc} due to the transepithelial transport of Na^+ that is cotransported with the D-glucose. Addition of ouabain significantly reduces the magnitude of this current indicating that the Na^+ flux, and therefore the D-glucose flux, has been decreased. This action of ouabain is presumably through inhibition of the Na^+-K^+-ATPase which will cause an increase in the level of intracellular Na^+ and thus a decrease in the magnitude of the Na^+-electrochemical gradient which drives Na^+-hexose cotransport.

Besides D-glucose, addition of a number of other hexoses to the apical bathing solution stimulate the I_{sc}. These hexoses act in a hierarchy similar to that observed for inhibition of concentrative α-meG uptake (Misfeldt and Sanders, 1981). These findings strongly suggest that at confluence LLC-PK_1 cells possess an asymmetric distribution of the two hexose transport systems which is identical to that observed in vivo.

The most useful model systems for study of development and differentiation are those in which the expression of some characteristic function can be modulated. A number of laboratories have studied the effects of various compounds on the regulation of dome formation as a marker for the presence of transepithelial salt and water transport. In MDCK cells (Leighton et al., 1969; Lever, 1979) and in LLC-PK_1 cells (unpublished observation) domes are usually not observed until the cells reach confluence. Valentich et al. (1979) have found that compounds which elevate intracellular cAMP levels enhance the number and size of domes in confluent MDCK monolayers. Lever (1979) has found that in

addition to compounds which elevate intracellular cAMP levels, compounds which induce differentiation of Friend erythroleukemia cells (Marks and Rifkind, 1978), e.g. DMSO and hexamethylene bisacetamide (HMBA), also stimulate dome formation. Dome formation is inhibited by cytoskeletal disrupting agents, cytochalasin B and vinblastine, by the protein synthesis inhibitors cycloheximide and puromycin, and by ouabain (Lever, 1979). However, in contrast to induction of hemoglobin synthesis in Friend erythroleukemia cells (Marks and Rifkind, 1978), induction of dome formation in MDCK monolayers is a reversible process (Lever, 1979). Rabito et al. (1980) have shown that the number and size of domes in MDCK monolayers can be modulated by manipulating the strength of the cell-substratum adhesion. They suggest that most if not all modulation of dome formation by various compounds is due to the effect of these compounds on cell-substratum adhesion and not to an effect on expression of a differentiated transport function, i.e. vectorial salt and water transport. For this reason dome formation may not be a suitable marker for monitoring the regulation of epithelial cell differentiation in culture.

As described above, LLC-PK_1 cells express another transport function normally observed only in the terminally differentiated cell, Na^+-dependent hexose transport (Mullin et al., 1980; Rabito and Ausiello, 1980; Misfeldt and Sanders, 1981), in addition to trans-epithelial salt and water transport. Mullin et al. (1980) report that the level of this transport function is dependent on cell density, being absent in subconfluent cultures and increasing progressively with

increasing time at confluence. This finding suggests that LLC-PK_1 cells may modulate expression of Na^+-dependent hexose transport during growth in culture and thus could serve as a model system for the study of the acquisition of a differentiated epithelial transport system. This thesis describes such an analysis of the development of Na^+-dependent hexose transport during growth of LLC-PK_1 cells in culture.

Initially the level of Na^+-dependent hexose transport was examined during growth in culture, by monitoring the level of α-meG accumulation. In sparse cultures there was no evidence of concentrative hexose uptake. As the cells attained confluence the transport function first became evident and the level continued to increase with increasing time at confluence. The time course of appearance of the transport function could be modulated by addition of various compounds to the growth medium. Addition of compounds which elevate intracellular cAMP levels (dibutyryl cAMP, theophylline, and 1-methyl-3-isobutyl xanthine), as well as addition of the Friend cell inducer HMBA, accelerated the appearance of concentrative hexose transport. The appearance of the transport function was inhibited by addition of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to the growth medium. TPA is a potent tumor promotor that has been found to inhibit cellular differentiation in a number of systems (Diamond et al., 1978) but promote differentiation in other cell systems (Huberman et al., 1979).

In addition, the regulation of another transport function, Na^+-dependent amino acid transport, was examined during growth in culture. This transport function exhibited regulation reciprocal to

that of Na^+-dependent hexose transport. In particular, addition of TPA caused an elevation of the level of Na^+-dependent amino acid transport even after the cultures had decreased the level of the transport function. This effect was examined in more detail.

The development of Na^+-dependent hexose transport in a cell population was then analyzed using a mathematical model based on the electrical analog of the charging of a capacitor. To obtain a homogeneous cell population for use in this analysis the LLC-PK_1 cell line was cloned. One of the cloned lines obtained, Cl 4, was used in this study. The development of α-meG accumulating capacity by a Cl 4 cell population could be explained by only two of the five proposed mechanisms for development. Of these two, one could be ruled out on the basis of other data. Thus it was concluded that development of Na^+-dependent hexose transport in a Cl 4 cell population is through the acquisition of this transport function by an increasing proportion of the cell population.

Finally, using Na^+-dependent [^{3}H]phlorizin binding as a measure for the number of active Na^+-dependent hexose transporters, a method which has been established for in vivo systems, the change in the number of active transport molecules was examined during development of concentrative hexose transport. In parallel with this, the use of 4-azidophlorizin as a photoaffinity probe for the Na^+-dependent hexose transporter of Cl 4 cells was assessed.

CHAPTER II

DEVELOPMENT OF Na^+-DEPENDENT HEXOSE TRANSPORT IN LLC-PK_1 CELLS

Introduction

As discussed in Chapter I, Mullin et al. (1980) and Rabito and Ausiello (1980) have described a Na^+-dependent hexose transport mechanism in LLC-PK_1 cells. This transport process is a function normally found only in terminally differentiated epithelial such as kidney and intestine. Further, Misfeldt and Sanders (1981) have reported that these cells carry out unidirectional transepithelial hexose transport, indicating that these cells become not only morphologically but functionally polarized at confluence. Mullin et al. (1980) report that expression of this transport function is dependent on cell density, suggesting that LLC-PK_1 cells may modulate expression of Na^+-dependent hexose transport during growth in culture.

In this chapter the development in vitro of differentiated Na^+-dependent hexose uptake in LLC-PK_1 cells is described and it is shown to have many similarities to the Na^+-dependent hexose transport mechanism reported for the renal proximal tubule brush border membrane. In addition inclusion of a number of compounds in the growth medium can either accelerate or delay the time course of expression of this function. Using these compounds it is shown that elevated intracellular cyclic AMP may be a necessary but is not a sufficient effector for the development of the differentiated transport mechanism.

Materials and Methods

Cells and Culture

$LLC\text{-}PK_1$ cells were the kind gift of Dr. Robert Hull, Eli Lilly Laboratories, Indianapolis, Indiana. The cells were grown at 37°C in a humidified 5% CO_2 atmosphere. Stock cultures were grown in Falcon or Corning T-75 flasks containing 10 ml Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum (Grand Island Biological Corporation) plus 2 mM L-glutamine (complete medium). Confluent stock cultures were passaged by trypsinization and replating at 1:10 dilution. The medium was changed every 2 days.

For experiments, confluent stock cultures were trypsinized and plated in 35 mm Falcon plastic tissue culture dishes containing 2 ml complete medium or on collagen-coated Nucleopore filters (25 mm filter diameter, 5 μm pore diameter) attached to the bottom of Lab-Tek plastic petri dishes (100 mm × 100 mm) containing 20 ml complete medium. The density of cells was 5.5×10^3 cells/cm^2 for both plating protocols. Collagen-coated filters were prepared by the method of Rabito et al. (1978) and gas sterilized with ethylene oxide. Two days after the cells were plated, each filter was detached with forceps and floated in a 35 mm plastic petri dish containing 2 ml of either complete medium or medium with added compounds as indicated. The cells plated in tissue culture dishes were fed with 2 ml complete medium or medium with added compounds two days after plating. The medium was changed every 2 days.

Measurement of Uptakes

The procedure for measurement of uptake was essentially the same for all compounds assayed. Plates containing cells were removed from the incubator and placed on ice. The medium was poured off and the dishes were rinsed 3 times with cold Hanks balanced salt solution (HBSS) buffered to pH 7.4 with 10 mM Hepes, and 0.9 ml HBSS was added back to each dish. The plates were then placed in a water bath at 37°C for 2 minutes. The uptake assay was initiated by addition of 0.1 ml HBSS containing radioactive substrate; final concentrations of both [^{14}C]-α-methyl-D-glucopyranoside (α-meG, New England Nuclear) or [^{14}C]-3-O-methyl-D-glucose (meGlc, New England Nuclear) were 100 μM, 0.2 μCi/ml. Other compounds as indicated were included in the uptake-assay medium. The plates were incubated at 37°C for the desired time period. At the end of this time the medium containing the radioactive substrate was poured off and the dishes were rapidly rinsed 3 times in ice-cold Tris-buffered saline solution (TBS) at pH 7.4. The monolayers were then solubilized in 2 ml 0.2% sodium dodecyl sulfate (SDS, BDH Biochemical Company). One ml was added to a scintillation vial containing 9 ml ACS counting solution (Amersham) and the radioactivity was measured in a Packard liquid scintillation counter. Protein was measured using 0.5 ml of the SDS-solubilized cell solution by the fluorometric technique of Avruch and Wallach (1971). Error bars indicate ± one standard deviation on triplicate samples. Where only duplicate samples were taken, the points represent the average.

[^{3}H]Thymidine Incorporation

In the same procedure used for uptakes, cells were incubated in 1 μCi/ml [^{3}H]thymidine at 37°C for 60 min. After three TBS washes, 1 ml ice cold 10% trichloroacetic acid (TCA) was added to the plates which were then allowed to stand at room temperature for at least 10 min. The TCA was aspirated off, and the plates were rinsed twice with cold TCA and 3 times with cold 95% ethanol. The TCA-insoluble material was resolubilized in 1.5 ml 1 N NaOH. One ml was added to a scintillation vial and neutralized with 0.1 ml 10 N HCl. The radioactivity and protein were measured as described above.

As a check on whether α-meG and meGlc were metabolized, LLC-PK$_1$ cells were allowed in separate measurements to take up these compounds for 60 min. After the cultures were rinsed in cold TBS, more than 99% of the radioactivity could be extracted in 80% methanol. On paper chromatography with n-butanol:acetic acid:water (7:2:5) as solvent, a system that readily separates phosphorylated from nonphosphorylated hexoses, the extracted radioactivity comigrated with the authentic parent compound; no secondary peaks were detected. Both of these transported substrates therefore appear to remain unmetabolized in these cells.

Measurement of Intracellular Cyclic AMP Levels

The medium was poured off and the plates were rinsed 3 times with cold TBS. One ml 80% methanol was added, and the plates were allowed to stand at room temperature for at least 10 minutes. The methanol soluble material was removed to a 1.5 ml Sorvall plastic microfuge tube, and the

tube incubated at 37°C until the methanol had evaporated. Fifty µl of Tris-EDTA buffer was added to each tube, and the cyclic AMP (cAMP) was assayed with a cAMP Assay Kit (Amersham) according to the directions provided by the manufacturer. The cAMP assay was not affected by the test compounds 1-methyl-3-isobutyl xanthine (MIX), HMBA, or TPA at ten times the medium concentrations.

Other Materials

Phorbol, 12-O-tetradecanoylphorbol-13-acetate (TPA), and 4-O-methyl TPA were the generous gift of Dr. T. J. Slaga. Hexamethylene bisacetamide (HMBA) was prepared by the method of Germinario et al. (1977). α-Methyl-D-glucopyranoside, 3-O-methyl-D-glucose, 1-methyl-3-isobutyl-xanthine, theophylline, and dibutyryl cyclic AMP were from Sigma. All other chemicals were reagent grade.

Results

Properties of Concentrative α-meG Uptake in Confluent LLC-PK_1 cells

The time course of α-methyl-D-glucopyranoside (α-meG) uptake by just-confluent LLC-PK_1 cells in the presence and absence of Na^+ is shown in Fig. II-1a. Na^+-free medium was prepared by substituting an equimolar concentration of choline. In the absence of Na^+ the uptake was linear for less than 10 minutes and approached a steady intracellular concentration by 60 minutes. Uptake in the presence of Na^+ was linear over the first 20 minutes and again approached a steady level at about 60 minutes. Consequently the 60-min uptake has been used routinely

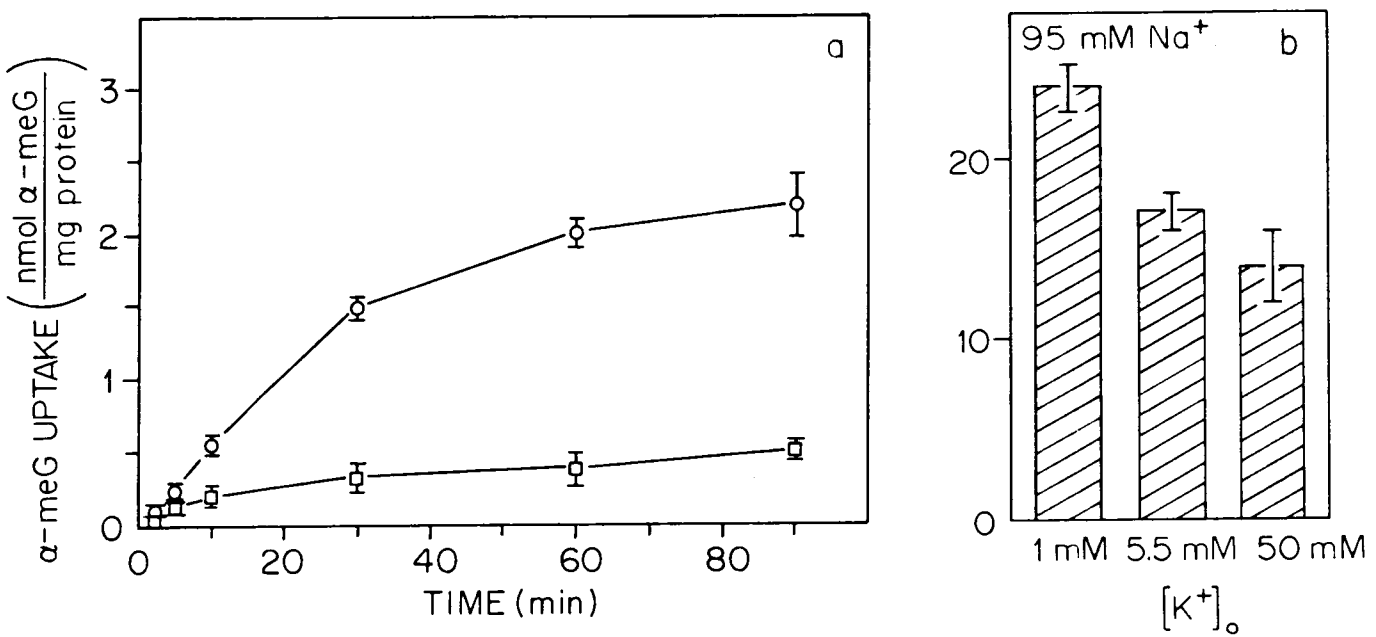


Fig. II-1. Dependence of α-meG accumulation on the Na^+-electrochemical gradient. (a) Time course of uptake by just-confluent LLC-PK_1 cells in the presence (circles) or absence (squares) of 165 mM Na^+. Na^+-free medium was made up by the equimolar substitution of choline chloride. α-MeG concentration in the medium was 100 μM. (b) Sixty-minute accumulation of α-meG in medium with 95 mM Na^+ and various concentrations of K^+, constant osmolarity being maintained with choline chloride. α-MeG in the medium was 100 μM. This was a separate experiment from (a), performed several days after the cells had reached confluence, and the control cells in normal 5.5 mM K^+ had a much higher concentrating capacity despite the reduced Na^+.

throughout the remainder of this study. In the presence of Na^+ the magnitude of the steady-state level was substantially increased, confirming the Na^+-dependence of this process (Rabito and Ausiello, 1980; Silverman, 1976). In some experiments the Na^+ concentration of the medium was held constant at 95 mM and the K^+ concentration varied, osmolarity again being maintained with choline. This maneuver is expected to alter the membrane potential with the cells being hyperpolarized in low K^+ and partially depolarized in high K^+. The 60 minute hexose uptake varied inversely with the K^+ concentration (Fig. II-1b). The result is consistent with the assumption that the driving force for accumulation is the Na^+-electrochemical gradient.

To verify that the Na^+-dependent α-meG uptake is a concentrative process, the 60-min uptake of α-meG was compared to that of 3-O-methyl-D-glucose (meGlc). The latter is poorly or not at all transported by the renal Na^+-dependent hexose transport mechanism (Silverman, 1976), but readily transported by the Na^+-independent facilitated diffusion mechanism found in most cell types. Since the latter is not a concentrating system, at steady-state the meGlc intracellular concentration approaches the extracellular concentration and can be used as a marker for cell water. Fig. II-2 shows that in Na^+-containing medium with both sugars at the same external concentration the 60-min uptake of α-meG was markedly greater than that of meGlc, indicating concentrative accumulation of the former. In Na^+-free medium, the uptake of α-meG was slightly less than that of meGlc; this is the result expected on consideration that the Na^+-gradient may actually be reversed in these cells.

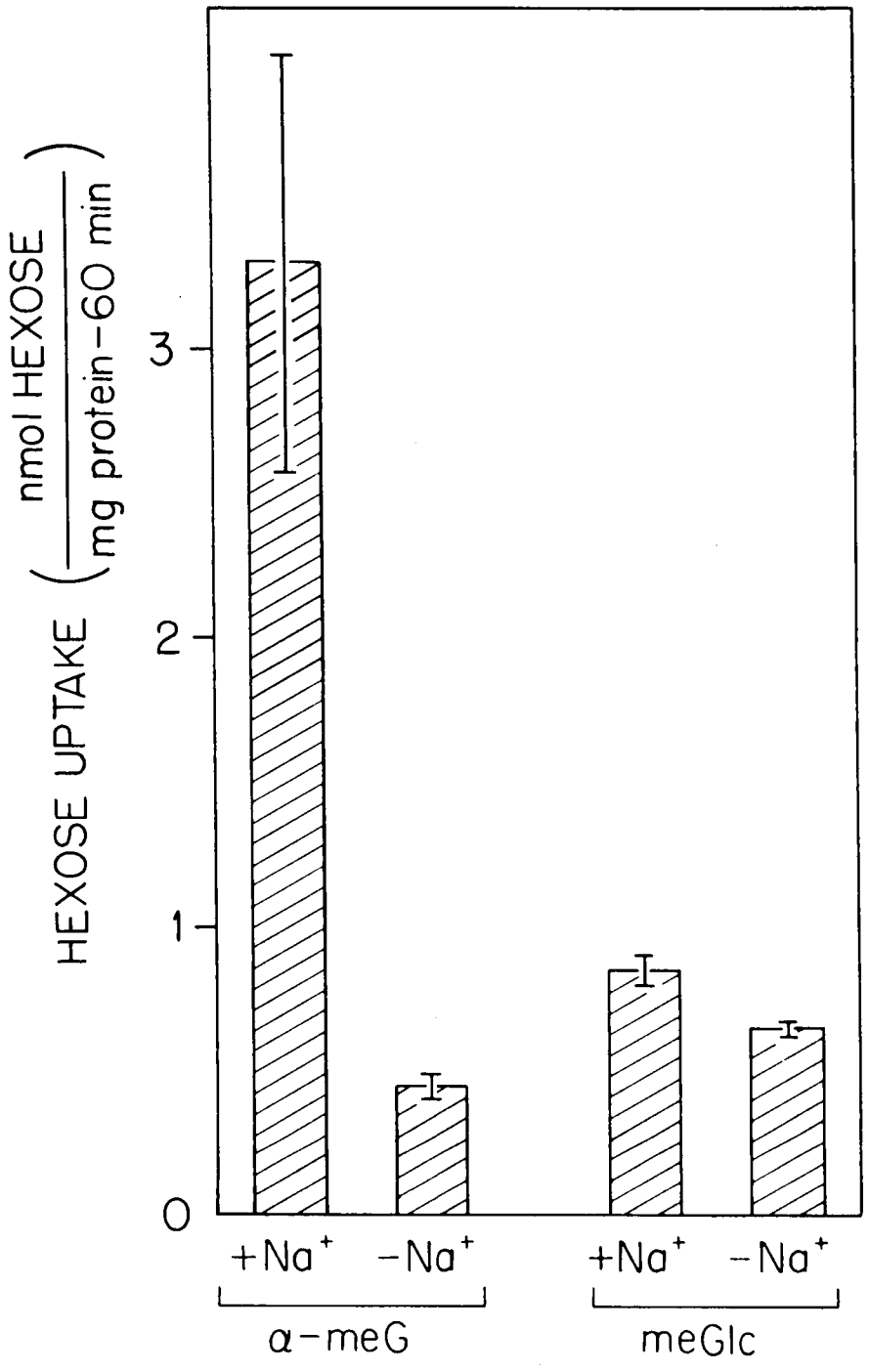


Fig. II-2. Sixty-minute accumulation by just-confluent LLC-PK_1 cells of α-meG and meGlc in the presence and absence of 165 mM Na^+. Osmolarity in Na^+-free media was maintained by the equimolar substitution of choline chloride. Both hexoses were present at 100 μM in the medium. The cultures in Na^+-containing medium had domes; in Na^+-free medium the domes had collapsed.

In addition Fig. II-2 shows that the uptake of either hexose in the absence of Na^+ was less than the uptake of meGlc in the presence of Na^+. It has been observed microscopically that in Na^+-free medium the domes disappear within a few minutes. Most of the decrease of the meGlc uptake in the absence of Na^+ is attributed to a decrease in the available water space under the monolayers (see Discussion); hexose transported through the monolayer and trapped under the domes will be measured as uptake. Thus the meGlc space in the presence of Na^+ appears to include both cell water and dome water, and is the reference for the volume into which α-meG is being transported. As can be seen in Fig. II-2 the difference between the "$+Na^+$" and "$-Na^+$" meGlc spaces is not large. Assuming that the latter is the more accurate estimate of cell water volume, a water/protein ratio of approximately 6 µl/mg is obtained, a value consistent with observations in other cell types.

In renal tubular cells Na^+-dependent hexose transport is strongly inhibited by phlorizin (Diedrich, 1966; Vick et al., 1973). In Fig. II-3 the phlorizin sensitivity of α-meG concentrative uptake was confirmed, and further it was shown that under the conditions of the experiment the inhibitory $K_{I,0.5}$ is about 0.2 µM. This value is of the same order of magnitude as the calculated K_d for Na^+-dependent phlorizin binding to isolated brush border membranes from a variety of sources (Frasch et al., 1970; Glossmann and Neville, 1972; Silverman and Black, 1975) and to the K_d for Na^+-dependent phlorizin binding to a clone derived from the LLC-PK_1 cell line (see Chapter V). Ten µM phlorizin had no effect on the 60-min uptake of meGlc (not shown).

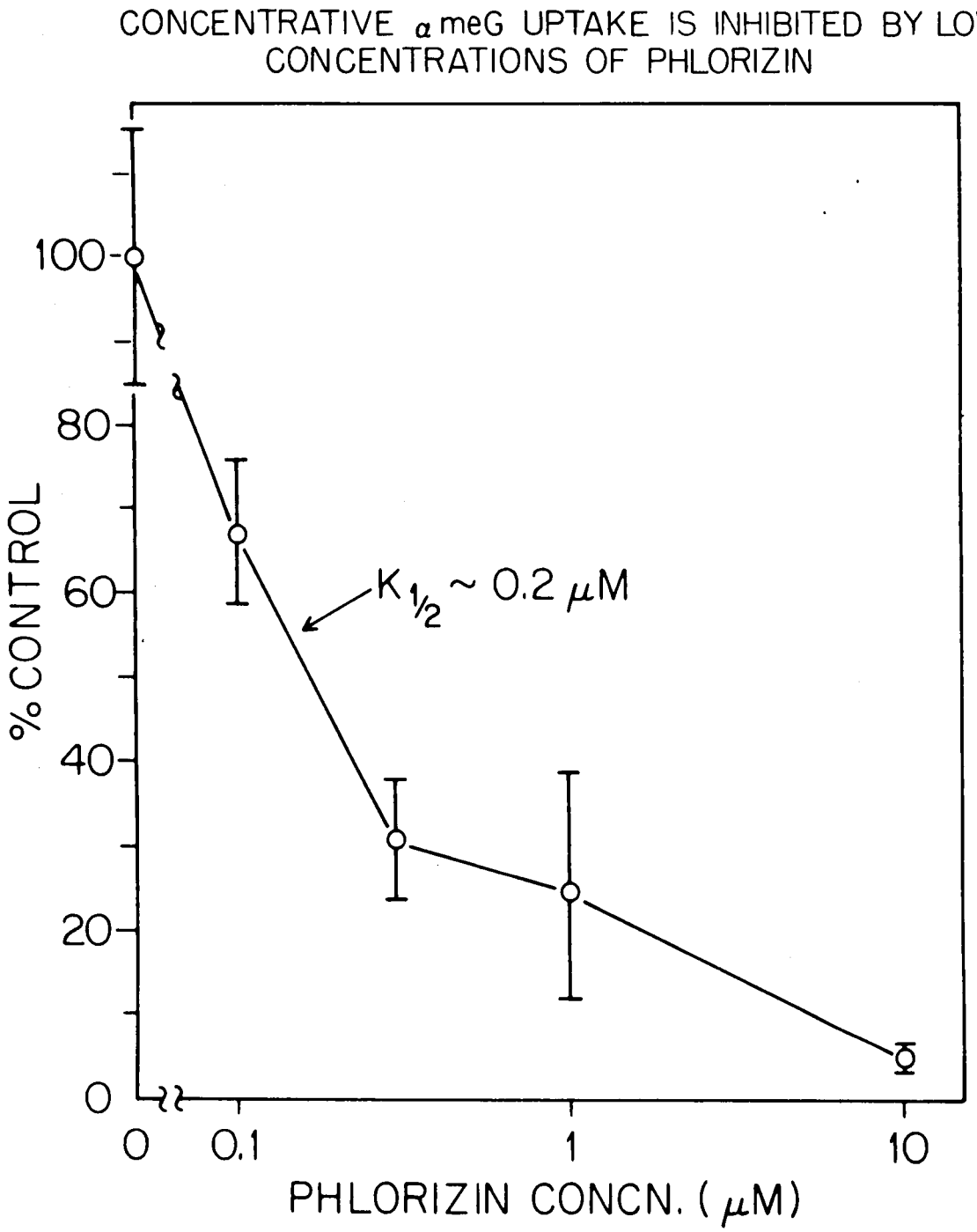


Fig. II-3. Effect of phlorizin on 60-min uptake of α-meG. Concentration of α-meG in medium was 100 μM, and of Na^+ was 165 mM. Phlorizin was added with the hexose and was present throughout the uptake period. The control uptake was 1.6 ± 0.3 nmol α-meG/mg protein-60 min.

Development of α-meG Concentrative Capacity in LLC-PK_1 Cells

The time course of appearance of α-meG concentrative capacity was examined as a function of growth in culture (Fig. II-4). LLC-PK_1 cells were plated at low cell density and periodically the 60-min uptakes of α-meG and meGlc were assayed. For the first several days these uptakes were indistinguishable. On day 5 domes had started to appear in the cultures (note the small rise in meGlc uptake) and the α-meG/meGlc uptake ratio rose to about 4. This ratio continued to rise over the next several days. Since the time of appearance of concentrative capacity in untreated cells corresponds roughly to the attainment of confluence (Mullin et al., 1980), the length of time in culture required for the expression of this activity is an inverse function of the cell density on plating and to some extent of the dispersion of cells in the plating suspension. In all experiments therefore untreated cultures were included as internal controls.

Since the process of dome formation in MDCK cells can be accentuated by compounds that also induce erythroid differentiation in Friend erythroleukemic cells (Lever, 1979) similar effects on development of α-meG concentrating capacity in LLC-PK_1 cells were looked for. For this study one of the more potent Friend cell inducers, hexamethylene bisacetamide (HMBA) was chosen. The cells were plated 2 days before treatment and allowed to establish their normal growth pattern. On day 0 of the treatment, the medium was changed and the desired chemicals were added. Fresh medium, with the same additives, was replenished

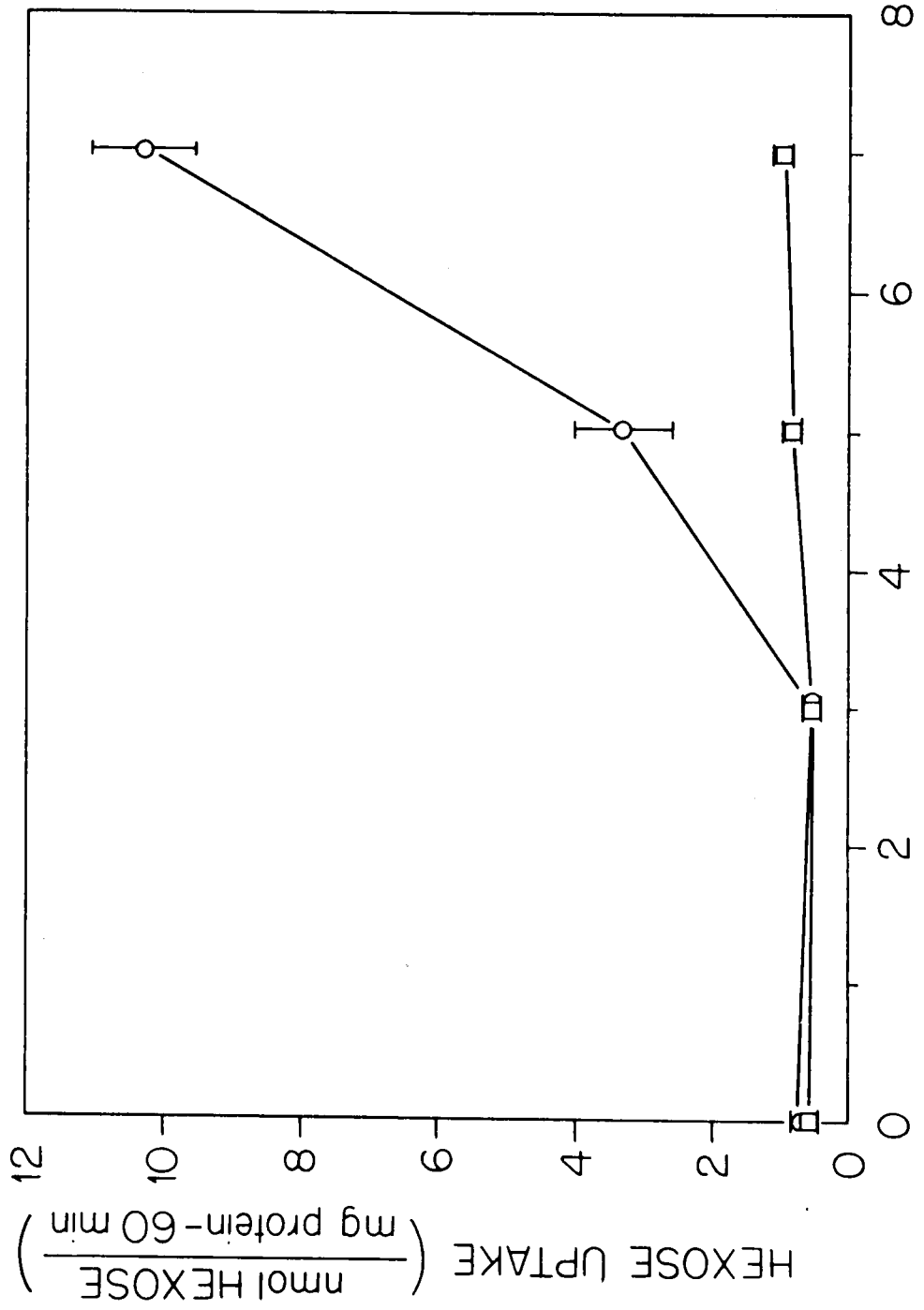


Fig. II-4. Sixty-minute uptakes of α-meG (circles) and meGlc (squares) by LLC-PK$_1$ cells as a function of days in culture. Both hexoses were assayed at a medium concentration of 100 μM.

every 2 days thereafter. Assays were performed 2 days after the last medium change. The assay medium did not contain the additives. As seen in Fig. II-5a, 2 mM HMBA markedly accelerated the development of concentrative transport in these cultures.

The effects of HMBA on Friend cell induction can be at least partially countered by the tumor promoter 12-O-tetradecanoylphorbol--13-acetate (TPA) (Marks and Rifkind, 1978; Becker and Cook, 1981). In addition, the expression of a number of other differentiated characteristics by cell lines in culture can be blocked by TPA (Diamond et al., 1978). In LLC-PK_1 cells the inclusion of TPA at low concentration (0.1 μM) with HMBA completely blocked the development of α-meG concentrating capacity, even beyond the time when marked accumulation could be observed in the controls (Fig. II-5a). TPA alone is cytotoxic to these cells and consequently was not used alone in these studies. The effects of TPA were observed down to 10^{-9} M, but 10^{-7} M produced the maximal effect without excessive cytotoxicity and was therefore used throughout these studies.

The effects of the additives on cell growth were assessed by the 60-min incorporation of [^{3}H]thymidine into acid-insoluble material (Fig. II-5b). In the controls there was a small increase between day 0 and day 2, but this subsequently dropped to low levels as the cultures became confluent. In HMBA-treated cultures the drop was already apparent on day 2. In HMBA+TPA-treated cells, thymidine incorporation was markedly stimulated on day 2, but subsequently fell to levels close to control or HMBA-treatment values. In Fig. II-5a the number in

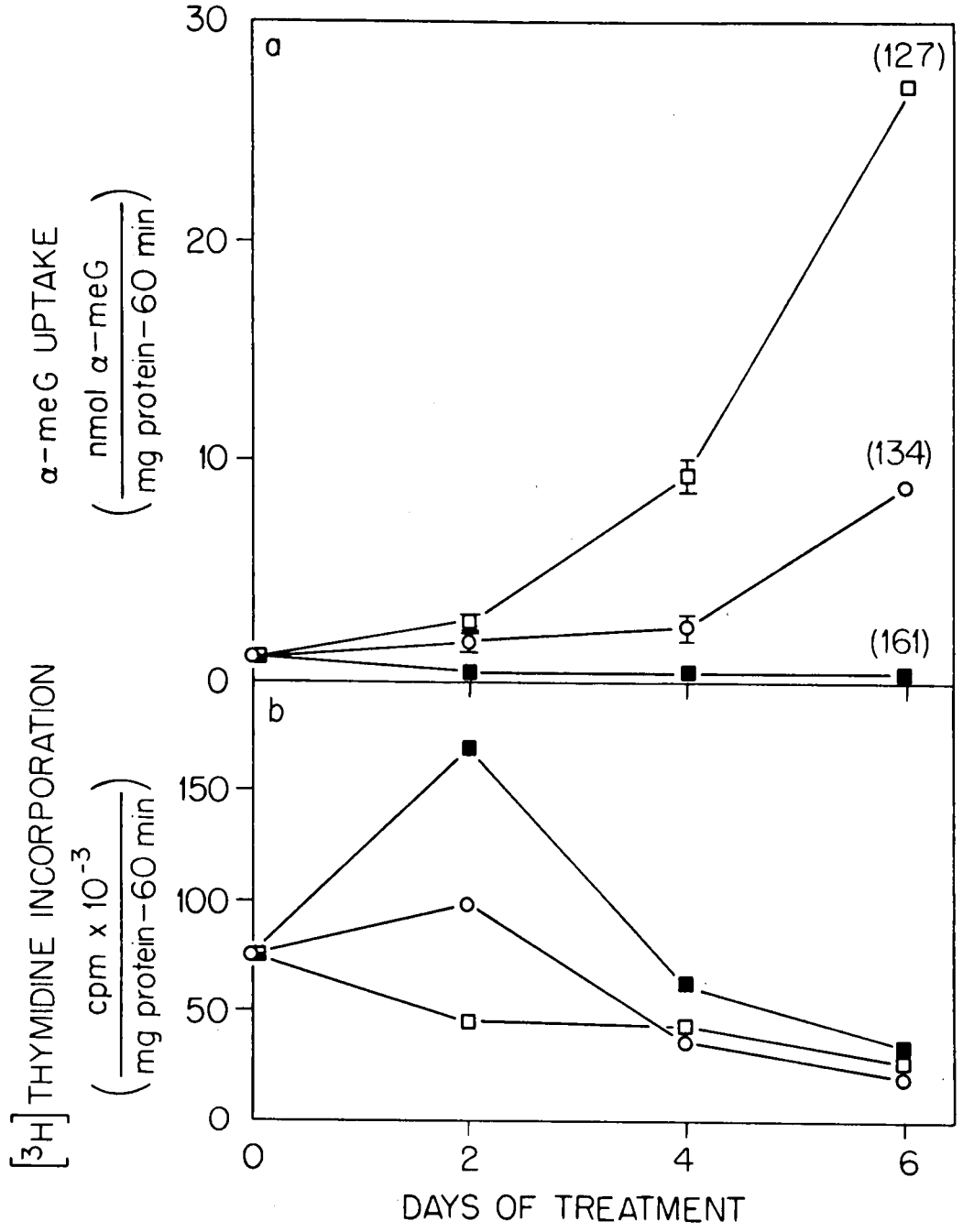


Fig. II-5. Effects of HMBA and HMBA+TPA on LLC-PK_1 cells. The cells were plated 2 days prior to onset of the various treatments. HMBA was used at 2 mM, and TPA at 0.1 μM. Media were changed every 2 days. (a) Sixty-minute uptake of α-meG. Numbers next to the 6-day points give culture densities in μg protein/cm^2. (b) Sixty-minute incorporation of [^{3}H]thymidine into TCA-insoluble material. Thymidine in the medium was 1 μCi/ml. Open circles, controls; open squares, 2 mM HMBA; closed squares, 2 mM HMBA + 0.1 μM TPA.

parentheses next to each data point on day 6 is the cell concentration expressed as μg protein/cm^2. Comparison of Figs. II-5a and II-5b shows that neither acceleration of growth, nor high cell density, nor growth arrest are adequate stimuli per se to the accelerated development of α-meG concentrating capacity.

The relationship of the TPA effect to its known tumor promoting properties was considered by using together with HMBA the inactive (in tumor promotion) analogs phorbol and 4-0-methyl TPA (Fig. II-6). As before, HMBA was an effective accelerator of the response; the inactive analogs failed to inhibit the acceleration. In many reported cases the transition of cells from a growth phase into a differentiating phase is accompanied by a rise in intracellular cyclic AMP (cAMP). That this is also true in the present case is shown in Fig. II-7. Cells were plated on day -2 and treatment with HMBA or HMBA+TPA was started on day 0. By day 1 cAMP levels had risen in all cultures, with HMBA-treated cultures showing the greatest rise. Throughout the experiment HMBA-treated cultures showed an elevated level of cAMP when compared to either control cultures or to cultures treated with both HMBA and TPA. This same trend was observed in the levels of α-meG uptake. By day 2 the HMBA-treated cultures exhibited concentrative hexose uptake and the level continued to rise thereafter. In contrast, control cultures did not display concentrative hexose uptake until day 3 and over the rest of the experiment the increase observed was more gradual than that seen in HMBA-treated cultures. HMBA+TPA-treated cultures showed depressed hexose uptake over the entire course of the experiment. The results

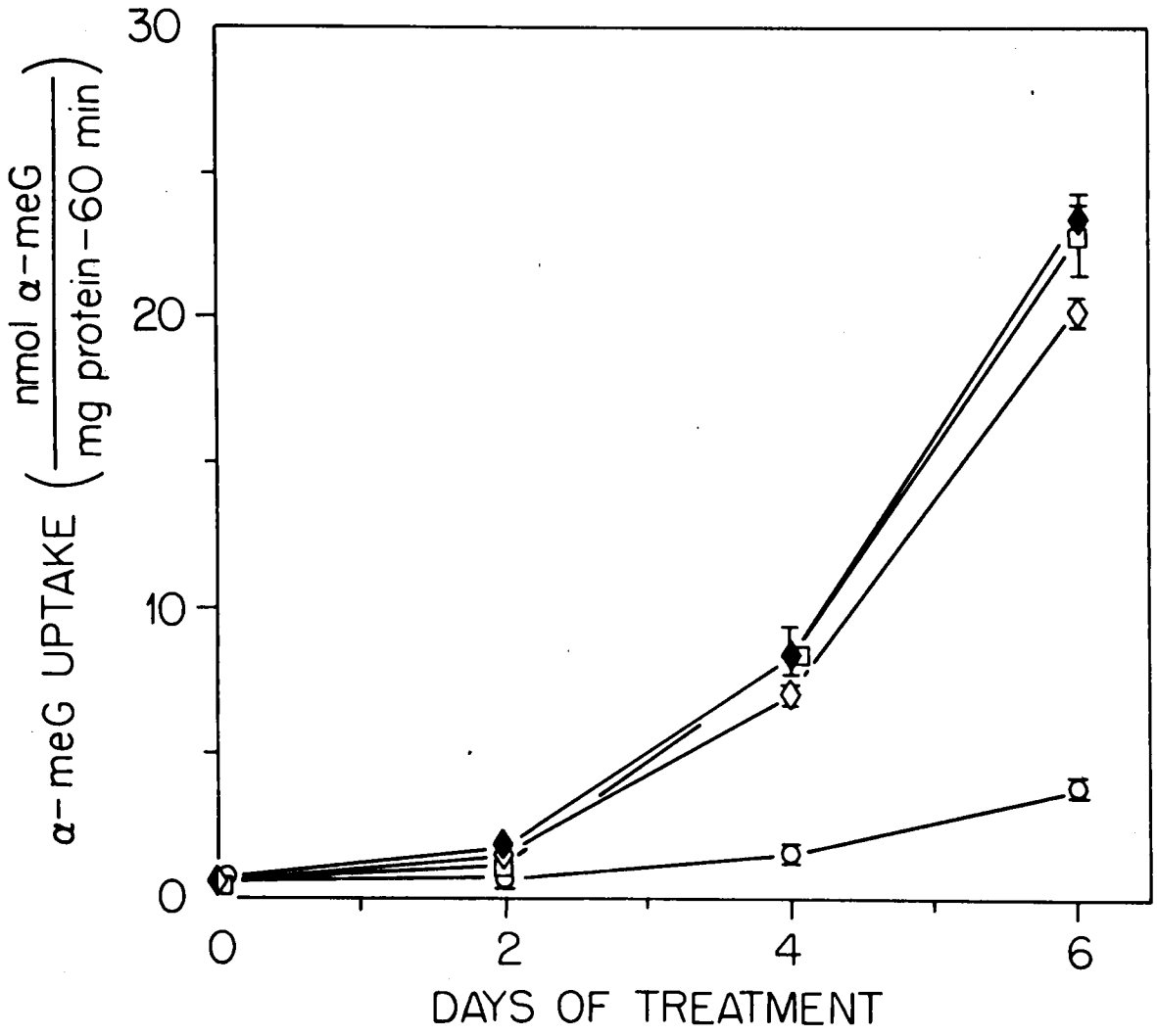


Fig. II-6. Failure of the TPA analogs 4-O-methyl TPA and phorbol to prevent the HMBA-accelerated development of α-meG concentrating capacity in LLC-PK_1 cells. Cells were plated 2 days prior to the onset of the treatments, and 60-min accumulations of α-meG were measured on the days indicated. HMBA in the medium was 2 mM; and 4-O-methyl TPA and phorbol were present at 0.1 μM. Open circles, controls; open squares, HMBA; open diamonds, HMBA + phorbol; closed diamonds, HMBA + 4-O-methyl TPA.

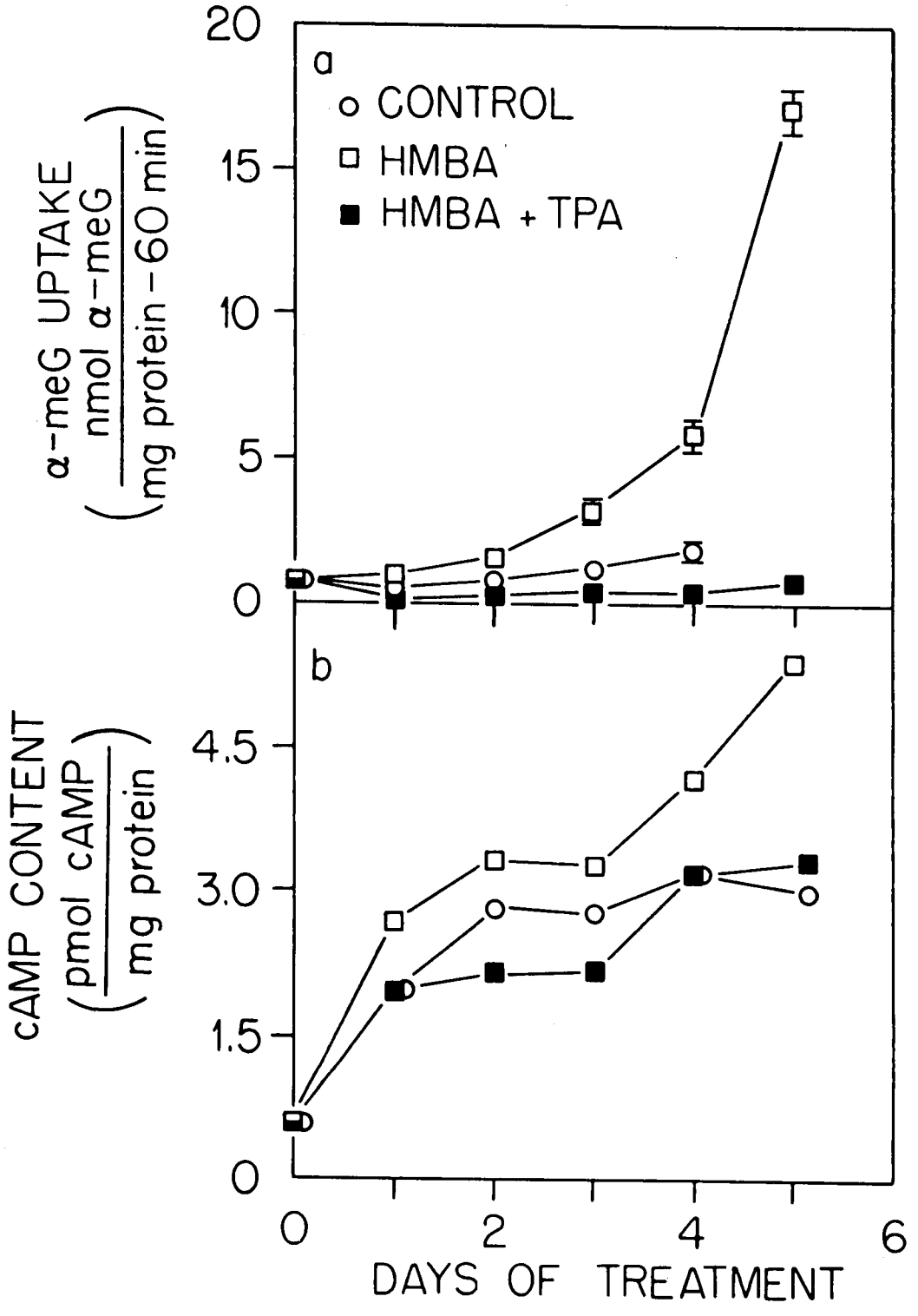


Fig. II-7. Effects of HMBA and HMBA+TPA on development of α-meG accumulation capacity and on intracellular cyclic AMP. Conditions as in Fig. II-5. (a) Sixty-minute α-meG uptake. (b) Intracellular cyclic AMP. Open circles, controls; open squares, 2 mM HMBA; closed squares, 2 mM HMBA + 0.1 μM TPA.

suggest that cAMP may be correlated with α-meG accumulating capacity, but that the responses are not coincidental in time.

To investigate this question more directly, the cells were treated with compounds known to elevate cAMP through the inhibition of phosphodiesterase. First tested was the combination of dibutyryl cyclic AMP (0.25 mM) and theophylline (1 mM). As seen in Fig. II-8, this combination was effective in accelerating the development of α-meG concentrating capacity. In a second set of experiments theophylline alone, the phosphodiesterase inhibitor 1-methyl-3-isobutyl-xanthine (MIX; 1 mM), and each of them in combination with 0.1 μM TPA were used (Fig. II-9). The cells were plated fairly heavily in the experiment shown, and when the treatments were started 2 days later (day 0 in the figure) the 60-min uptake of α-meG was already at 4 nmol/mg protein or about 8-10 times the level usually observed in sparse cultures. Over the next 11 days this value increased further in the controls to 18 nmol/mg protein. In theophylline-treated cultures the development of α-meG concentrating capacity was substantially accelerated. TPA not only blocked the theophylline-supported acceleration, but in theophylline+ TPA-treated cultures the 60-min α-meG accumulation actually fell back to the levels characteristic of sparse cultures.

MIX was even more potent than theophylline as an inducer of the differentiated transport system and caused even greater acceleration of its development (Fig. II-9). In the presence of MIX, 0.1 μM TPA was again strongly inhibitory for a prolonged period of time, but eventually MIX appeared to override the TPA block and the 60-min α-meG uptake rose

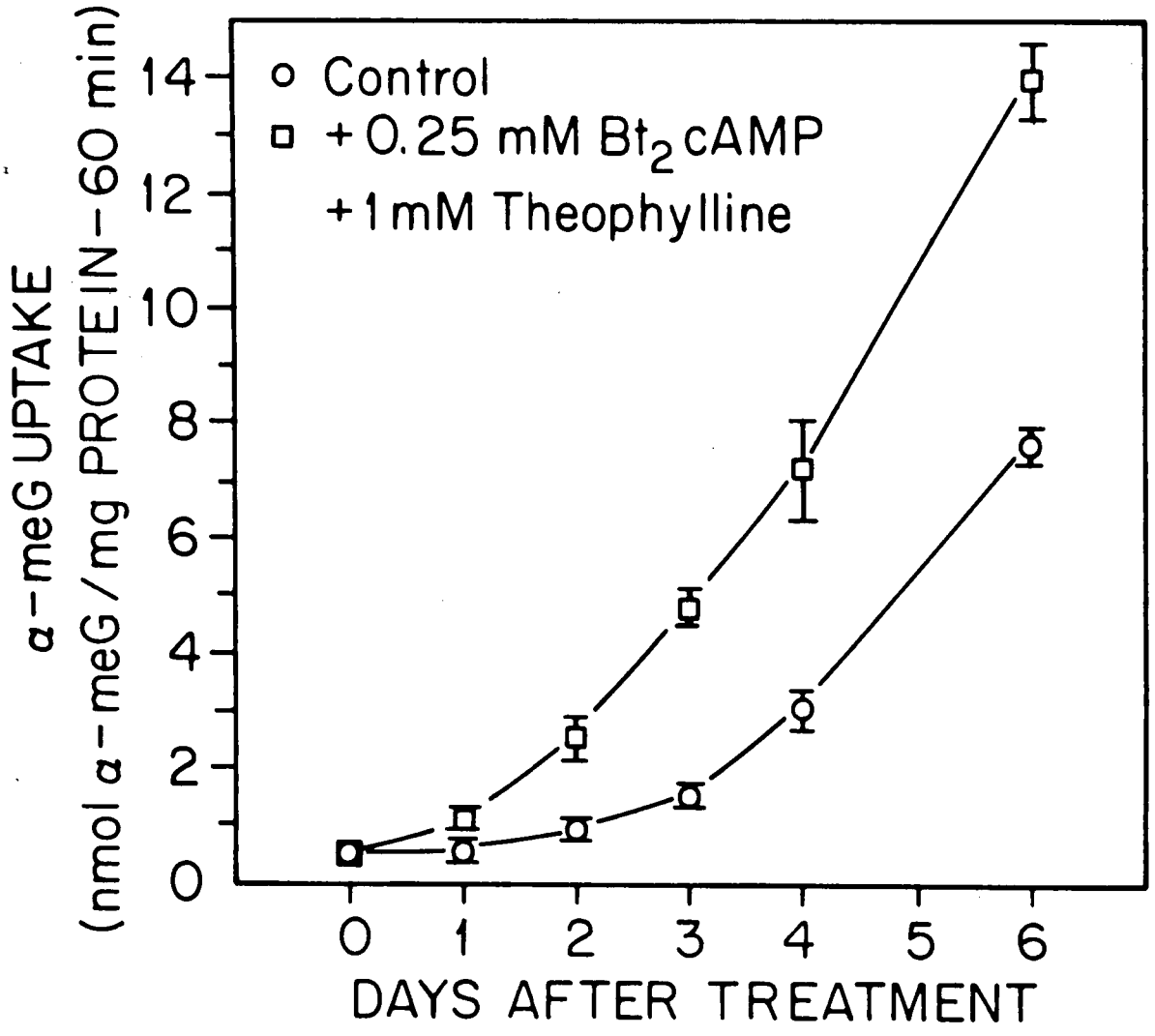


Fig. II-8. Effect of 0.25 mM dibutyryl cyclic AMP + 1 mM theophylline (squares) on the development of α-meG concentrating capacity in LLC-PK_1 cells, relative to untreated controls (circles). Cells were plated on day -2, and the treatment was begun on day 0. Sixty-minute uptake of α-meG from a medium concentration of 100 μM was assayed on the days indicated.

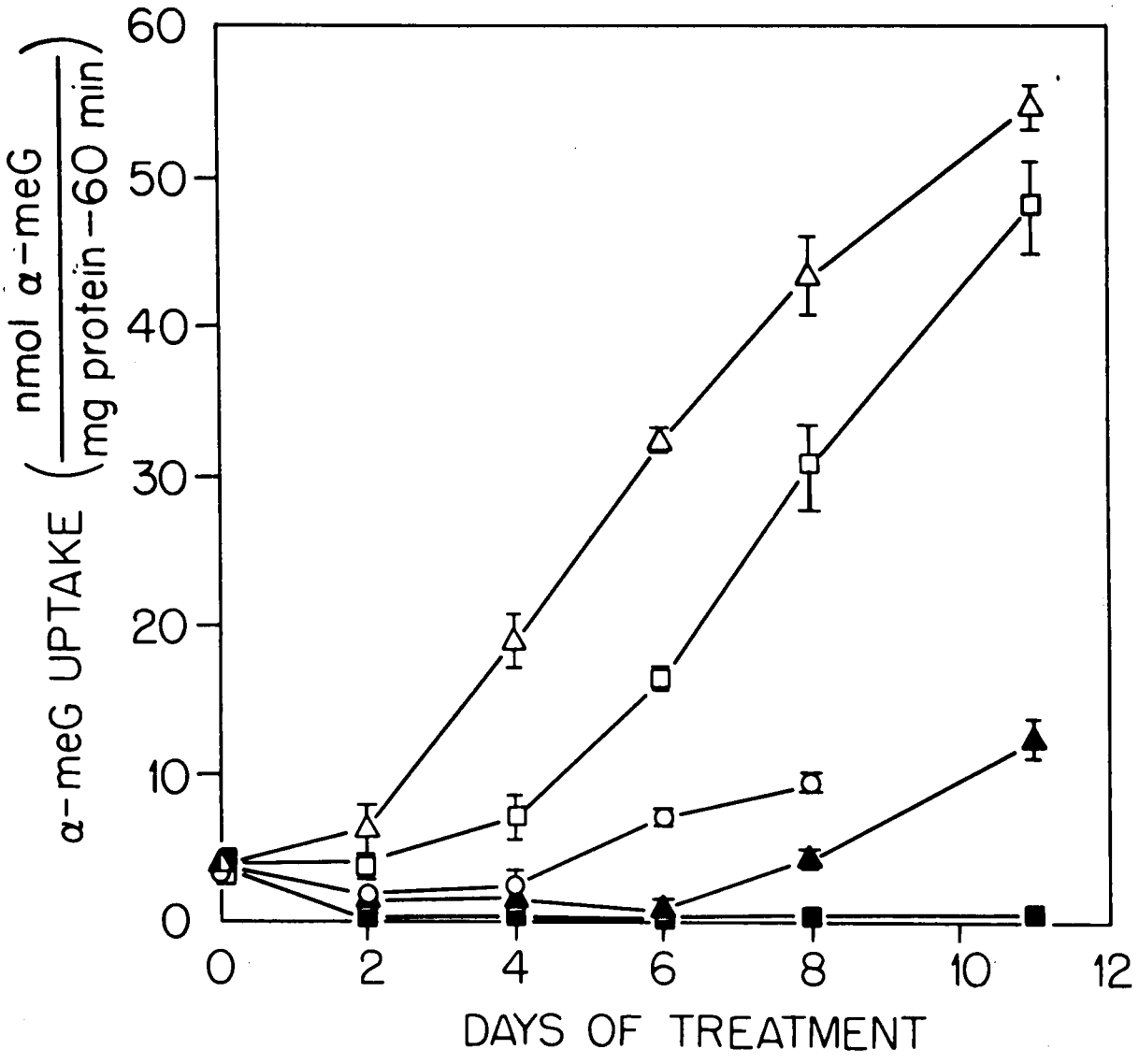


Fig. II-9. Effects of MIX, theophylline, MIX+TPA, and theophylline+ TPA on development of α-meG concentrating capacity. Conditions as in Fig. II-5 (p. 23). MIX and theophylline were present at 1 mM, and TPA at 0.1 μM. Open circles, controls; open squares, 1 mM theophylline; closed squares, 1 mM theophylline + 0.1 μM TPA; open triangles, 1 mM MIX; closed triangles, 1 mM MIX + 0.1 μM TPA.

to significant levels over meGlc uptake after 8-11 days of the combined treatments.

Fig. II-10 shows the cAMP levels in the same experiment. Four trends are evident. First, MIX and theophylline both initially elevate cAMP to levels greater than those seen in controls, with MIX again being the more potent agent. Second, TPA inhibited, but relatively weakly, the rise of cAMP in MIX-treated cultures. Data for theophylline+TPA-treated cultures are not included because cAMP levels were below the limits of detection. Third, the rise of cAMP in MIX+TPA-treated cells was greater than in either theophylline-treated cultures or controls, but the development of α-meG accumulating capacity was greatly delayed in these MIX-TPA-treated cultures with respect to the other two. Finally, in all treatments the maximum cAMP levels were observed at 4 days and tended downward thereafter, although the α-meG accumulating capacity continued to increase over the next 7 days. This pattern of a peak level of cAMP followed by a decline was consistently observed, although the exact time course varied among the individual experiments.

Discussion

The strain of LLC-PK_1 cells used in this work displays Na^+-dependent hexose uptake similar to that observed by others using strains from this cell line (Mullin et al., 1980; Rabito and Ausiello, 1980; Misfeldt and Sanders, 1981). Reduction of external Na^+ reduces uptake. In addition, the driving force on the Na^+ ion, to which the hexose flux is coupled, is the Na^+ electrochemical gradient, and thus change of the membrane

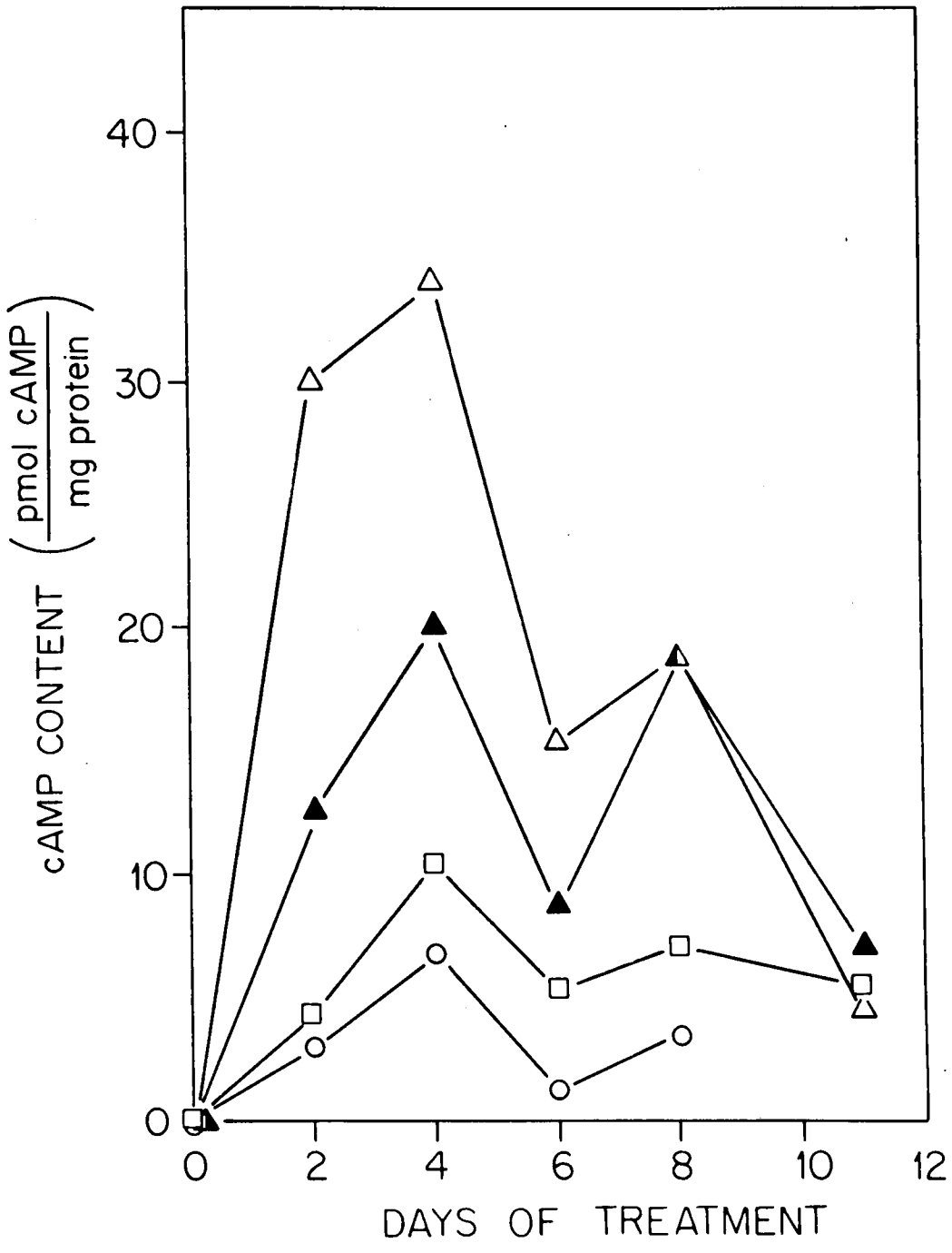


Fig. II-10. Effects of MIX, theophylline, and MIX+TPA on cyclic AMP levels. Same experiment as Fig. II-9. In theophylline+TPA-treated cultures cyclic AMP was below the level of detection, Open circles, controls; open squares, 1 mM theophylline; open triangles, 1 mM MIX; closed triangles, 1 mM MIX + 0.1 μM TPA.

potential at constant external Na^+ concentration would also be expected to affect the hexose accumulating capacity. Depolarization and hyperpolarization were attempted, although no direct measurements of the membrane potential were made, by altering the external K^+ concentration. Reduction of the external K^+ to a value near its K_m for Na,K-ATPase (1 mM) may reduce whatever electrogenicity the pump may have in these cells, but this effect would not be expected to exceed a few millivolts. The enhanced K^+ diffusion potential appears to predominate (Fig. II-1b, p. 15). Attempts to accentuate the role of the K^+ diffusion potential with the K^+-ionophore valinomycin were not successful since this ionophore is highly toxic to these cells even at low concentrations. The expected changes in α-meG accumulation were observed in the altered K^+ media, although no quantitative interpretations can be drawn from this qualitative experiment.

The standard assay used in these studies contained 100 μM α-meG in the medium. This is well below the uptake K_M for α-meG of 750 μM as determined by Rabito and Ausiello (1980). Under these conditions, uptake approached a steady state in 60 min. This result is in contrast to that of Mullin et al. (1980) who, working with a 10-fold higher concentration, did not find a clear-cut plateau even after 120 minutes.

As described in the Results, it is believed that a minor part of the α-meG uptake observed in these cultures on plates is accumulation in the fluid of the domes. For this reason a precise estimate of the extent of maximal accumulation within the cells cannot be made from the present data. With cells grown on collagen-coated Nucleopore filters,

where doming does not occur, observed accumulation ratios for α-meG in excess of 100-fold over the uptake of meGlc (data not shown) have been obtained. No enhancement of α-meG accumulation was found in the presence of 1 μM cytochalasin B, in contrast to the results of Rabito and Ausiello (1980) who enhanced α-meG accumulation by blocking its efflux with phloretin. The lack of sensitivity to cytochalasin B suggests that α-meG is poorly transported on the facilitated diffusion carrier for hexoses that is sensitive to this drug. Thus, once α-meG is taken up by the Na^+-dependent transporter, it does not appear to be escaping to any great extent by the cytochalasin B-sensitive pathway. A small amount may nevertheless diffuse through the tight junctions of this leaky epithelium.

The situation with meGlc is somewhat more complex. In the absence of Na^+, meGlc uptake is slightly depressed (Fig. II-2, p. 17), a fact that is attributed at least in part to the collapse of the domes under these conditions. One μM cytochalasin B enhances the uptake of meGlc as much as 3-fold in cultures capable of concentrating α-meG over 10-fold. This result suggests that meGlc may be weakly transported on the Na^+-dependent system (Misfeldt and Sanders, 1981) and concentrated when its efflux is blocked (Kimmich et al., 1977). Since in the absence of cytochalasin B the meGlc space is about 6 μl per mg protein, a value similar to the water space in many mammalian cells, any accumulation of meGlc via the Na^+-dependent system would appear to be rapidly vitiated by the facilitated diffusion carrier. Considering that nevertheless meGlc may be slightly concentrated in these cells, estimates of

concentrating capacity for α-meG based on the α-meG/meGlc ratio are minimum estimates.

The determination of a true $\underline{k}_i$, or set of $\underline{k}_i$'s, for phlorizin would require a complete kinetic analysis at various external Na^+ concentrations, since phlorizin binding in the kidney is known to be Na^+-dependent, as well as at various external concentrations of α-meG. The determination of a $K_{I,0.5}$ of 0.2-0.3 μM refers to its efficacy under the conditions of these experiments: 142 mM Na^+ and 100 μM α-meG. The conditions are similar to physiological conditions under which phlorizin has been used in renal studies, and the inhibitory potency is similar to that observed in such in vivo studies (Silverman, 1976).

It has been observed previously (Mullin et al., 1980) as is noted in Fig. II-4 (p. 21) that the development of α-meG concentrative capacity is associated with attainment of confluency of the cultures. That the relationship may be more complex than this can be seen in the responses to various chemical treatments. HMBA stimulates growth and accelerates both the attainment of confluency and of α-meG accumulation capacity. But hexose accumulation is accelerated even more by the phosphodiesterase inhibitors theophylline and, especially, MIX and these compounds are growth inhibitory. Treated with these compounds the cultures develop a high degree of α-meG concentrative capacity at substantially lower cell densities than is true of the controls (Fig. II-5, p. 23). Since the cells grow in islands (Mullin et al., 1980), they are in contact with their neighbors even at relatively low cell densities, but such contact is nevertheless not an adequate

stimulus to induce the maximum α-meG accumulation in the controls. Conversely, cultures treated with HMBA+TPA were briefly stimulated in their growth, becoming very dense before growth subsided. Even at these high cell densities, development of α-meG accumulating capacity was markedly inhibited by the TPA. It is possible that normal cell-cell communication is also disrupted by TPA (Yotti et al., 1979).

Several of these compounds were chosen for this study because they influence a differentiated function, dome formation, in MDCK cells (Lever, 1979). In microscopic observations (not shown), HMBA enhanced dome formation in LLC-PK_1 cells as it does in MDCK cells. Dibutyryl cyclic AMP + theophylline, theophylline alone, and MIX also enhanced the appearance of domes in islands in subconfluent cultures, but inhibited dome formation in the confluent cultures. In TPA-treated cultures, with TPA added either alone or with any of the other compounds, the normal polygonal cell morphology was lost; the cells became elongated and dendritic and overgrew each other. In TPA the appearance of domes was totally blocked.

In addition to the phosphodiesterase inhibitors theophylline and MIX, and presumably dbcAMP as well, the differentiation inducer HMBA effects increases in intracellular cAMP. The increase is attenuated by TPA. Control cells on reaching confluence also show a cAMP increase. Despite the apparent association between elevated cAMP and development of α-meG accumulating capacity, it seems unlikely that cAMP participates in a direct modification of the membrane so as to confer on it the property of Na^+-dependent hexose uptake. Several factors make this

doubtful. In the first place, the rise in cAMP precedes the rise in hexose accumulation by several days. In MIX-treated cultures an increased cAMP level is observed as early as one hour after MIX addition and continues to increase over the next 24 hours (unpublished data). Second, even in the continued presence of the effectors, cAMP levels reach their peak in a day or two and then start to decrease. Even as the cAMP levels are falling, the α-meG accumulating capacity continues to rise to very high levels (Figs. II-9 and II-10, pps. 29 and 31). Finally, in the presence of either HMBA or MIX, TPA attenuates but does not abolish the cAMP rise. In these cases the cAMP levels equal or exceed those observed in the controls as they become confluent. Yet in both instances the treated cultures are severely inhibited with respect to the controls in the development of concentrative α-meG uptake. A tentative conclusion is that elevated cAMP may be a necessary early step in the development of Na^+-dependent hexose transport, but high intracellular cAMP is not a sufficient condition. As appears to be true in the case of cells treated with TPA, such differentiation may be blocked at a later step.

CHAPTER III

GROWTH-DEPENDENT AIB AND meAIB UPTAKE IN LLC-PK_1 CELLS

Introduction

LLC-PK_1 cells develop the capacity to carry out Na^+-dependent concentrative hexose transport during growth in culture (Mullin et al., 1980; Rabito and Ausiello, 1980; Misfeldt and Sanders, 1981; Chapter II). The rate of acquisition of this differentiated property can be modulated by a number of compounds. The Friend cell differentiation inducer HMBA (Marks and Rifkind, 1978) and the cyclic AMP phosphodiesterase inhibitors dibutyryl cAMP, theophylline, and MIX are all accelerators, while the tumor promoter TPA is a potent inhibitor. The onset of expression of the concentrative hexose transport function is always associated with elevated intracellular cAMP and decreased thymidine incorporation. These phenomena are commonly associated with growth arrest, and differentiation, in many cell systems (Prescott, 1976).

In contrast to this pattern of increasing Na^+-dependent hexose uptake in growth-arrested LLC-PK_1 cells, in a number of cell types the Na^+-dependent uptake of amino acids by the A-system (alanine-preferring) declines with growth arrest (Foster and Pardee, 1969; Oxender et al., 1977; Villereal and Cook, 1978) and increases again on growth stimulation. In part these changes in A-system transport are due to a changing membrane potential and consequently a changing Na^+-electrochemical gradient (Villereal and Cook, 1977; Becker and Cook, 1981). Since this gradient is the driving force for hexose accumulation in LLC-PK_1 cells,

it was of interest to explore the growth associated changes in A-system activity in this cell line and compare them to the simultaneously changing pattern of Na^+-dependent hexose transport.

Materials and Methods

Cell Culture and Measurement of Uptake

These procedures were performed essentially as described in Chapter II. Final concentrations of both [^{3}H]aminoisobutyric acid (AIB) and [^{14}C]methyl-aminoisobutyric acid (meAIB) in the assay medium were 100 µM. The final radioactivity of [^{3}H]AIB was 1 µCi/ml and of [^{14}C]meAIB was 0.2 µCi/ml.

Short-Term Effects of TPA

For experiments examining the short-term effects of TPA in the presence of other compounds, stock solutions of the various compounds were prepared in either HBSS or 95% ethanol. The stock solutions were then combined as required and further diluted with HBSS to yield 10X working stock solutions. Two hundred µl of the working stock was added to the culture dish with filter without a medium change. Control cultures received 200 µl HBSS containing the appropriate concentration of ethanol, so that each dish in an individual experiment received the same total volume of addition and the same concentration of ethanol. At these dilutions ethanol had no effect on the measured parameters.

Measurement of Protein and RNA Synthesis

The procedure for measuring [^{35}S]methionine or [^{3}H]uridine incorporation into acid-insoluble material was the same as the procedure described in Chapter II for measuring [^{3}H]thymidine incorporation. Incorporations were assayed using trace quantities of radioactive methionine at an activity of 1 μCi/ml, of uridine at a radioactivity of 0.5 μCi/ml, and of thymidine at 1.0 μCi/ml. In the first two cases incorporation was allowed to proceed for 15 min, in the latter case for 60 min.

Chemicals

[^{3}H]AIB, [^{14}C]meAIB, and [^{35}S]methionine were obtained from New England Nuclear, and [^{3}H]uridine from Schwarz/Mann. AIB, meAIB, and ouabain were obtained from Sigma. All other chemicals were of reagent grade.

Results

Characteristics of AIB and meAIB Uptake in LLC-PK$_1$ Cells

Figure III-1 shows time courses of uptake of AIB and meAIB by rapidly growing (sparse) LLC-PK$_1$ cells, together with the Na^+-dependence of the respective uptakes. Under the conditions of these experiments, with the test substrate at 100 μM in the medium, equilibrium between medium and cell water is achieved at an uptake of 0.5-0.8 nmoles/mg protein, see Chapter II. In normal Na^+-medium uptake of both compounds was linear for 120 min. Although this rate of uptake is a function of

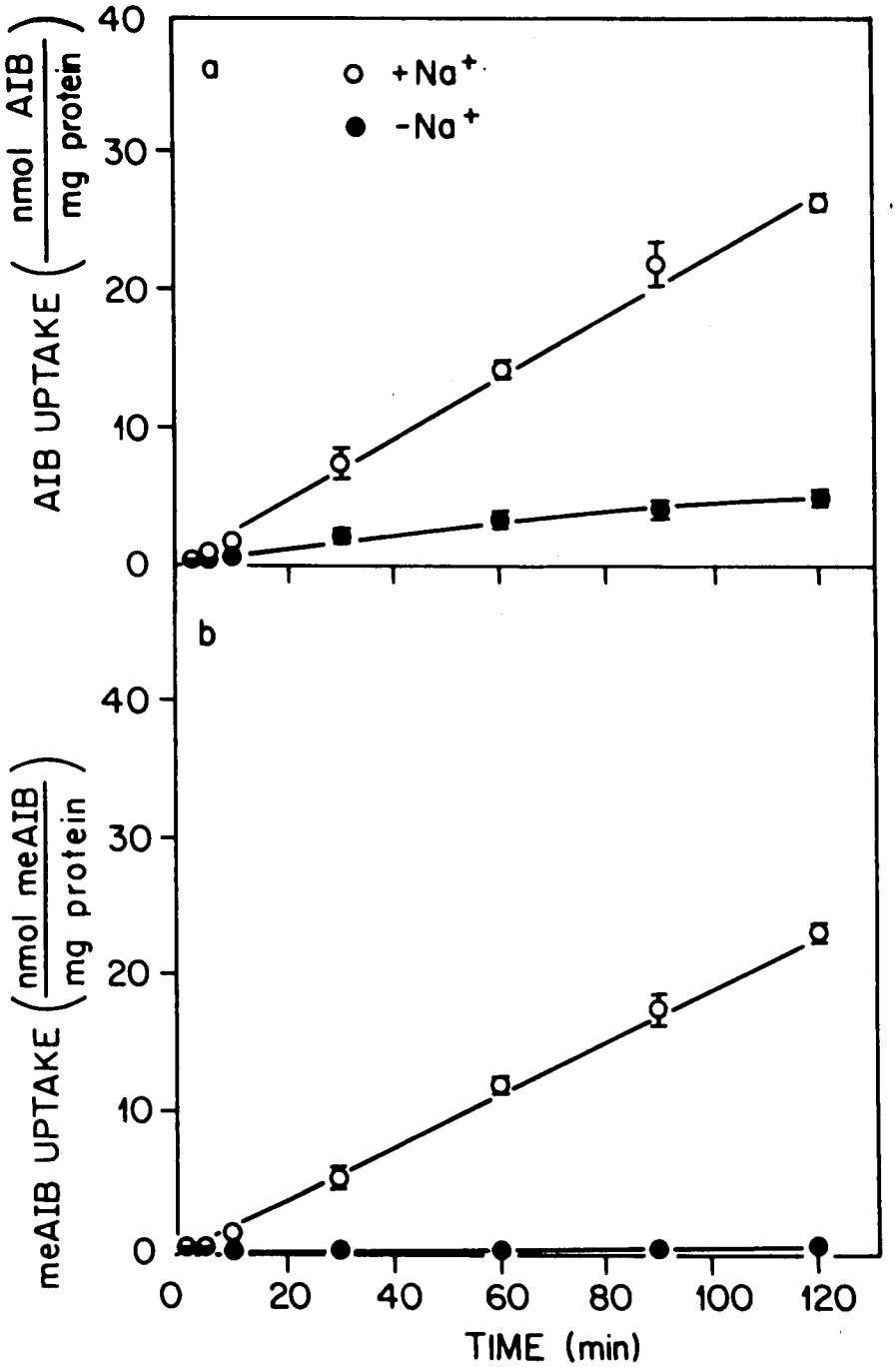


Fig. III-1. Time course of uptake of AIB (a) and meAIB (b) by rapidly growing LLC-PK_1 cells. Uptake of the amino acid analogs was measured in the presence (open circles) or absence (closed circles) of Na^+ as described in Methods. LLC-PK_1 cells were plated on collagen-coated Nucleopore filters and uptake was measured after 1 day in culture.

growth state (see below), the linearity for more than 60 min was always observed. Comparison of Figs. III-1a and III-1b shows that AIB is taken up more rapidly than meAIB, and at 120 min was concentrated in growing cells nearly 50-fold with respect to the medium.

The Na^+-dependencies for uptake of the two analogs are different. In Na^+-free medium, Na^+ being replaced with equimolar concentration of choline, meAIB was not detectably concentrated in the cells even at 120 min. In this case the Na^+-dependence for concentrative uptake was virtually absolute. In contrast, in Na^+-free medium AIB was concentrated slightly, though in the presence of Na^+ the rate of uptake was increased about five times over that observed in the absence of Na^+. AIB uptake could also be depressed about 30% by the addition of excess leucine to the assay medium (not shown). These results are in accord with earlier observations that AIB in several cell types may be concentrated to some extent by exchange with leucine or other amino acids over other transporters (reviewed in Christensen, 1975, 1979).

In the experiments described below the standard assay has been 60-min uptake of either AIB or meAIB in Na^+-medium. It is thus a rate measurement. Because of its absolute Na^+ dependence, meAIB uptake is taken to be the more specific assay for A-system activity, but the changing uptake patterns described below were in each case observed with both amino acid analogs.

AIB Uptake During Growth in Culture and Effects of Modulating Compounds

The uptake of AIB was examined during the growth of LLC-PK_1 cells in culture (Fig. III-2a). When the control cells were sparse the level of uptake was high, approximately 10 nmoles/mg protein-60 min in the experiment shown, and declined as the cells approached confluence. At confluence the uptake rate had fallen to 4-5 nmoles/mg protein-60 min. If the cells were grown in 2 mM HMBA, the decline in uptake rate was accelerated. Conversely, if the tumor promoter TPA was added together with HMBA, the uptake was initially stimulated with respect to the controls and even after 6 days remained above the level of the confluent controls. Fig. III-2b shows cell growth as assessed by the incorporation of [^{3}H]thymidine in the same experiment. There was a striking parallel between the modulation of this parameter and the uptake of AIB, i.e., depression by growth in HMBA and stimulation by growth in HMBA+TPA. At its optimum effective concentration (0.1 μM) TPA alone is cytotoxic to these cells and was therefore always added in combination with HMBA or the phosphodiesterase inhibitors (Chapter II).

A more extensive time-course of the step-down effect is shown in Fig. III-3. Here the cells were seeded at somewhat sparser densities than in Fig. III-2, and the AIB uptake rate on day 0 was somewhat greater, 15-16 nmoles/mg protein-60 min. As before, at confluence the uptake fell to a new low steady-state rate and remained there for the duration of the experiment. In this experiment, the modulating compounds added were the cyclic AMP phosphodiesterase inhibitors theophylline and MIX. Acceleration of stepdown by the former was evident at 2 days, and

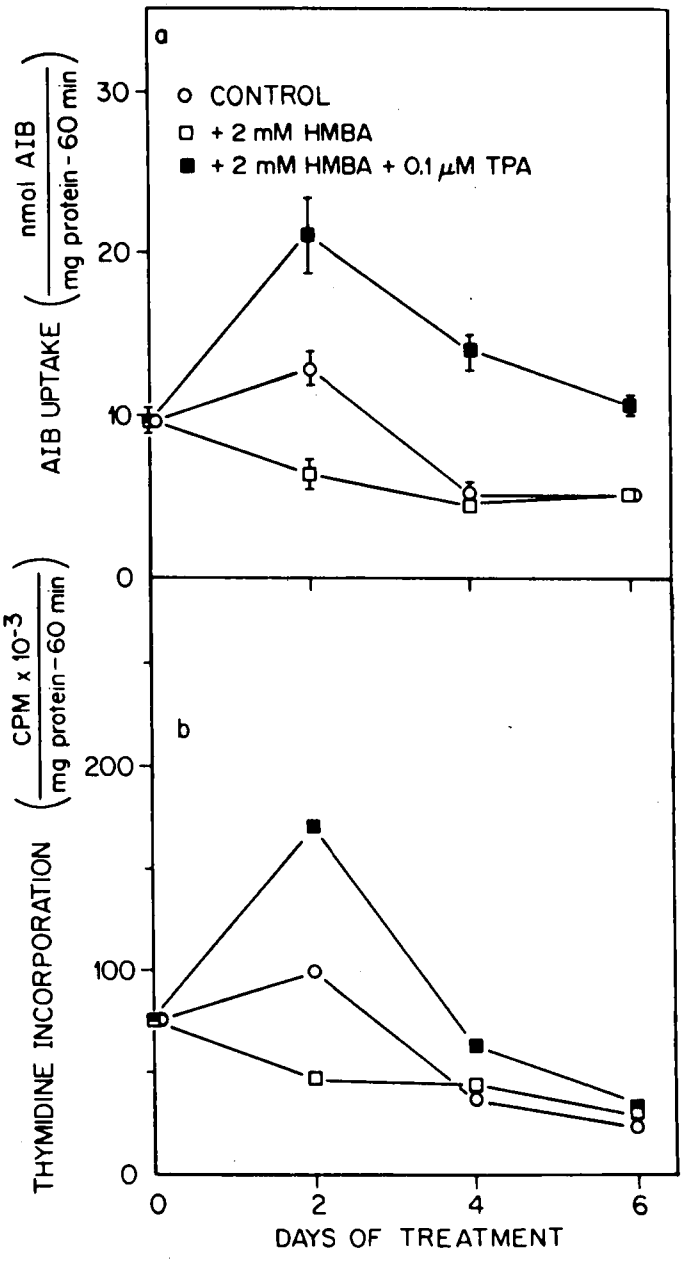


Fig. III-2. Regulation of AIB uptake (a) and [^{3}H]thymidine incorporation (b) in LLC-PK$_1$ cells grown in complete medium (open circles), or complete medium plus 2 mM HMBA (open squares) or plus 2 mM HMBA + 0.1 μM TPA (closed squares). LLC-PK$_1$ cells were plated on collagen-coated Nucleopore filters. Two days later (day 0 in the figure) the filters were transferred to individual 35 mm petri dishes containing 2 ml complete medium or complete medium plus the various compounds. Cultures were refed every 2 days. AIB uptake and [^{3}H]thymidine incorporation were measured 2 days after the last medium change as described in Methods.

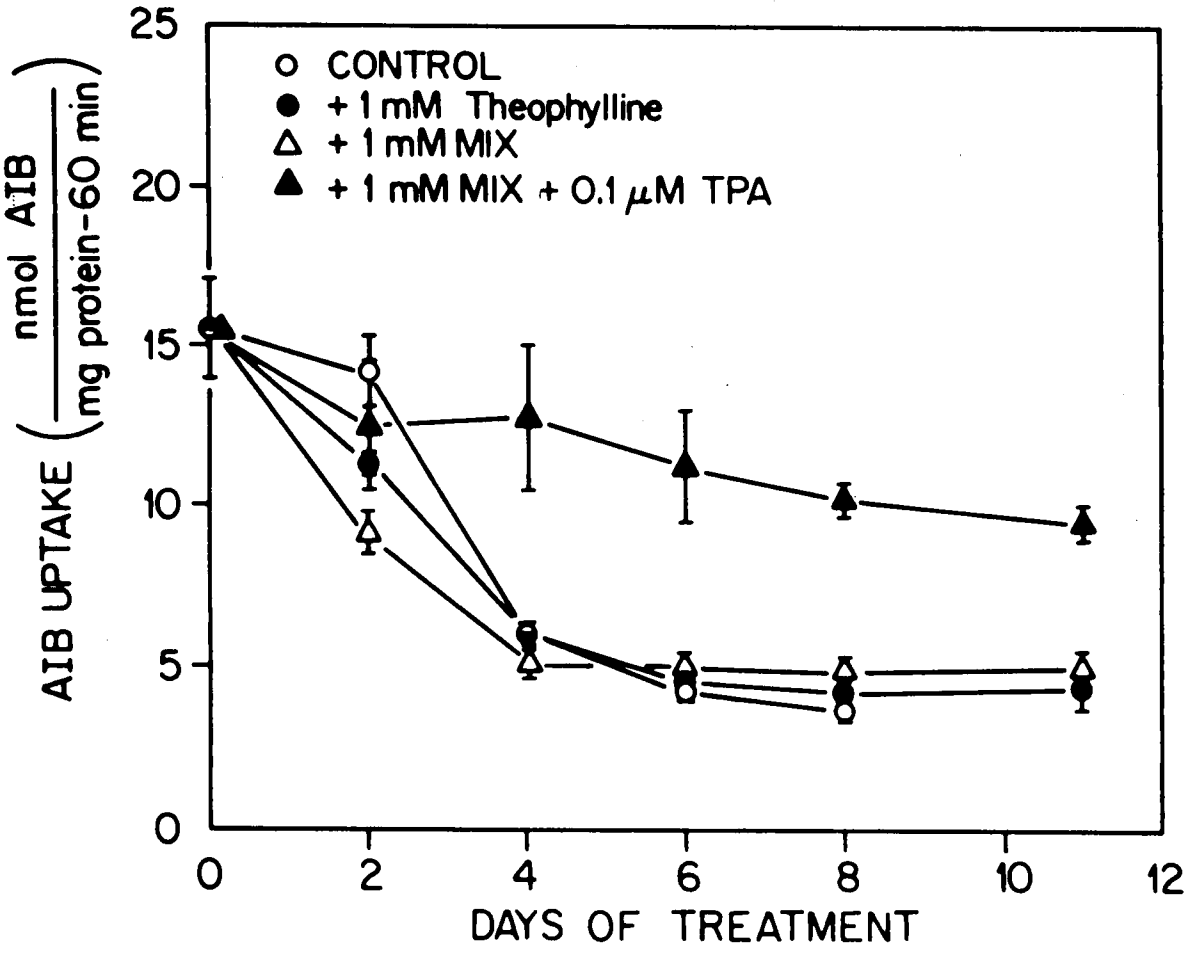


Fig. III-3. Regulation of AIB uptake in LLC-PK_1 cells by growth in phosphodiesterase inhibitors with or without TPA. LLC-PK_1 cells were seeded on collagen-coated Nucleopore filters and 2 days later (day 0 in figure) were transferred to complete medium (open circles), or complete medium plus 1 mM theophylline (closed circles), plus 1 mM MIX (open triangles), or plus 1 mM MIX + 0.1 μM TPA (closed triangles).

was even more evident in the presence of the more potent MIX. In both cases the final steady-state value achieved was the same as that for the controls. As with HMBA, addition of TPA to the MIX cultures markedly inhibited the stepdown even with respect to the controls.

In parallel experiments (not shown) either phorbol or 4-O-methyl TPA, compounds chemically related to TPA but inactive in tumor promotion (Baird and Boutwell, 1971; Hecker, 1978) were included with HMBA in the growth medium. They were also ineffective in modulating the kinetics of AIB uptake as a function of growth state.

The TPA effect is slowly reversible. The consequences of removal of TPA from the growth medium are shown in Fig. III-4. LLC-PK_1 cells were grown in HMBA+TPA for 6 days, at which time AIB uptake was still high (0 time in the figure) at 13-14 nmoles/mg protein-60 min. At the outset of the experiment, the medium was aspirated off all cultures and replaced with complete medium containing either HMBA alone or HMBA+TPA as before. The uptake of AIB was assayed periodically over the next 48 h.

There was an initial small acceleration of the rate of AIB uptake observed in all cultures, apparently associated with the medium change, that reached its maximum at the first assay point at 2 h. Both groups returned to pre-medium-change uptake rates by about 6 hours, and in the HMBA+TPA treated cultures this high rate was maintained until the termination of the experiment. In the HMBA-medium without TPA the same results were observed for the first 24 h after TPA removal, but over the next 24 h the uptake fell toward the rate characteristic of confluent

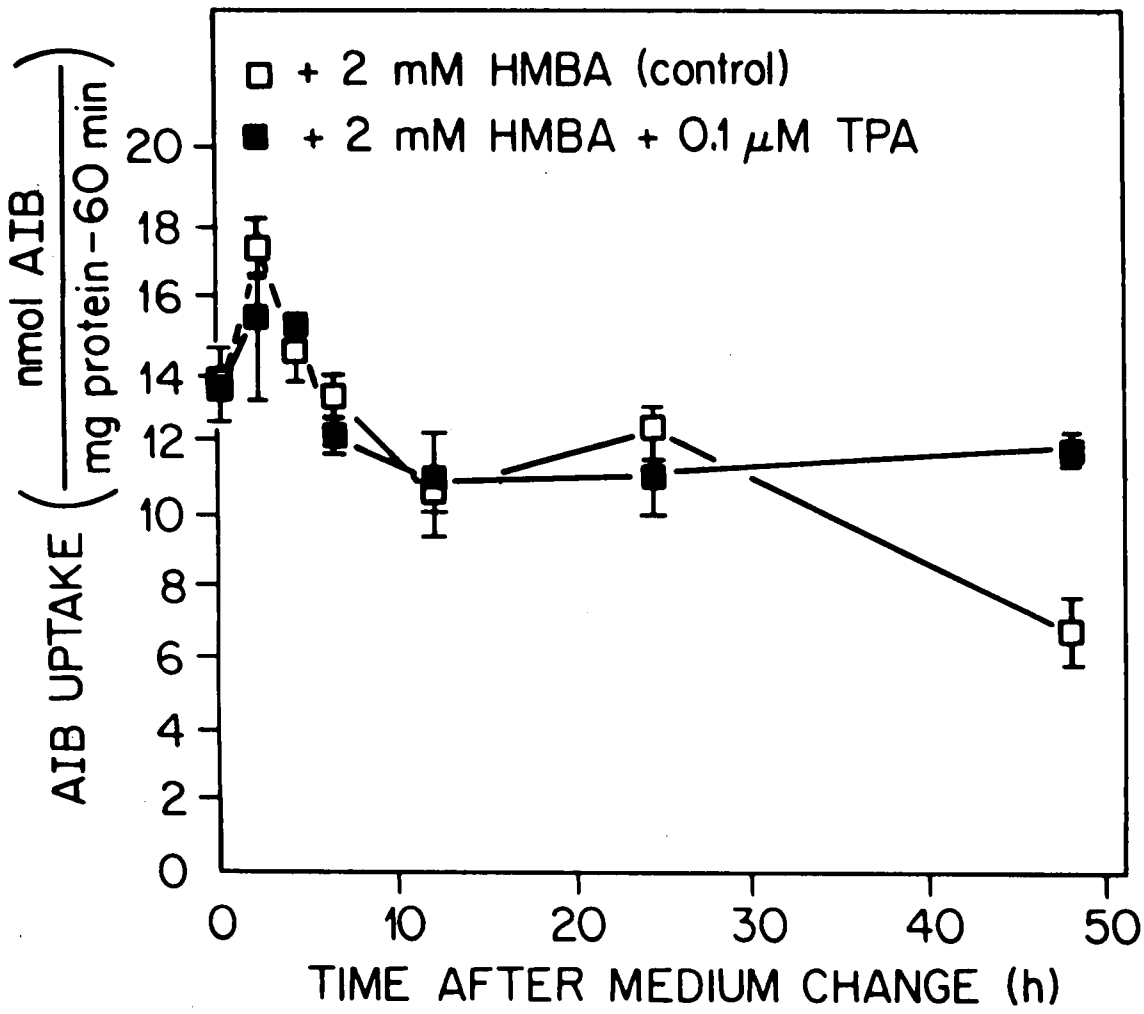


Fig. III-4. Reversibility of the effect of TPA on AIB uptake in LLC-PK$_1$ cells. LLC-PK$_1$ cells were plated on 35 mm tissue culture dishes and grown in complete medium plus 2 mM HMBA plus 0.1 μM TPA for 6 days. The medium in all cultures was then aspirated and replaced with 2 ml complete medium plus 2 mM HMBA plus 0.1 μM TPA (closed squares) or complete medium plus 2 mM HMBA (open squares) at 0 hour in the figure. The 60-min uptake of AIB was assayed at various times after this medium change.

cultures. The TPA effect is thus slowly reversible, but the reversal is not seen until after the time required for one cell cycle (about 22 h).

Effects of TPA on AIB and meAIB Uptake in Confluent Cells

In contrast to the slow reversibility observed after TPA removal, the response of already stepped-down cells to addition of TPA is rapid and dramatic. For the experiment shown in Fig. III-5a, LLC-PK_1 cells were grown to confluence in HMBA (6 days) at which time the AIB uptake rate had stabilized at its characteristic low level. TPA was added at 0 h in the figure without a medium change, and the level of uptake assayed periodically over the next 56 h. Within 2 h of TPA addition the rate of AIB uptake had risen substantially, and by 6 h reached a value approximately 2-fold higher than that observed in control (HMBA only) cultures. The disparate levels observed in the 2 regimens were maintained over the next 50 h. Similar results (not shown) were found when the cells were pre-grown in the presence of MIX instead of HMBA and then treated with TPA.

The same response to TPA in stepped-down cultures is seen with the more specific A-system analog, meAIB (Fig. III-5b). The protocol for this experiment was the same as in Fig. III-5a except for the transported substrate. As noted (Fig. III-1, p. 40), the initial uptake rates for meAIB were somewhat lower than for AIB, but the TPA response was very similar.

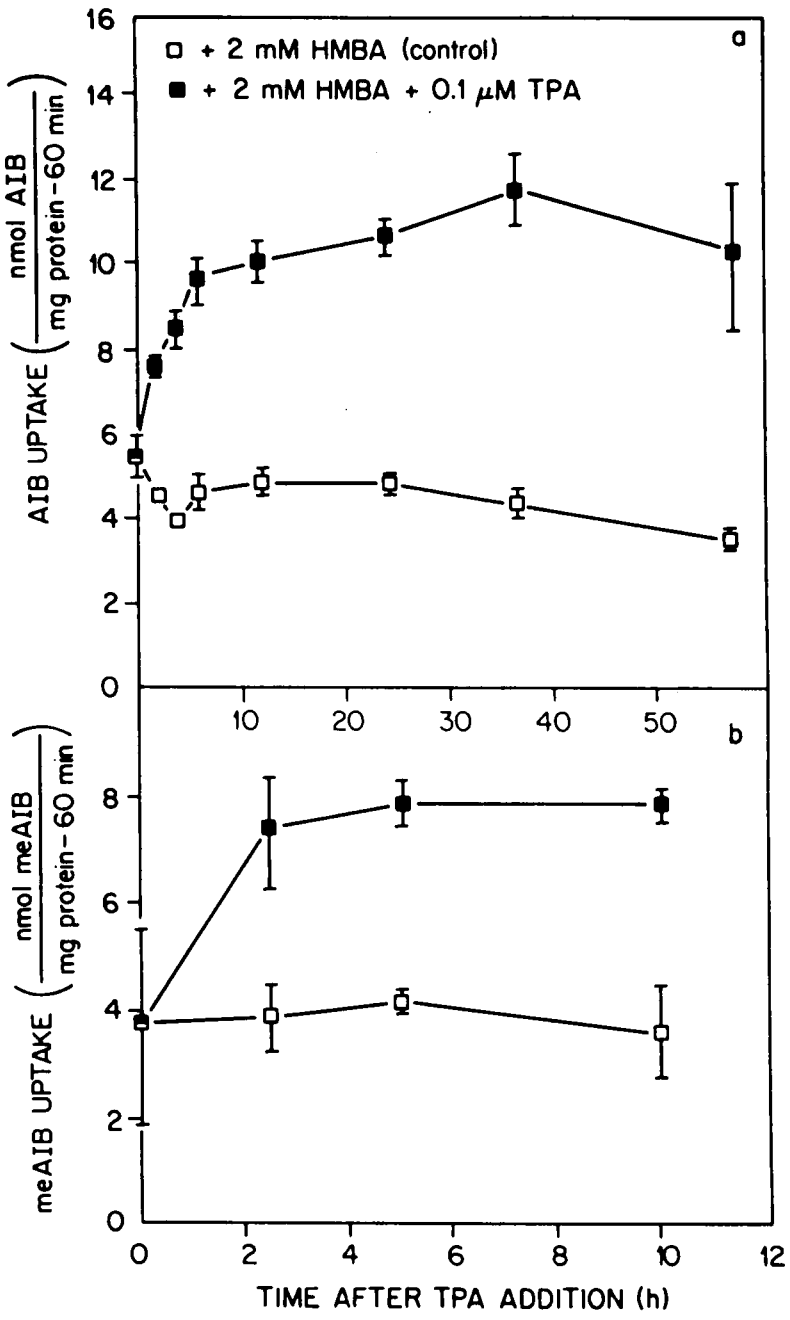


Fig. III-5. Short-term effects of TPA on AIB (a) and meAIB (b) uptake in "stepped-down" LLC-PK_1 cells. LLC-PK_1 cells were grown in complete medium plus 2 mM HMBA for 6 days. At 0 hour in the figures 200 μl HBSS containing ethanol (open squares) or ethanol plus TPA (final concentration 0.1 μM; closed squares) was added to each culture. The 60-min uptake of the amino acid analogs was assayed at various times after this addition. In (a) cells were seeded on 35 mm tissue culture dishes; in (b) on collagen-coated Nucleopore filters.

Role of Macromolecular Synthesis in the TPA Response

The requirements for protein and RNA synthesis in the rapid TPA response were examined with appropriate inhibitors of these functions. In order to identify more specifically the effects on the A-like transport system, meAIB was used as the test substrate. As before, the cells were grown for 6 days in HMBA-medium so that the transport system would be stepped down at the outset of each experiment.

In Fig. III-6, the enhancement of meAIB uptake by TPA is again demonstrated, but the effect is blocked in the presence of cycloheximide. The degree of inhibition, which was total in this experiment, was somewhat variable among separate experiments, but inhibition was always observed. Under the conditions used, treatment of LLC-PK_1 cells with 10 µg/ml cycloheximide for 2 h reduced [^{35}S]methionine incorporation into acid-insoluble material by about 30%. Higher levels of cycloheximide were not used because its effects were clearly demonstrable at this dose and cycloheximide had cytotoxic effects in these relatively long-duration experiments. It should be noted in Fig. III-6 that cycloheximide alone added to control (HMBA-treated) cultures caused a slight depression in transport.

Similar effects were also observed with the RNA-synthesis inhibitor, actinomycin D (Fig. III-7). Under these conditions, actinomycin D at 0.1 µg/ml for 2 h inhibited [^{3}H]uridine incorporation into acid-insoluble material by 80%. The TPA-enhanced uptake of meAIB was slightly inhibited by actinomycin D at 2 h and very markedly so at later times. Again the inhibitor depressed uptake in the control (HMBA) cells. As with

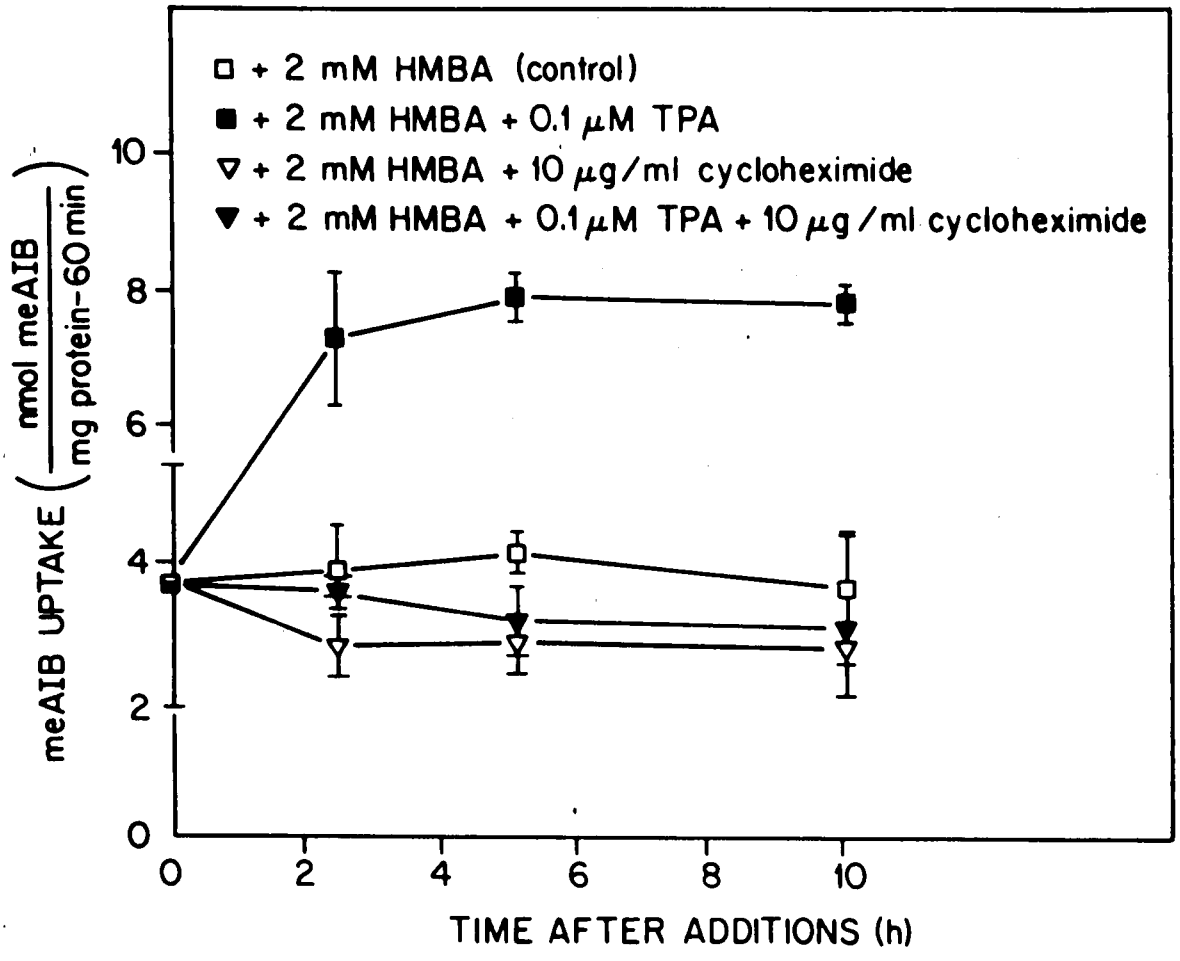


Fig. III-6. Inhibition of short-term TPA effect on meAIB uptake by cycloheximide. LLC-PK_1 cells were seeded on collagen-coated Nucleopore filters and grown for 6 days in complete medium plus 2 mM HMBA. At 0 hour in the figure 200 μl HBSS containing ethanol (open squares), or ethanol plus TPA (final concentration 0.1 μM; closed squares), ethanol plus cycloheximide (final concentration 10 μg/ml; open inverted triangles), or ethanol plus TPA plus cycloheximide (closed inverted triangles) was added to the cultures. The 60-min uptake of meAIB was measured at various times after the additions.

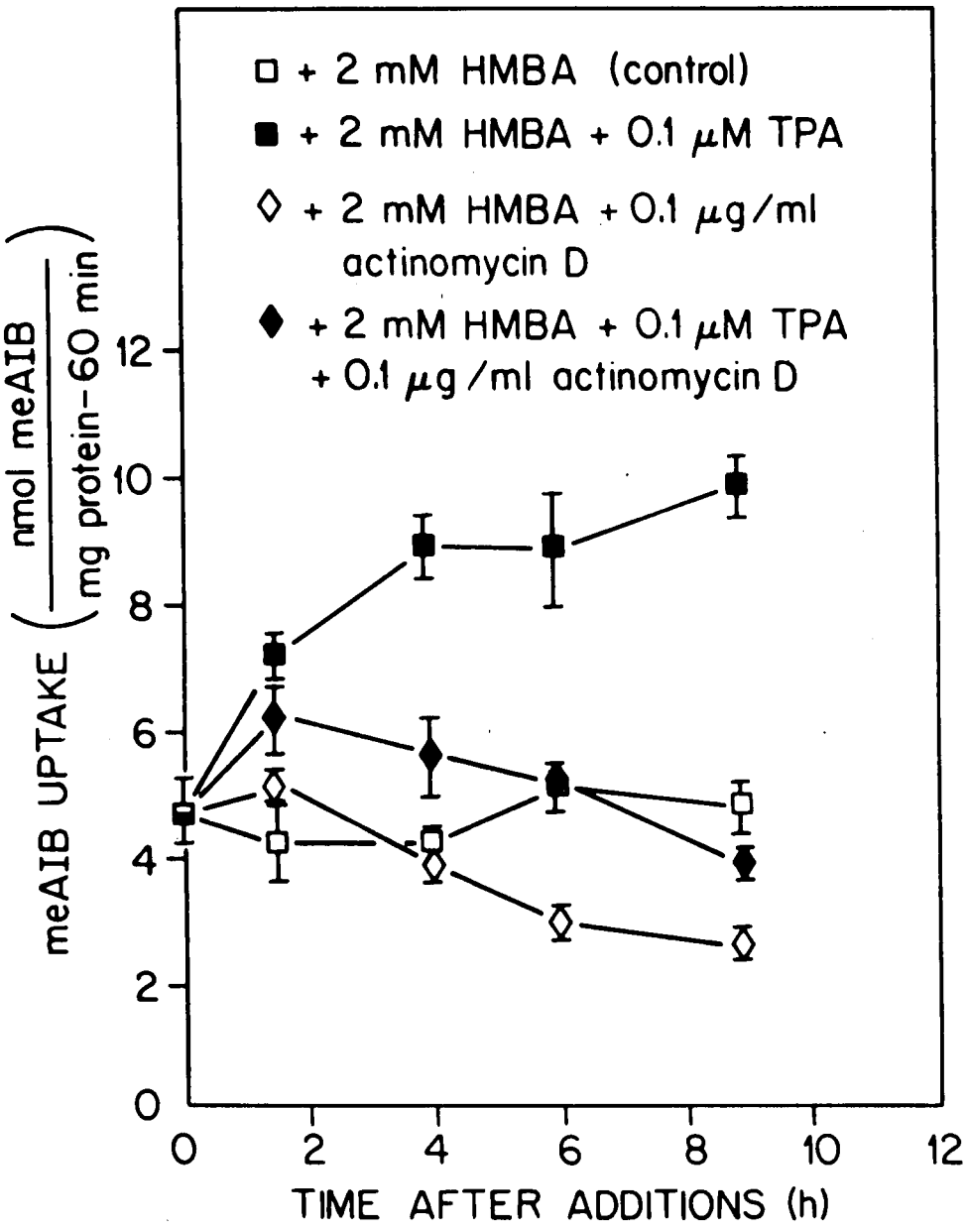


Fig. III-7. Inhibition of short-term TPA effect on meAIB uptake rate by actinomycin D. LLC-PK_1 cells were seeded on filters and grown in complete medium containing 2 mM HMBA for 6 days. At 0 hour in the figure 200 µl HBSS containing ethanol (open squares), ethanol plus TPA (0.1 µM; closed squares), ethanol plus actinomycin D (final concentration 0.1 µg/ml; open diamonds), or ethanol plus TPA plus actinomycin D (closed diamonds) was added to the cultures. The 60-min uptake of meAIB was measured at various times after the additions.

cycloheximide, actinomycin D effects were quantitatively variable among separate experiments, but always evident.

Effect of TPA on the Ouabain-Sensitive Component of meAIB Uptake

If the Na^+-K^+-ATPase is electrogenic it may contribute to the electrical gradient driving Na^+-dependent meAIB uptake in addition to maintaining the Na^+ chemical gradient. TPA stimulation of uptake might then be ascribable to an enhanced electrogenicity of the Na^+,K^+ transporter. To investigate this possibility, the experiment shown in Fig. III-8 was performed. Here the cells were again stepped down in HMBA-medium for 6 days, and at the beginning of the experiment TPA was added to half the cultures. Ouabain was added only during the uptake assays. The concentration of ouabain used (0.1 mM) is approximately 2 orders of magnitude greater than its k_i for ^{86}Rb uptake in LLC-PK_1 cells in the same medium (data not shown). The uptake period in the assays was shortened to 20 min to minimize the effects of ouabain in dissipating the Na^+ chemical gradient. In the control (HMBA) cells, ouabain inhibited meAIB uptake 10-30% at all time points. The TPA stimulating effect, with respect to controls, was again evident, and the TPA-enhanced meAIB uptake was also inhibited by ouabain 10-35% at all time points. If the effect of TPA were solely on the electrogenicity of the Na^+,K^+ transporter, the uptake in the presence of ouabain would be expected to be the same in control and TPA-treated cultures, but this was not observed. Thus TPA stimulation of meAIB uptake in LLC-PK_1 cells

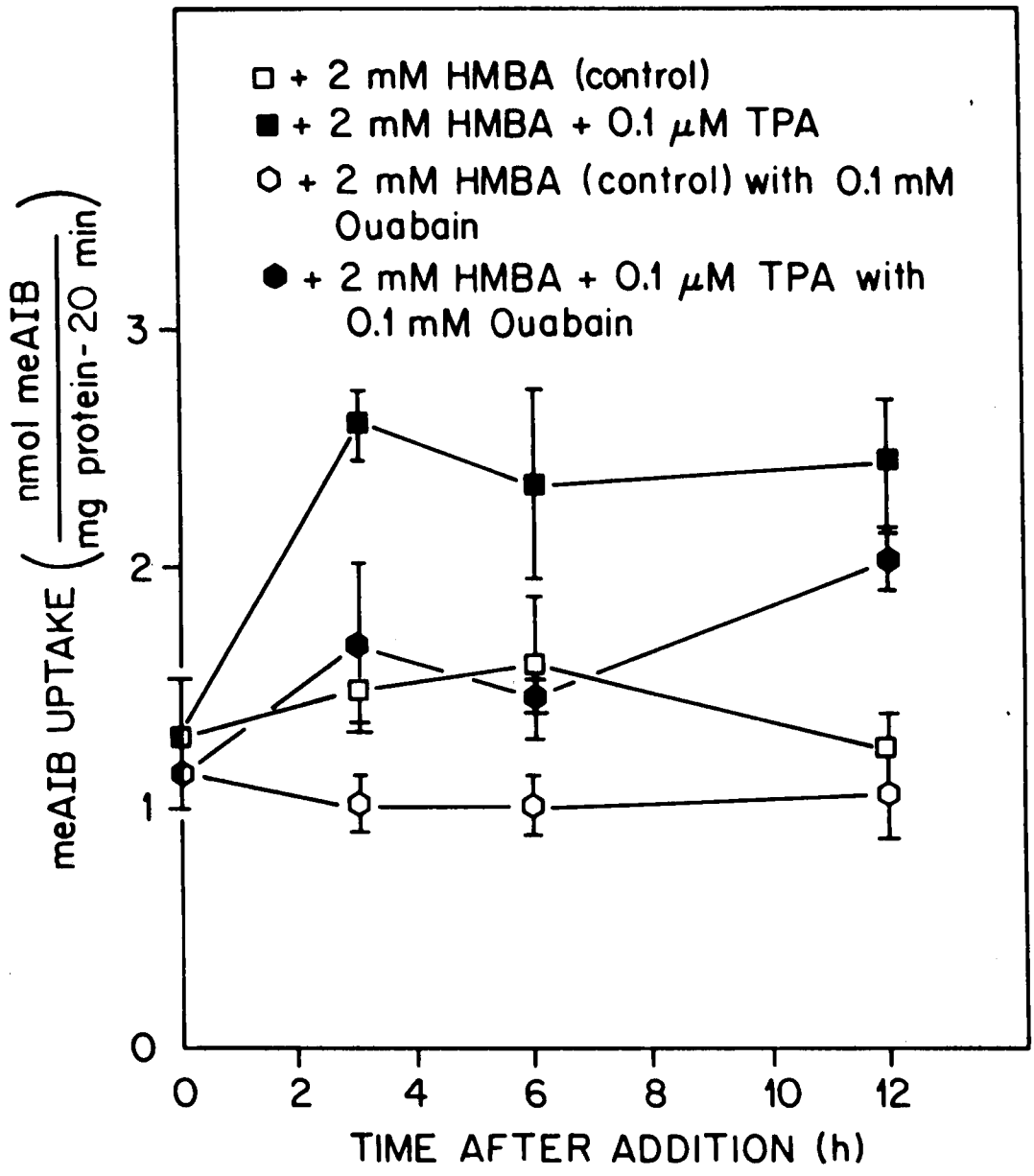


Fig. III-8. Effect of ouabain on control and TPA-enhanced meAIB uptake rate. Cells were seeded on filters and grown in complete medium containing 2 mM HMBA for 6 days. At 0 hour in the figure either 200 µl HBSS containing ethanol (open symbols) or 200 µl HBSS containing ethanol plus TPA (final concentration 0.1 µM; closed symbols) was added to the cultures. The uptake of meAIB after 20 min was measured in the absence (squares) or presence (hexagons) of 0.1 mM ouabain. The assay period was shortened to 20 min to eliminate secondary effects on meAIB uptake due to ouabain-induced depletion of ion gradients.

does not appear to be due to an increase in the electrogenic component of Na^+,K^+ transport.

Discussion

In addition to Na^+-dependent hexose transport described in Chapter II, LLC-PK_1 cells also exhibit Na^+-dependent amino acid transport. This amino acid transport function bears many similarities to the A-system described in other cell types. Concentrative meAIB uptake is absolutely dependent on the presence of Na^+ in the incubation medium, Fig. III-1 (p. 40). In the absence of this ion meAIB is taken up to the same extent as hexoses in Na^+-free medium, and for reasons discussed in Chapter II reaches diffusional equilibrium with the medium meAIB concentration. AIB, on the other hand, can be concentrated slightly in the absence of Na^+, but only one fifth as well as in its presence. As others have shown, this may be due to accelerative exchange of AIB for intracellular amino acids via the L-like transporter (Christensen, 1975; Christensen, 1979). In support of this, AIB uptake by LLC-PK_1 cells is partially inhibited (about 30%) by addition of excess leucine to the uptake medium (unpublished observation).

As in the cases of other cultured cell lines cited in the Introduction, LLC-PK_1 cells regulate the rate of Na^+-dependent amino acid transport during growth in culture, the rate being highest in sparse cultures and declining to a lower, but still concentrative, level when the cells approach confluence and growth slows.. There is a notable parallelism between AIB uptake rate and [^{3}H]thymidine incorporation

(Fig. III-2, p. 43). This parallelism is still observed when the Friend cell differentiation inducer HMBA (Marks and Rifkind, 1978) is added to the cultures.

In addition to HMBA, the phosphodiesterase inhibitors theophylline and MIX accelerate the stepdown of Na^+-dependent amino acid transport, an effect that is strongly countered by inclusion of TPA in the growth medium. TPA-treated cultures, even when MIX is also added to the medium, maintain high levels of meAIB uptake even at high cell densities. In all respects, the transport response is the reciprocal of that seen for Na^+-dependent accumulation of α-meG. Since the driving force for both transport functions is the Na^+-electrochemical gradient, a change in only one of the two transport processes can be attributed to a change in the driving force for uptake. A previous study with human fibroblasts has shown that the driving force for AIB uptake decreases as the cells reach quiescence (Villereal and Cook, 1977). About half of the decrease in uptake is due to a decrease in the membrane potential and a corresponding elevation in K_m for the transport system. The other half is due to a decrease in V_{max} and presumably reflects a smaller number of transporters in quiescent as compared to growing cells (Villereal and Cook, 1978). It is possible that similar events might account for the stepdown in meAIB uptake in LLC-PK_1 cells. The analysis of mechanisms in the human cells was done by "clamping" the membrane potential with the K^+-ionophore valinomycin and manipulating the K^+ concentration of the medium. A similar analysis with LLC-PK_1 cells has not been possible

because the cells are extraordinarily sensitive to the cytotoxic effects of the ionophore, as was indicated in Chapter II.

TPA has the effect of either maintaining a high level of meAIB uptake when present throughout the growth of the culture or of stimulating uptake in stepped-down cultures. Neither the proximate effect of TPA in inducing the cells' pleiotypic responses nor ultimate effect on A-system-like transport are known. Gene expression is probably required, since both cycloheximide and actinomycin D can inhibit the TPA effect on stimulation of transport in stepped-down cell cultures. It is of interest that the TPA stimulation of meAIB uptake in stepped-down cells does not fully restore this function to the level seen in rapidly growing cells. By analogy with the studies on human fibroblasts cited above, a tentative explanation may be that in confluent cells both the driving force for transport and the number of transporters are decreased, and the short-term effects of TPA are only in enhancing the driving force, possibly through alteration of the membrane potential. In this respect, Becker and Cook (1981) showed that in Friend erythroleukemia cells TPA similarly generated a cycloheximide-sensitive enhancement of uptake of AIB with a similar time-course to the TPA effect observed in LLC-PK_1 cells. A major difference, however, is that the enhancement in the Friend cells is entirely in the ouabain-sensitive component of AIB uptake. In the case of Friend cells, it was concluded that a major component of the TPA effect on AIB transport resulted from an enhanced electrogenicity of the Na^+-K^+-ATPase. There is no change in the ouabain-sensitive component of meAIB uptake in LLC-PK_1 cells following

TPA treatment. Therefore, although an increased membrane potential driving meAIB uptake remains a possible mechanism for the effects in LLC-PK_1 cells, it is not the result of a change in the electrogenicity of the Na^+-K^+-ATPase activity.

Na^+-dependent hexose transport has been localized to the apical surface of LLC-PK_1 monolayers (Misfeldt and Sanders, 1981; Rabito, 1981; E. M. Wright, personal communication). A-system amino acid transport, as defined by meAIB inhibitability, is not found in rabbit kidney brushborder vesicles (Mircheff et al., 1981). Misfeldt and Sanders (1981) reported that addition of AIB to the apical surface of monolayers of LLC-PK_1 cells does not increase the short-circuit current, suggesting that there is no AIB-associated Na^+ uptake at the apical surface. These findings suggest that A-system amino acid transport occurs at the basolateral surface of LLC-PK_1 cells. Therefore these two transport functions may be present at different surfaces of the polarized LLC-PK_1 cell. The reciprocal regulation of the two transport functions may be a manifestation of a general differential regulation of components of the two cell surfaces. On the other hand, LLC-PK_1 cells may specifically regulate the levels of these two transport functions. Preliminary measurement of the level of Na^+-K^+-ATPase during differential regulation of the two transport functions indicates that the level of this membrane component, which is localized to the basolateral membrane following cell polarization (Mills et al., 1979; Misfeldt and Sanders, 1981), does not vary during growth in culture (unpublished observation). This result argues against a coordinate regulation of all membrane components at

either surface and suggests that the reciprocal regulation of Na^+-dependent hexose transport and Na^+-dependent amino acid transport in LLC-PK_1 cells is due to the specific modulation of these two functions.

CHAPTER IV

ANALYSIS OF THE DEVELOPMENT OF Na^+-DEPENDENT HEXOSE UPTAKE IN A CLONED CELL LINE DERIVED FROM THE LLC-PK_1 CELL LINE

Introduction

Characterization of the regulation of α-meG uptake and meAIB uptake in LLC-PK_1 cells has provided a number of insights into the mechanism by which this cell line acquires Na^+-dependent hexose uptake during growth in culture. However, from the data presented to this point there are a number of possible mechanisms by which this developmental process could occur.

In the Appendix a model for development of concentrative hexose uptake has been developed by J. S. Cook. A mathematical expression describing this process is derived. In its simplest form this equation is:

$$C_t = C_{max}(1-e^{-kt}) \qquad (1)$$

C_t is the intracellular hexose concentration measured in the total cell population at time t, C_{max} is the maximal intracellular α-meG concentration which can be obtained in the cell population, k is the rate constant for uptake of α-meG, and t is the time period for the uptake measurement. From the Appendix it can be seen that C_{max} is a function of f, the proportion of the cell population that is capable of accumulating hexose against a concentration gradient, and $(S_i/S_o)^*_{max}$, the maximal

accumulation capacity in accumulating cells. This maximal accumulation capacity is in turn a function of the Na^+-electrochemical gradient. The rate constant k is a function of g, the conductance which is primarily a function of the number of transporters per unit of accumulating volume under the assay conditions used here, and C, the maximum accumulation in accumulating cells at a given Na^+-electrochemical gradient. Variation of factors that affect the rate constant for uptake, g and C, will change the rate of hexose uptake but will not alter the maximal level of hexose accumulation whereas variation of factors that affect C_{max}, f and $(S_i/S_o)^*_{max}$, will alter the maximal accumulation of hexose but will not change the rate constant for uptake.

A number of mechanisms could produce development of the concentrative hexose transport function but these various possibilities would yield this development through modulation of various parameters. In one possible mechanism the cells may independently develop the full ability to accumulate α-meG in a time scale that is short with respect to the experiment. The slow increase in uptake in the cell population would be due to the recruitment of an increasing fraction of the cell population into the accumulating mode (variation in f). A second alternative suggests that all cells in a given population acquire the ability to concentrate α-meG at the same time, but the number of transporters per cell, and thus the rate of uptake, increases slowly over the time course of the experiment (variation in g). A third possibility is that all cells in a population acquire the ability to accumulate hexose at some specific point during growth in culture, e.g.

confluence, but the driving force for hexose accumulation, the Na^+-electrochemical gradient, continually increases during growth in culture [variation in $(S_i/S_o)^*_{max}$]. In a fourth possibility for the development of this transport process, the cells may always possess Na^+-dependent hexose transporters but the rate of hexose leakage from the cells might decrease during growth in culture. Since the measured accumulation level is dependent on both the rate of uptake and the rate of leakage, a decrease in the rate of hexose leakage would produce an increase in the accumulation capacity of the cells. As a final alternative explanation for this developmental process, a subpopulation of cells within the LLC-PK_1 cell line may acquire the ability to accumulate hexoses during growth in culture. Then other cells within the population which cannot concentrate hexoses may become physically coupled to these accumulating cells, increase in f and decrease in g. This process would also give the appearance that the level of α-meG accumulating capacity was increasing in the cell population with time in culture.

Using the mathematical model, theoretical sets of data were generated for each of the five alternative explanations. The experimental data obtained from analysis of the time course of hexose uptake during development of hexose accumulation in the cell population could then be analyzed to determine which of the various alternatives best describes the actual developmental process. However, one of the assumptions of the mathematical model is that the cell population is homogeneous. The LLC-PK_1 cell line has never been cloned and may contain a variety of differing cell types. Therefore the LLC-PK_1 cell

line was cloned and one of the clones, Cl 4, was used in the analysis. It was found that the rate constant for uptake did not vary during development of accumulating capacity but there was a continual increase in the maximal level of hexose accumulation in the cell population. Only two of the five alternatives could account for these results: (1) development of accumulating capacity occurs independently in each cell and there is a progressive recruitment of cells into this accumulating population; and (2) the increasing level of hexose accumulation observed during growth in culture is due to a progressive increase in the driving force for uptake. The latter of these two possibilities has been ruled out by other observations. It is therefore proposed that development of hexose accumulation in a Cl 4 cell population is due to the recruitment of cells into the accumulating population during growth in culture.

Materials and Methods

Cell Culture and Measurement of Uptake

These procedures were performed essentially as described in Chapter II.

Cloning of the LLC-PK_1 Cell Line

LLC-PK_1 cells were removed from a confluent flask with trypsin-EDTA. One hundred cells were seeded in a 150 mm Falcon tissue culture dish containing 10 ml complete medium. The culture was grown for 12 days in a humidified 5% CO_2 atmosphere, the medium being changed

every 3 days. At the end of 12 days, well-defined and well-separated colonies of cells were identified and marked using a felt-tip pen to mark the underside of the dish underneath each of the colonies. Then the medium was aspirated and 5 ml trypsin-EDTA was added to the dish. The dish was incubated at room temperature for 2 minutes and then the excess trypsin-EDTA was removed by aspiration. The dish was incubated a further 5 minutes at 37°C. This procedure caused the cells to round-up and become loosely adherent to the culture dish. Each colony was picked up using a sterile pasteur pipet and placed in an individual 35 mm tissue culture dish containing 2 ml complete medium. The dishes containing the individual colonies were grown for 15 days, the medium being changed every 2 days. At the end of this period the dishes were checked to determine which contained cells. The dishes containing cells were grown to confluence and then each of the clones was detached with trypsin-EDTA and seeded in a T-75 flask containing 10 ml complete medium. Each of the flasks was grown to confluence and the clones were then treated in the same manner as the parent line. Aliquots of cells from each of the three clones obtained through this procedure have been frozen and stored in liquid nitrogen.

Results

Development of α-meG Accumulating Capacity in Clones Isolated from the LLC-PK$_1$ Cell Line

The isolated clones, designated Cl 1, Cl 2, and Cl 4, were studied to determine which of the clones were capable of developing Na^+-dependent

hexose accumulating capacity. In addition, the effect of MIX, HMBA, and HMBA + TPA on the development of the transport function in each of the clones was analyzed. Cells from each of the clones were seeded on collagen-coated Nucleopore filters and grown for 5 days in complete medium or complete medium containing 2 mM HMBA, 1 mM MIX, or 2 mM HMBA + 0.1 μM TPA. The level of α-meG uptake in each clone under each treatment was then assessed (Fig. IV-1). Cl 1 cells grown in the absence of any inducing compound displayed a low but significant level of concentrative hexose uptake, equilibrium between medium and intracellular water being ≅0.5 nmoles α-meG/mg protein at this substrate concentration (see Chapter II). The level of uptake was slightly enhanced by addition of MIX to the culture medium, but was greatly enhanced by the addition of HMBA to the medium. Cl 2 cells grown in the absence of an inducing compound also displayed a low level of concentrative uptake. There was also a small enhancement in this level of uptake when the cells were grown in the presence of MIX. However, in contrast to the large enhancement of uptake when Cl 1 cells were grown in the presence of HMBA, Cl 2 cells grown with HMBA showed no enhancement of uptake when compared to control, untreated cultures. Cl 4 cells, as was true of the other two clones, developed a low level of concentrative hexose uptake when grown for 5 days in the absence of any inducing compounds. This clone displayed a slight stimulation of uptake when grown in the presence of HMBA, but showed a much greater enhancement when grown in the presence of MIX. In all three of the clones, addition of 0.1 μM TPA to the

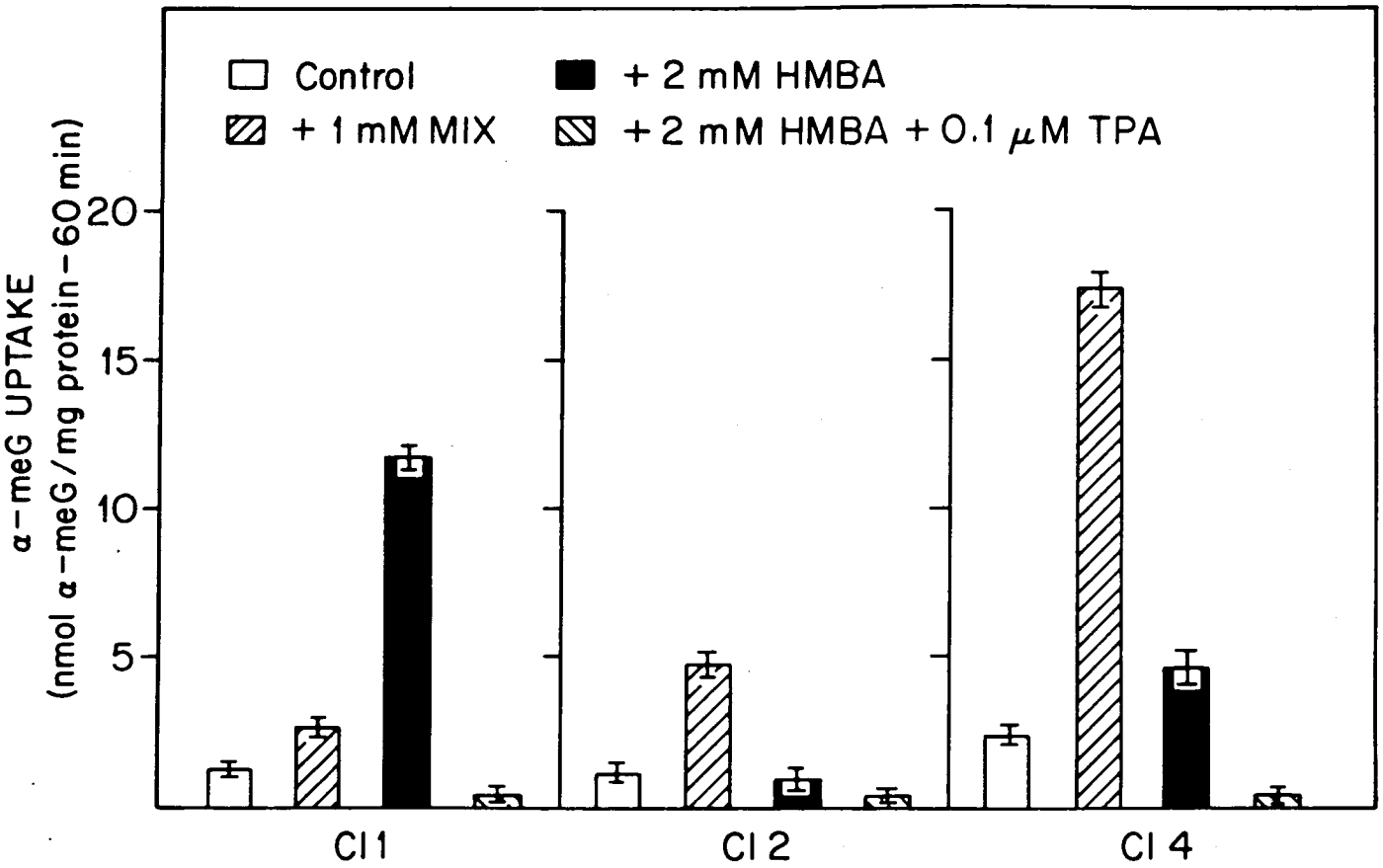


Fig. IV-1. Development of α-meG accumulating capacity in clones derived from the LLC-PK_1 cell line. Cells from each clone were seeded on collagen-coated Nucleopore filters and grown in complete medium or complete medium plus various compounds for 5 days. α-MeG uptake was measured as described previously.

culture medium along with 2 mM HMBA totally inhibited expression of α-meG accumulating capacity.

The effect on α-meG uptake of growth of Cl 2 and Cl 4 cells in the various compounds was examined in more detail (Fig. IV-2). Cl 2 cells (Fig. IV-2a) grown in the absence of any inducing compound slowly increased the level of α-meG uptake over the period of the experiment. Inclusion of 2 mM HMBA in the growth medium had no effect on the appearance of the transport function over the entire course of the experiment. In contrast, inclusion of 1 mM MIX in the growth medium accelerated the appearance of concentrative α-meG uptake when compared to untreated control cultures. Addition of 0.1 μM TPA along with 2 mM HMBA retarded appearance of the transport function. Cl 4 cells (Fig. IV-2b) grown in the absence of any inducing compounds also slowly increased the level of α-meG uptake over the period of the experiment. Inclusion of either 2 mM HMBA or 1 mM MIX in the growth medium accelerated appearance of concentrative hexose uptake when compared to untreated control cultures. Though both compounds accelerated appearance of the transport function when compared to untreated control cultures, MIX was more potent than HMBA at accelerating expression. Addition of 0.1 μM TPA along with 2 mM HMBA severely retarded appearance of the transport function although the cells did eventually express concentrative hexose uptake toward the end of the experiment (data not shown).

These data indicate that appearance of α-meG accumulating capacity occurred in all of the clones. This appearance could be modulated by addition of various compounds to the culture medium, although the

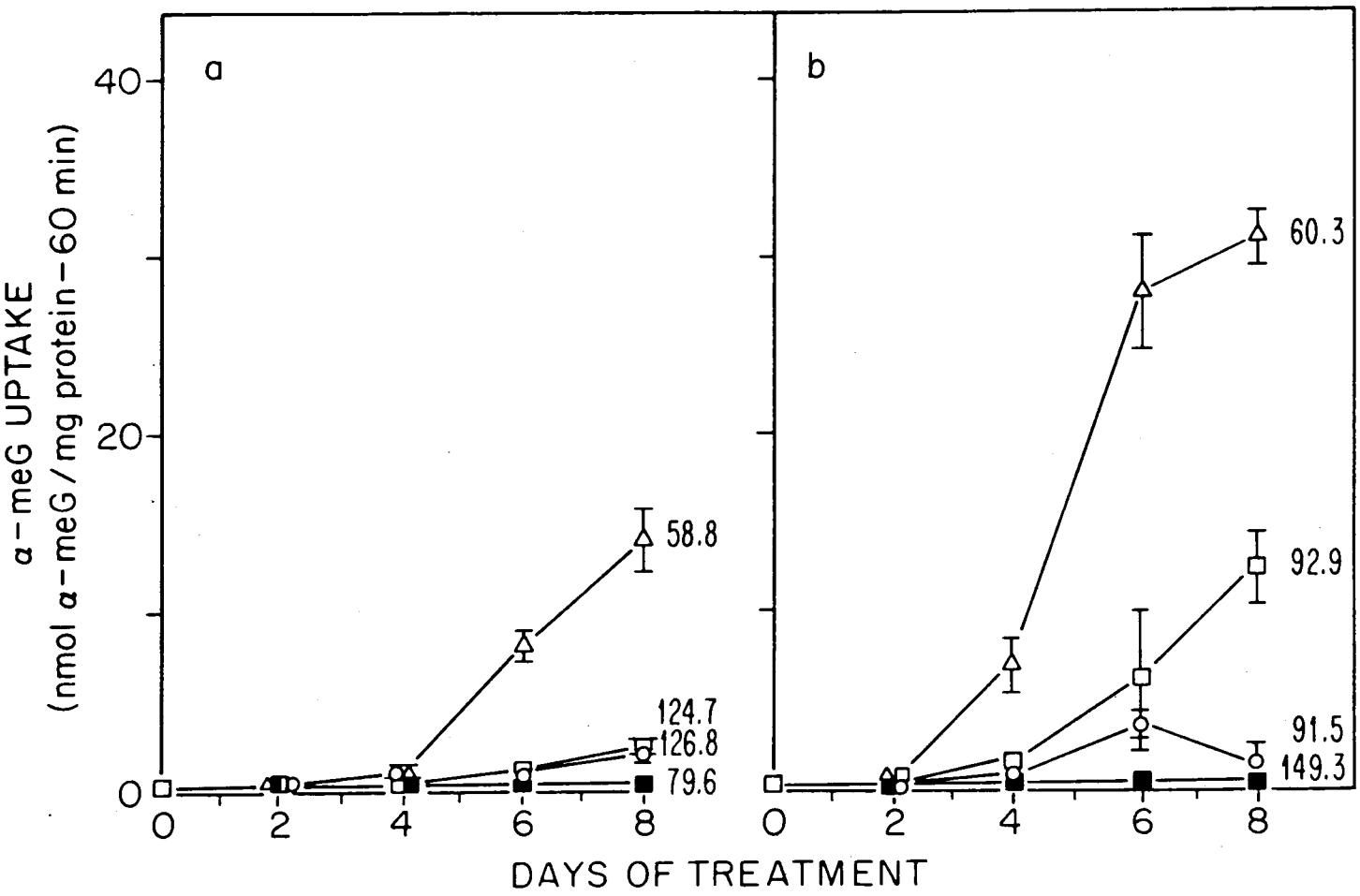


Fig. IV-2. Effect of various compounds on the development of α-meG accumulating capacity in Cl 2 (a) and Cl 4 (b) cells. Cells were seeded on collagen-coated Nucleopore filters. Two days later the filters were placed in petri dishes containing 2 ml complete medium (open circles), or complete medium plus 1 mM MIX (open triangles), plus 2 mM HMBA (open squares), or plus 2 mM HMBA + 0.1 μM TPA (closed squares). Medium was changed every two days.

response of each clone to a given compound was not the same. The major focus of this research project is an analysis of the mechanism of development of Na^+-dependent hexose uptake capacity. Since the expression of this transport function appeared to be the most susceptible to external manipulation in Cl 4 cells this clone was chosen for further analysis.

Analysis of the Development of α-meG Accumulating Capacity in Cl 4 Cells

Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown in complete medium containing 1 mM MIX. After various numbers of days in culture the time course of uptake of α-meG was determined (Fig. IV-3). As was described previously (see Fig. IV-2b) growth of Cl 4 cells in medium containing MIX produced a progressive increase in the level of α-meG uptake after 60 minutes.

Using the mathematical model, the experimental data were fit by generation of theoretical curves. Each set of curves was generated by variation of a single parameter. The curves shown in Fig. IV-3 were generated from the mathematical model by varying C_{max}, a function of f and $(S_i/S_o)^*_{max}$, and keeping k constant. Variation of k did not generate curves displaying the characteristics of the experimental data. Thus the experimental results can be accounted for by only two of the proposed mechanisms. They are: (1) the fraction of the cell population capable of α-meG accumulation is increasing with time in culture; or (2) all cells in the population develop the full capacity for hexose accumulation at the same time but the driving force for uptake increases with

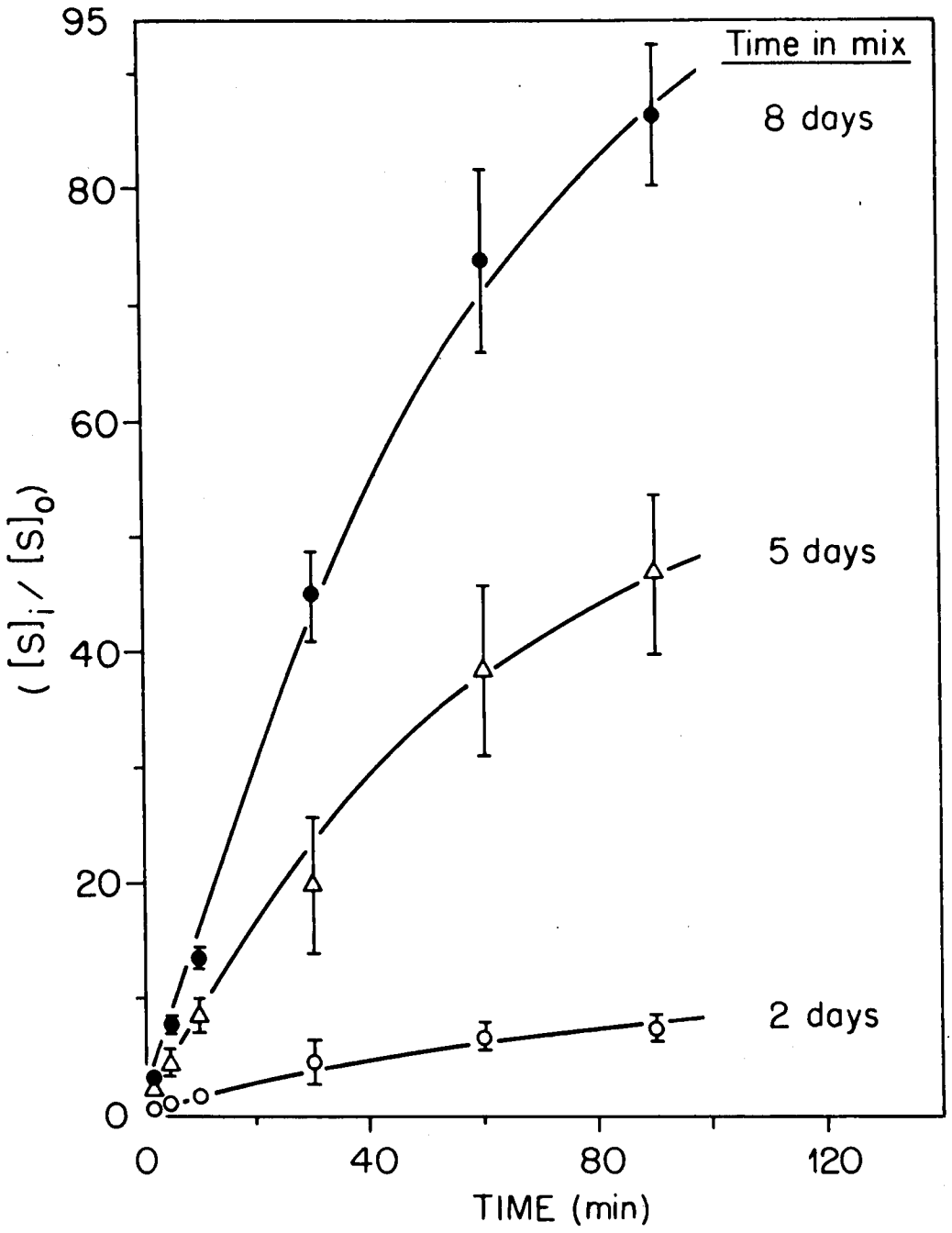


Fig. IV-3. Uptake of α-meG by populations of Cl 4 cells that had been growing for the designated number of days in medium containing 1 mM MIX. The curves are fit to the data according to equation (1), with the assumptions: (i) C_{max} = 10.4 on day 2, = 60.2 on day 5, and = 112 on day 8; and (ii) the time constant k = $(60\ min)^{-1}$ on all three days.

increasing time in culture. For reasons explained in the Discussion the latter of these two alternatives has been ruled out. Therefore it is proposed that development of Na^+-dependent hexose transport in Cl 4 cells is due to a progressive recruitment of cells capable of accumulating hexose.

Reversibility of Expression of Concentrative Hexose Uptake

If development of accumulating capacity in a cell population is due to the progressive recruitment of an increasing proportion of the cell population into the accumulating mode then the level of α-meG uptake in a cell population is directly proportional to, and can be used as a relative measure of, the fraction of the cell population which possesses the ability to accumulate α-meG. Using the level of α-meG uptake as a monitor for the relative proportion of the cell population expressing concentrative hexose uptake the reversibility of expression of the transport function has been examined. The level of α-meG uptake was examined after short, 24 hours, and long, 5 days, exposure of Cl 4 cells to MIX. This level of uptake was compared to the level in cultures which had been grown in medium containing MIX throughout the experiment and to untreated control cultures (Fig. IV-4).

Cl 4 cells were seeded on collagen-coated Nucleopore filters and 2 days later were placed in 35 mm petri dishes containing either complete medium or complete medium plus 1 mM MIX. After 24 hours one group of cultures which had received complete medium plus MIX was refed with complete medium without MIX. Another group of cells was grown in

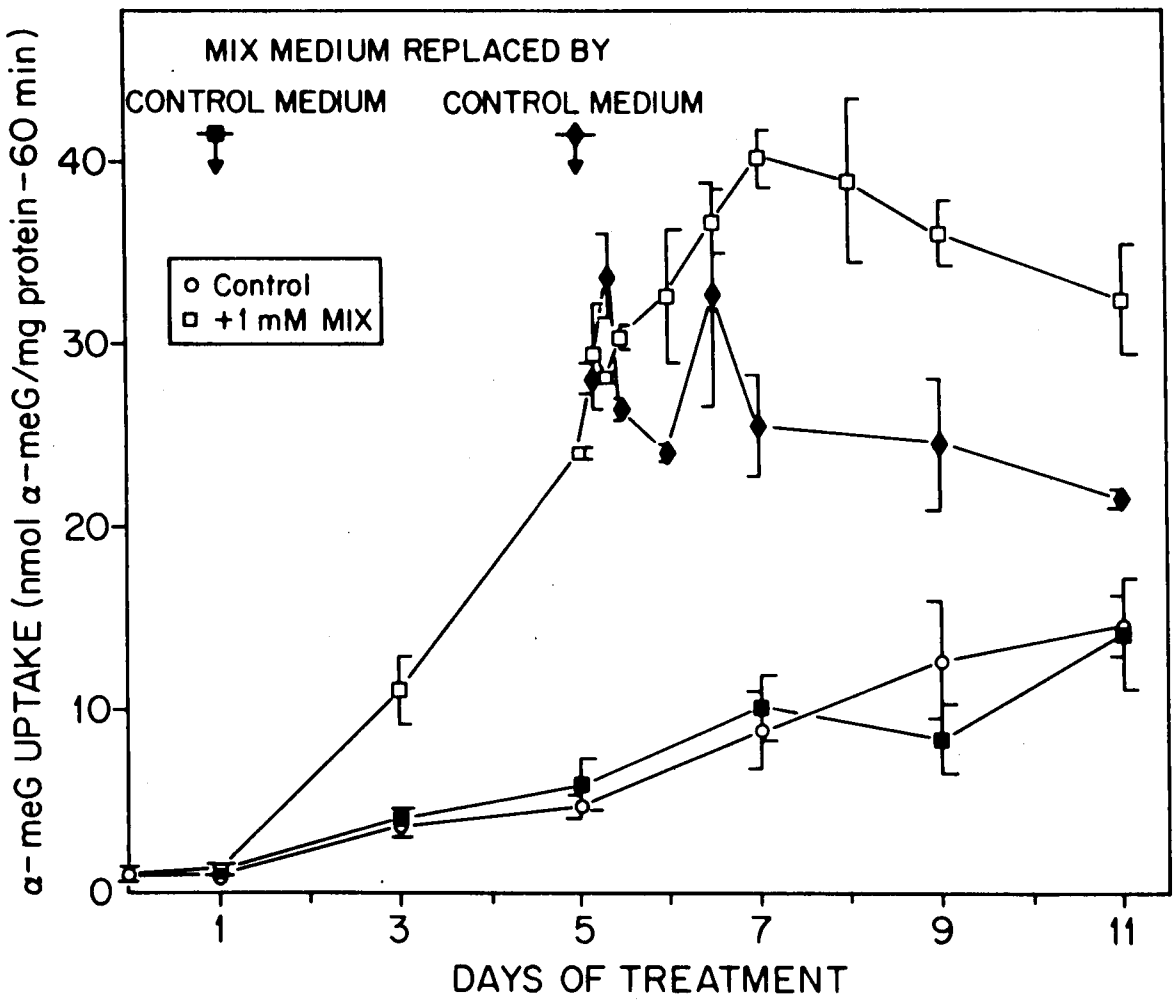


Fig. IV-4. Commitment and reversibility of expression of concentrative hexose uptake in Cl 4 cells. Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown in complete medium without (open circles) or with 1 mM MIX (open squares). Some cultures that originally received complete medium containing MIX were then refed with complete medium without MIX (arrows) after 1 day (closed squares) or 5 days (closed diamonds). Uptake of α-meG was measured periodically over the course of the experiment.

medium containing MIX for 5 days and then refed with medium not containing MIX. In all cases, unless otherwise stated, cultures were refed every 2 days. All cultures were refed on the same days.

A pulse exposure of Cl 4 cells to 1 mM MIX, 24 hours, followed by continuous culture in medium not containing MIX did not affect expression of concentrative hexose uptake when compared to control cultures. If the exposure to MIX was continued longer than 24 hours the cultures exhibited an accelerated appearance of the transport function. This stimulation of expression in the presence of MIX continued throughout the entire course of the experiment, as has been observed in previous experiments (see Fig. IV-2b, p. 67). Cl 4 cells grown in the presence of MIX for 5 days exhibited a substantial enhancement of α-meG uptake when compared to untreated cultures. If the MIX-treated cells were refed with complete medium not containing MIX after 5 days exposure to MIX the level of α-meG uptake transiently increased, maximum being reached 6 hours after the medium change, and then decreased, declining back to the original uptake level by 24 hours after medium change. Throughout the rest of the experiment the cells refed with complete medium without MIX maintained a constant level of α-meG uptake whereas the cells refed with medium containing MIX increased the level of hexose accumulation until day 7 of the experiment. After this time the level of uptake began slowly to decline.

Discussion

Characterization of the Clones Derived from the LLC-PK_1 Cell Line

The three clones obtained from the LLC-PK_1 cell line all are capable of developing Na^+-dependent hexose transport. However, the regulation of the expression of this transport function appears to be different in the various clones. Cl 1 cells are highly responsive to treatment with HMBA but are only slightly responsive to MIX. The rate of expression of concentrative hexose uptake in Cl 2 cells is somewhat enhanced by growth of the cells in medium containing MIX but is completely unaffected by the presence of HMBA in the medium. The fact that both of the clones are capable of developing Na^+-dependent hexose transport but respond differently to the two compounds suggests that the two compounds may not stimulate the appearance of the transport function through the same mechanism. It is possible that a number of stimuli may lead, through a variety of cellular mechanisms, to appearance of the transport function. Lack of responsiveness of a clone to one of the inducing compounds may indicate an alteration at some step in one of the pathways leading to appearance of the transport function. Future studies on these two cell lines may prove very useful in identifying the various cellular mechanisms capable of stimulating expression of concentrative hexose uptake. Cl 4 cells respond to both MIX and HMBA, although there is a greater response when cells are grown in the presence of MIX. All three clones respond to TPA by retarding the appearance of the transport function, as is true of the parent cell line.

Analysis of the Kinetics of Development of Hexose Accumulation Capacity in Cl 4 Cells Using a Mathematical Model for the Process

Using the mathematical model developed by J. S. Cook for acquisition of Na^+-dependent hexose transport by a cell population, it has been shown that the experimental data are compatible with only two of the five explanations presented. The first possible alternative asserts that development of Na^+-dependent hexose transport capacity in a cell population is due to the independent and complete development of this capacity in each cell. The recruitment of an increasing proportion of the cell population into this accumulating mode produces the experimentally observed slow increase in hexose accumulation. The second possible explanation states that all cells in a population acquire the capacity for hexose accumulation simultaneously but that the driving force for uptake increases during growth in culture or in response to the inducing compounds. The latter alternative appears unlikely on the basis of two experimental observations. C. Shaffer of this laboratory has measured the membrane potential of LLC-PK_1 cells during growth in culture using a lipophilic cation which distributes across the membrane on the basis of the membrane potential. The data indicate that the membrane potential decreases as the cells become growth arrested, at which time the level of α-meG uptake is increasing (personal communication). This result is in agreement with data from other cell types (Villereal and Cook, 1977) and also with the changes observed for AIB and meAIB uptake in LLC-PK_1 cells and Cl 4 cells (Fig. III-2a, p. 43 and unpublished observation).

These data suggest that the driving force for α-meG accumulation may actually be decreasing during the time when the level of α-meG uptake is increasing. Thus the appearance of concentrative α-meG uptake in cell populations and the increasing level of uptake observed with increasing length of time in culture or following treatment with an inducing compound, e.g. MIX, is probably due to an increasing proportion of the cell population which is capable of concentrating hexoses. Since the analysis of development of the transport function was carried out using the Cl 4 cell line the possibility of multiple cell types with differential rates of expression can be ruled out, thus eliminating possible ambiguities in interpretation of the data.

The random and independent "commitment" of Cl 4 cells to acquire Na^+-dependent hexose uptake bears striking similarities to other differentiation events studied in culture. Initiation of differentiation in Friend erythroleukemia cells (Marks and Rifkind, 1978) and the onset of myotube formation in cultured myoblasts (Zalin, 1979) have both been described as random processes in which each cell independently decides to "commit" to the differentiation pathway. In each of these experimental systems the "commitment" to differentiate is correlated with the irreversible entrance of the cells into a quiescent state (Marks and Rifkind, 1978; Zalin, 1979). The appearance of concentrative hexose transport in LLC-PK_1 cells (Fig. II-5, p. 23) and Cl 4 cells (unpublished observation) is also associated with decreased cell growth. In addition, the two compounds that accelerate appearance of the transport function, MIX and HMBA, also accelerate entrance of LLC-PK_1

cells into the quiescent state. Entry of tissue culture cells into a quiescent state has itself been described as a probablistic event in which each cell acts independently (Pardee et al., 1978). It is therefore tempting to propose that the development of α-meG accumulating capacity in a single cell is intimately associated with the entrance of that cell into quiescence. Such a mechanism would also correlate with the possible involvement of cAMP in development of the transport function since the elevated cAMP levels commonly associated with quiescence would result in activation of cAMP-dependent protein kinases which would stimulate specific phosphorylation events by these enzymes. In addition, in BHK cells the level of cAMP-dependent protein kinase type II isozyme increases at quiescence (McClung and Kletzien, 1981). If a similar event occurs in Cl 4 cells then the level of cAMP-dependent protein kinase activity would be further increased at quiescence. Whether cAMP stimulates appearance of α-meG accumulating capacity solely by stimulating entry of cells into quiescence or acts in a more direct manner to affect expression is not clear. As discussed in Chapter II, a number of factors make it unlikely that cAMP directly affects the membrane to modulate the level of Na^+-dependent hexose transport.

The effect of TPA on expression of α-meG uptake provides further insight into the complexity of the mechanism leading from stimulation to appearance of the transport function. LLC-PK_1 cells treated with HMBA plus TPA are transiently stimulated for cell growth, but eventually the cells enter a quiescent state. In cells treated with HMBA plus TPA intracellular cAMP levels are initially depressed but eventually rise to

levels at or above those attained in untreated cultures at quiescence. However, expression of α-meG accumulating capacity is still severely retarded in these cultures. The cAMP levels of cells treated with MIX plus TPA rapidly rise to levels far above those observed in control cultures yet expression of α-meG accumulating capacity is again delayed, although expression of the transport function does eventually occur. This suggests that TPA interferes with some step leading to expression of the transport function, but the cells are eventually able to overcome this interference and expression does occur. As was seen in Chapter II, expression does not occur until intracellular cAMP levels have risen, further supporting a crucial role for cAMP in regulation of expression of Na^+-dependent hexose transport capacity.

There are at least two alternative explanations for the effect of TPA on expression of α-meG accumulating capacity. The inhibition of appearance of the transport function by TPA may be due to the interaction of TPA or a product of TPA at some step in the pathway leading from the onset of quiescence to expression of concentrative hexose uptake. Alternatively, TPA may decrease the probability for a cell to enter the quiescent state and thus initiate expression. The fact that TPA-treated cells do become quiescent argues against the latter alternative, but it is possible that there are multiple quiescent states only one of which allows expression of concentrative hexose uptake. TPA could then restrict entry into this specific quiescent state without blocking entry of the cells into other quiescent states. Again, cAMP could be involved through stimulation of cell entry into the appropriate quiescent state.

The effect of TPA does not appear to be a direct interaction with the transporter, i.e. some sort of direct inactivation of transporter molecules, since addition of TPA to cells which are rapidly developing α-meG accumulating capacity does not cause a complete or even a large decrease in the level of α-meG uptake (unpublished observation). Rather, there is at most a small decline in the level of uptake after addition of TPA. Cells not treated with TPA continue to increase the level of α-meG uptake during the course of the experiment. These results suggest that TPA inhibits further recruitment of cells into the population of cells capable of accumulating hexoses, but does not significantly affect those cells which have already developed the transport function.

Reversibility of Expression of Concentrative Hexose Uptake

From Fig. IV-4, p. 71, it is apparent that continuous exposure to the inducing stimulus, 1 mM MIX, is required for continuous development of concentrative hexose uptake. Brief exposure, 24 hours, of nonaccumulating cells to the inducing stimulus does not significantly alter the rate of expression of the transport function when compared to control cultures that have never been treated with MIX.

In Friend erythroleukemia cells brief exposure, 12-16 hours, of cells to the inducing stimulus produces a maximal level of differentiation a number of days later (Marks and Rifkind, 1978). This has been interpreted as indicating that Friend cells undergo a "commitment" to differentiate which occurs long before the actual expression of the

differentiated characteristic. By analogy Cl 4 cells do not commit to develop Na^+-dependent hexose transport until just prior to or at the same time as expression of the transport function. If there was a commitment event which occurred substantially before the actual expression of the transport function the level of uptake would be expected to continue increasing for some length of time after removal of the inducing stimulus. This does not occur. The fact that exposure to MIX for 24 hours has no effect on the rate of expression of the transport function suggests that the commitment of any Cl 4 cells to begin expressing concentrative hexose uptake requires greater than 24 hours exposure to the inducing stimulus. Cells that have not begun to express concentrative hexose uptake by the time the inducing stimulus is removed will not express the transport function at a later time, unless another stimulus is applied.

In addition, Fig. IV-4, p. 71, indicates that the expression of Na^+-dependent hexose transport is an irreversible process. Removal of the inducing stimulus halts further recruitment of transporting cells, but the proportion of cells expressing concentrative hexose transport remains constant. Thus, Cl 4 cells appear irreversibly to develop Na^+-dependent hexose transport. An alternative, though less likely, interpretation of the data suggests that a specific length of treatment with an inducing stimulus causes a specific level of concentrative hexose uptake to be expressed in the population, but any single cell will be capable of reversible acquisition of the transport function. Though this seems unlikely it cannot be ruled out on the basis of the

present data. To determine directly the reversibility of expression in Cl 4 cells it would be necessary to identify and isolate cells that possess Na^+-dependent hexose transport capability. Then these cells could be directly monitored to determine at a cellular level whether an individual cell can differentiate and dedifferentiate repeatedly during maintenance in culture.

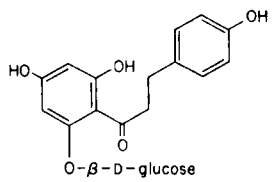
CHAPTER V

CHARACTERIZATION AND ANALYSIS OF [^{3}H]PHLORIZIN BINDING TO C1 4 CELLS

Introduction

It was concluded from the data presented in Chapter IV that development of concentrative hexose uptake in C1 4 cells is due to the progressive recruitment of cells into the concentrating population. Since the appearance of the transport function is believed to be through the appearance of active Na^+-dependent hexose transporters in the cell membrane, the increasing level of concentrative hexose transport should be paralleled by an increasing number of Na^+-dependent hexose transporters in the cell population. This correlation has been investigated directly by measuring the number of Na^+-dependent phlorizin binding sites in the cell population and comparing this to the level of α-meG accumulation in that population.

Phlorizin is a compound composed of two benzene rings (phloretin) attached to a D-glucose moiety. This compound, at low concentrations



(μM range), inhibits Na^+-dependent D-glucose transport in vivo (Lotspeich and Woronkow, 1958; Diedrich, 1966; Silverman et al., 1970; Chapter II) and in vitro (Turner and Silverman, 1977). A high affinity, Na^+-dependent, D-glucose inhibitable component of phlorizin binding can

be detected both in vivo and in vitro and has been shown to be due to binding of phlorizin to a site which is at or very near to that part of the Na^+-dependent D-glucose transporter that is exposed at the luminal face of the brushborder membrane in the renal proximal tubule (Silverman and Black, 1975). Thus measurement of the number of Na^+-dependent phlorizin binding sites provides a method for determining the number of active Na^+-dependent hexose transporters.

Initially it was determined that intact Cl 4 cells possess Na^+-dependent phlorizin binding sites. The characteristics of these sites are very similar to those measured for Na^+-dependent phlorizin binding to isolated brushborder membrane fragments from renal proximal tubular cells. Determination of the parameters for Na^+-dependent phlorizin binding, K_d (dissociation constant) and N (total number of binding sites), to intact Cl 4 cells exhibiting various levels of α-meG uptake indicates that there is a good correlation between the level of α-meG uptake and N. The K_d for binding did not vary significantly during development of the transport function.

Materials and Methods

Cell Culture and Measurement of Uptake

These procedures were performed essentially as described in Chapter II.

Measurement of [^{3}H]Phlorizin Binding

Measurement of [^{3}H]phlorizin binding to intact Cl 4 cells was performed using a procedure identical to that used for measurement of α-meG uptake.

Results

Na^+-Dependence of [^{3}H]Phlorizin Binding to Intact Cl 4 Cells

The time course of [^{3}H]phlorizin binding to Cl 4 cells in the presence or absence of Na^+ is shown in Fig. V-1. Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown for 5 days in complete medium containing 200 μM MIX, at which time the cells displayed a significant level of concentrative hexose uptake. Phlorizin binding was measured after various lengths of incubation at 37°C. In both the presence and absence of Na^+ phlorizin binding was already approaching a maximal level after 1 minute incubation. The slow increase in binding in the presence of Na^+ observed in this experiment was not a consistent observation. The level of phlorizin binding in both the presence and absence of Na^+ was found to be identical after 3 minutes incubation and after 200 minutes incubation. Since phlorizin binding reaches a maximum by 3 minutes of incubation all further experiments were carried out using a 3 minute incubation.

Concentration Dependence of [^{3}H]Phlorizin Binding to Intact Cl 4 Cells

In vitro analysis of [^{3}H]phlorizin binding to isolated fragments of brushborder membrane from renal proximal tubule indicates that the Na^+-dependent component of phlorizin binding is a saturable process, and that it is this component which represents binding to the Na^+-dependent D-glucose transporter. Therefore the concentration dependence of [^{3}H]phlorizin binding to intact Cl 4 cells was examined in the presence

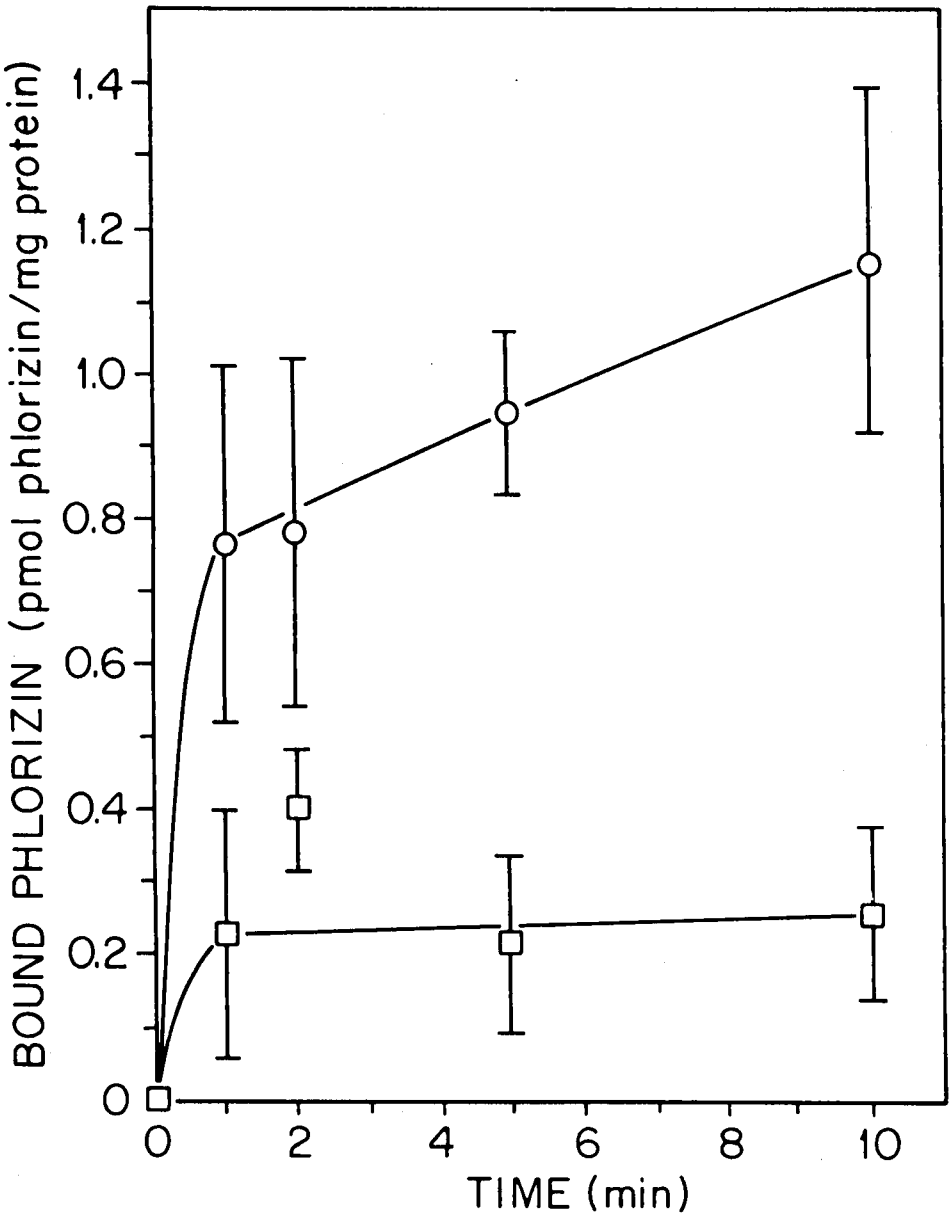


Fig. V-1. Time course of phlorizin binding to intact Cl 4 cells. Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown for 6 days in complete medium containing 200 μM MIX. [^{3}H]phlorizin was added to the cultures in the presence (circles) or absence (squares) of Na^+; final concentration 0.2 μM, 0.2 μCi/ml.

and absence of Na^+ (Fig. V-2). In Fig. V-2a the binding of phlorizin in the presence and absence of Na^+ is plotted against the free phlorizin concentration. In the absence of Na^+ the amount of phlorizin bound to the cells increased linearly with phlorizin concentration. These data are indicative of a nonsaturable binding activity. At all phlorizin concentrations tested the amount of phlorizin bound to cells in the presence of Na^+ was significantly greater than the amount bound in the absence of Na^+, indicating the presence of a Na^+-dependent component of binding. The binding of phlorizin in the presence of Na^+ was not a linear function of phlorizin concentration suggesting that this binding activity contains a number of components at least one of which is a saturable process.

The Na^+-dependent component of phlorizin binding was plotted according to Scatchard (Fig. V-2b). The data can be described by a single line, suggesting that the Na^+-dependent component of phlorizin binding is due to binding to a single homogeneous class of receptors. The slope of the line yields the dissociation constant, K_d, and the x-intercept corresponds to the total number of Na^+-dependent phlorizin binding sites, N. In the experiment presented in Fig. V-2 the K_d for Na^+-dependent phlorizin binding to intact Cl 4 cells is 51 nM and the total number of binding sites, N, equals 1.02 pmoles per milligram cell protein.

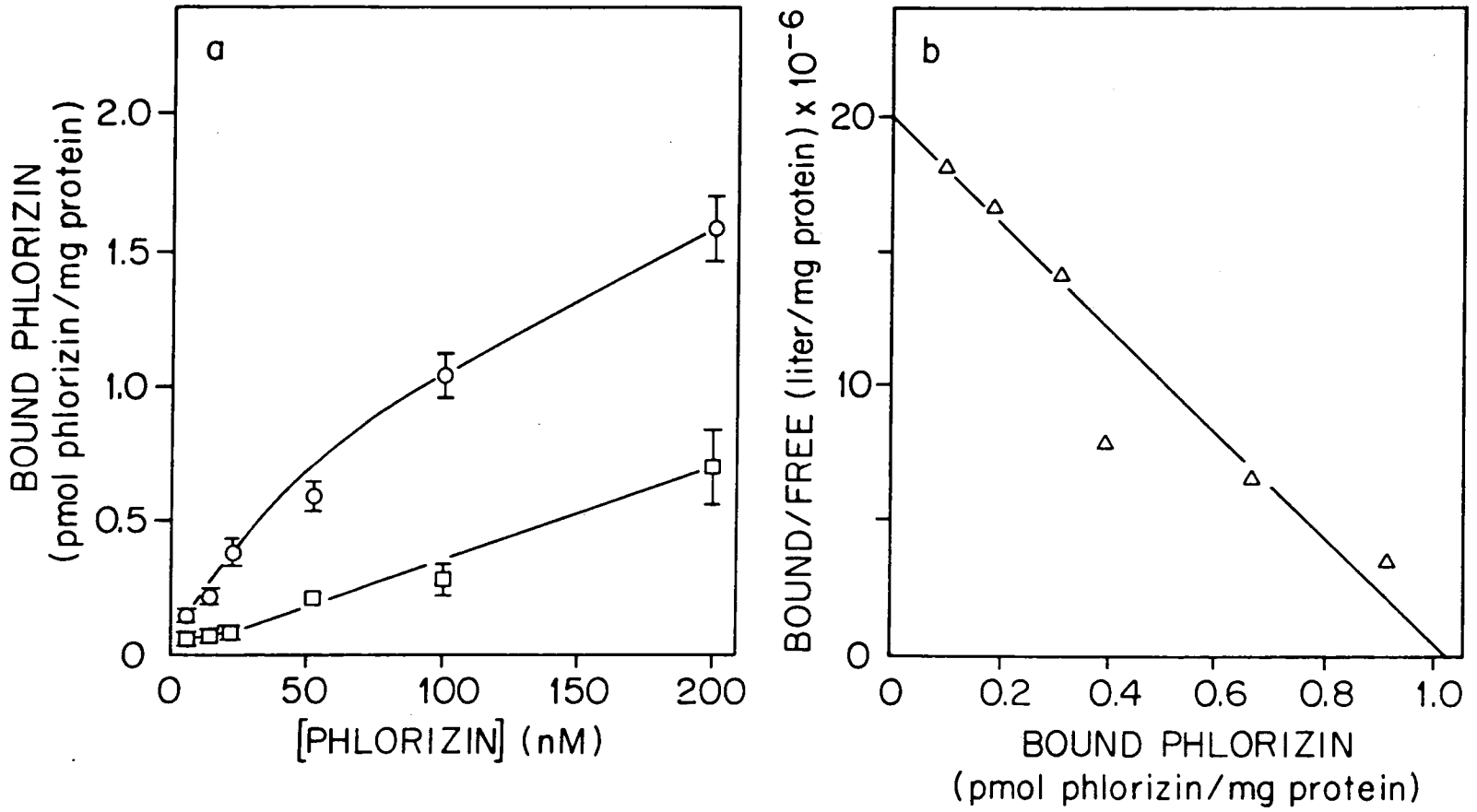


Fig. V-2. Concentration dependence of phlorizin binding to intact Cl 4 cells (a) and Scatchard analysis of the Na^+-dependent component of phlorizin binding (b). [^{3}H]Phlorizin was added in the presence (circles) or absence (squares) of Na^+ at a specific activity of 2.5 nCi/pmole. Na^+-dependent phlorizin binding (triangles) was calculated as the binding of phlorizin in the presence of Na^+ minus the binding in the absence of Na^+.

Correlation between Na^+-Dependent Phlorizin Binding and α-meG Uptake

Next, the characteristics of Na^+-dependent phlorizin binding were determined in cultures exhibiting various levels of α-meG uptake (Fig. V-3). The concentration dependence of phlorizin binding in the presence and absence of Na^+ was determined in four sets of cells exhibiting various levels of α-meG uptake. The uptake levels, as measured by the concentration ratio ($\alpha\text{-meG}_i/\alpha\text{-meG}_o$), ranged from 1, indicating no concentration of the hexose, to 33.48, indicating a high level of concentrative hexose uptake. In Fig. V-3a the concentration ratio for α-meG equaled 1 (upper left-hand panel of the graph) and the same amount of phlorizin was bound to Cl 4 cells in the presence and the absence of Na^+, i.e. there was no Na^+-dependent component of phlorizin binding measurable in these cultures. The level of α-meG accumulation in the next panel (Fig. V-3b) was 3.15 and the amount of phlorizin bound in the presence of Na^+ was slightly greater than the amount bound in the absence of Na^+. The third group of cultures had a 60 min accumulation ratio for α-meG of 19.2 and there was a corresponding increase in the amount of Na^+-dependent phlorizin binding measured (Fig. V-3c). Finally, the data presented in Fig. V-3d indicate that the cultures which exhibited the greatest concentration level, S_i/S_o = 33.5 for hexose also exhibited the greatest level of Na^+-dependent phlorizin binding.

The Na^+-dependent component of binding was calculated for each of the figures presented in Fig. V-3 and the data were plotted according to Scatchard (Fig. V-4). The binding parameters calculated for the various sets of data are tabulated in Table V-1. These data show that as the

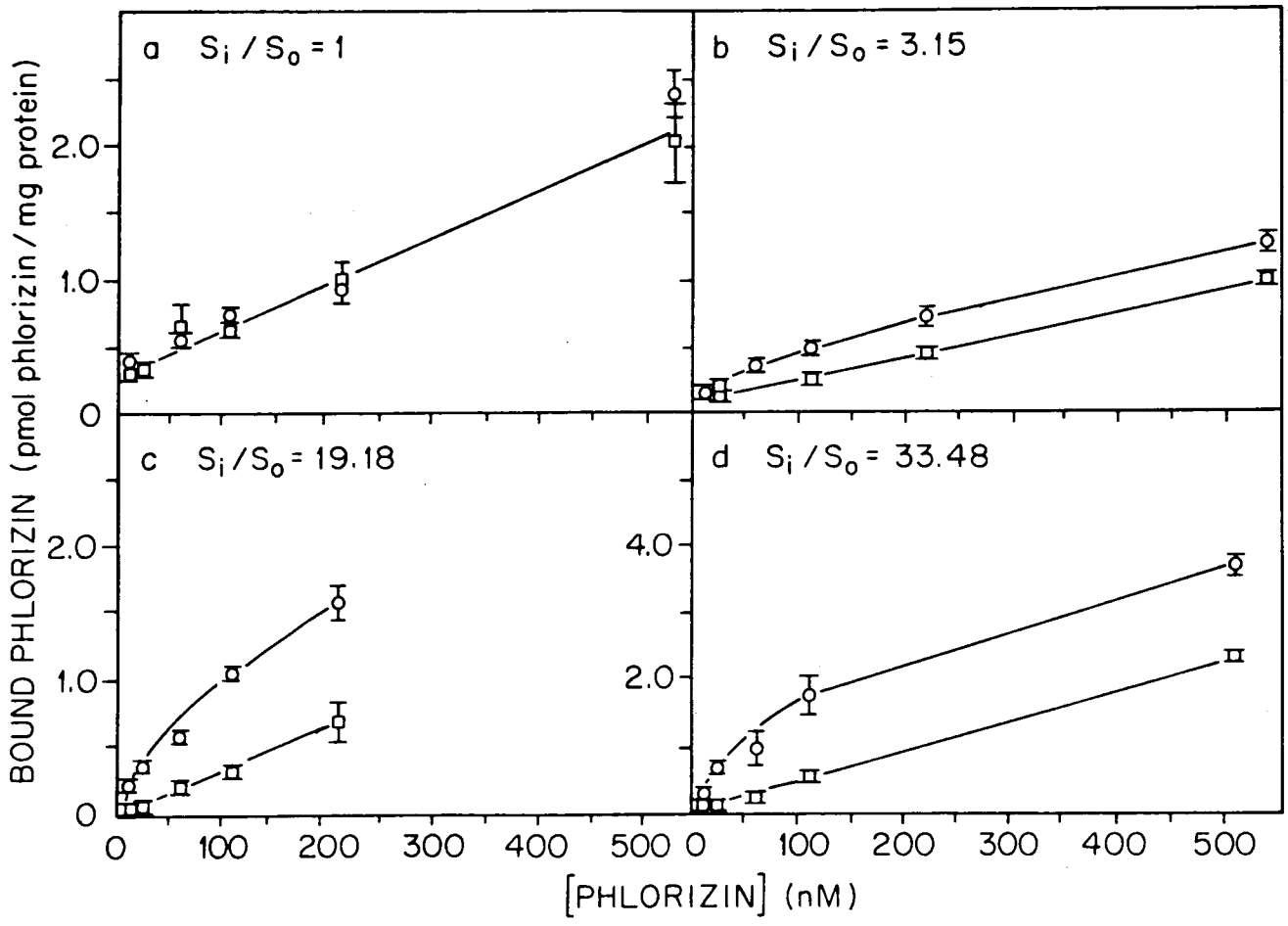


Fig. V-3. Concentration dependence of phlorizin binding to intact Cl 4 cells expressing various levels of α-meG accumulating capacity. The concentration ratio (S_i/S_o) is given in the upper left-hand corner of each panel. Binding of phlorizin was measured in the presence (circles) and absence (squares) of Na^+. The specific activity of [^{3}H]phlorizin was maintained at 2.5 nCi/pmole by serial dilution. Note scale change on the y-axis in panel (d).

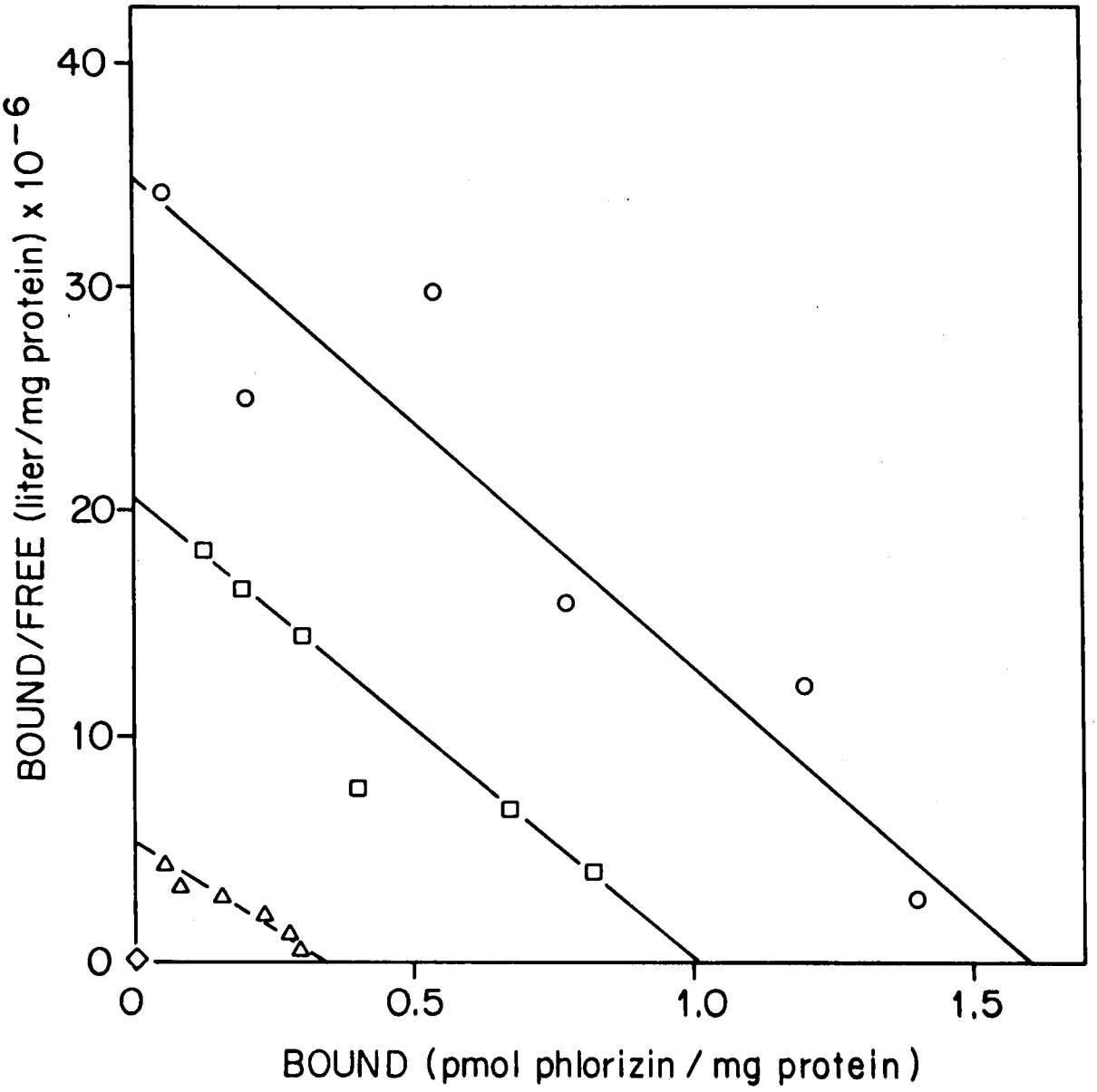


Fig. V-4. Scatchard analyses of the data presented in Fig. V-3. The data from Fig. V-3a are represented by the diamonds, Fig. V-3b by the triangles, Fig. V-3c by the squares, and Fig. V-3d by the circles. Note that all data points from Fig. V-3a are (0,0) because there is no Na^+-dependent component of phlorizin binding.

Table V-1. Binding parameters derived from Figure V-4.

Figure Number	K_d (nM)	N (pmol/mg protein)
V-3a	--	0
V-3b	62	0.34
V-3c	48	1.01
C-3d	46	1.60

Note: Values are obtained by inspection of lines. K_d is derived from the slope of the line and N is derived from the X-intercept.

level of Na^+-dependent hexose uptake increases there is a comparable increase in the total number of Na^+-dependent phlorizin binding sites. There is no consistent correlation between the level of α-meG uptake and the K_d for phlorizin binding.

The correlation between the level of hexose concentration and the number of Na^+-dependent phlorizin binding sites in Cl 4 cells is extended in Fig. V-5. In this figure the level of α-meG uptake is plotted against the total number of Na^+-dependent phlorizin binding sites for a large number of independent experiments. The level of Na^+-dependent phlorizin binding for each experiment was determined by the analysis used above. Data are presented for Cl 4 cells grown in complete medium containing 200 μM MIX (triangles) or complete medium containing 2 mM HMBA (squares). Over a wide range of levels of α-meG uptake there appears to be a linear correlation between the level of α-meG uptake and the total number of binding sites. However, at very low levels of concentrative hexose uptake the level of Na^+-dependent phlorizin binding was higher than would be predicted. At this time it is not known whether this represents a real deviation from the linear relationship or represents a high degree of experimental error that exists when trying to measure very low levels of phlorizin binding.

Discussion

Characterization of Na^+-Dependent Phlorizin Binding to Intact Cl 4 Cells

To test certain predictions made by the model for acquisition of concentrative hexose uptake presented in Chapter IV, and to directly

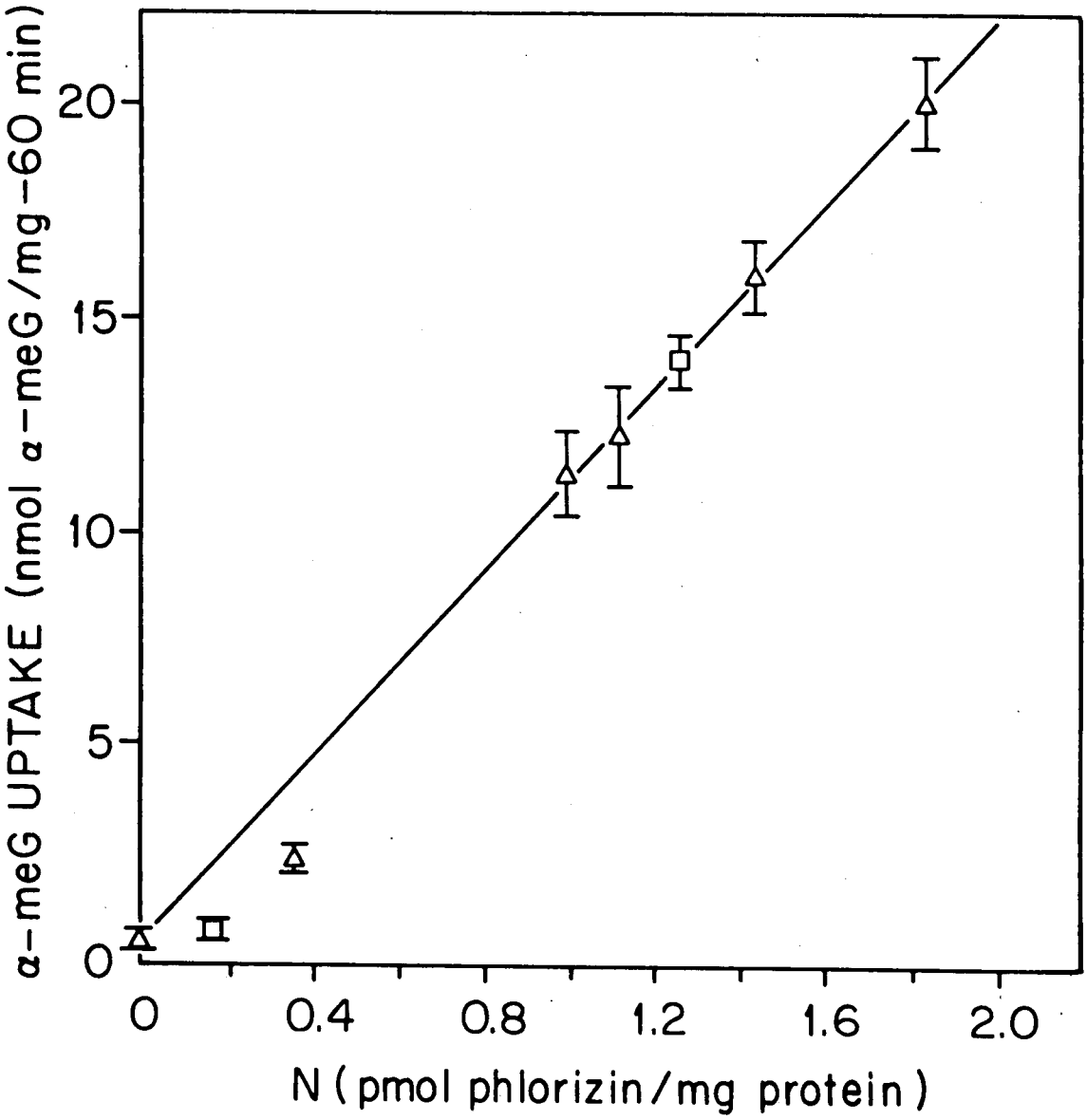


Fig. V-5. Level of α-meG uptake versus the total number of Na^+-dependent phlorizin binding sites, N. Each N is derived from analysis of the Na^+-dependent component of phlorizin binding as described previously. Data are presented for cultures grown in complete medium plus 1 mM MIX (triangles) and complete medium plus 2 mM HMBA (squares).

measure the level of Na^+-dependent hexose transporters in the plasma membrane of Cl 4 cells, [^{3}H]phlorizin binding to intact Cl 4 cells was analyzed. Binding of phlorizin in the presence or absence of Na^+ was extremely rapid, reaching a maximum within 1 to 2 minutes. To insure that a steady-state level of phlorizin binding had occurred, a necessary condition for analysis of binding parameters, a 3 minute incubation was used in all experiments.

The amount of phlorizin bound to Cl 4 cells was strongly dependent on the presence of Na^+ in the incubation medium. This Na^+-dependence of phlorizin binding has also been demonstrated for phlorizin binding to LLC-PK_1 cells (Rabito, 1981) and to isolated brushborder membrane fragments from renal proximal tubular cells (Toggenburger et al., 1978). Binding of phlorizin in the absence of Na^+ was a linear function of phlorizin concentration, indicating a nonsaturable process. This is in contrast to the findings of Rabito (1981) which indicated that even in the absence of Na^+ phlorizin binding to LLC-PK_1 cells exhibited a saturable binding component. He found that addition of Na^+ to the incubation medium resulted in a decrease in the K_d for the saturable component of binding but produced no changes in the total number of binding sites. One possible explanation for this difference in the nature of the Na^+-independent component of phlorizin binding in the two studies is that there was incomplete removal of Na^+ in the Rabito study. Aronson (1978) has reported that Na^+ modulates the affinity of phlorizin for the Na^+-dependent hexose transporter of rabbit renal brushborder membrane fragments without affecting the total number of binding sites

for phlorizin. However, in the complete absence of Na^+ no saturable component of binding exists. Therefore, the presence of a low concentration of Na^+ during binding of phlorizin to the LLC-PK_1 cells would yield results identical to those obtained by Rabito.

The K_d for Na^+-dependent phlorizin binding to Cl 4 cells has been determined to be approximately 80 nM. The values obtained from independent experiments vary between 40 nM and 120 nM. Aronson (1978) has shown that the K_d for Na^+-dependent phlorizin binding to brushborder membrane vesicles is dependent on the electrical potential across the membrane. Increasing the electronegativity of the intravesicular space by imposition of an inward-directed anion gradient or outward-directed K^+ or H^+ gradient, caused a stimulation of Na^+-dependent phlorizin binding. The stimulation of binding was shown to be the result of an increased association rate with no observed change in the dissociation rate. In the experiments on intact Cl 4 cells the membrane potential of the cells was not controlled, and it is therefore possible that the variations observed in K_d measurements between experiments were due to differences in the membrane potential of different cell populations. However, there was not a consistent variation in the K_d for Na^+-dependent phlorizin binding to cells as a function of the level of α-meG uptake. Since both membrane potential (personal communication, Carolyn Shaffer) and acquisition of α-meG accumulating capacity (Chapter II) have been correlated with entrance of the cells into a quiescent state it might be expected that an increasing level of α-meG uptake would correlate with an increasing K_d value due to a decreasing membrane

potential. However, in the Aronson study no quantitative measure of the change in membrane potential was made. Thus it is not clear that the range of the membrane potential variation in Cl 4 cells would be sufficient to produce changes in the K_d. Further, the absence of a consistent variation of K_d with the level of α-meG uptake argues against a significant variation in K_d due to differences in the membrane potential of the various cultures.

The value obtained for the K_d of Na^+-dependent phlorizin binding to Cl 4 cells is somewhat lower than the values obtained for binding to isolated renal brushborder membrane fragments from various sources (Silverman, 1976), 80 nM versus 200 nM to 800 nM, and is substantially lower than the value for Na^+-dependent phlorizin binding to LLC-PK_1 cells previously observed, 1.1 μM (Rabito, 1981). Substantial differences have been found in the characteristics of Na^+-dependent phlorizin binding to membrane fragments derived from various animal species. Thus quantitative, and possibly qualitative, comparison of binding characteristics between sources may not be valid. In addition, binding to Cl 4 cells is performed on intact living cells in contrast to measurement of binding to isolated membrane fragments. The experimental manipulations required to obtain purified brushborder membrane fragments may alter the binding characteristics, again making direct comparison of binding characteristics difficult.

The difference in the K_d for phlorizin binding to Cl 4 cells versus LLC-PK_1 cells is more difficult to account for. The experimental protocols employed in the two studies are very similar, making it

unlikely that the differences in binding characteristics are due to differences in experimental procedure. The Cl 4 cell line was derived from the LLC-PK_1 cell line, but it is conceivable that the Cl 4 cell line comprises only a small proportion of the LLC-PK_1 cell line and that the majority of cells in the parent population express a Na^+-dependent hexose transporter with significantly different phlorizin binding characteristics. It is also worth noting that the LLC-PK_1 cell lines used in the two laboratories were obtained from different sources. The cells used in this laboratory were obtained from Dr. R. N. Hull, who originally isolated the line. The cells used by Rabito were obtained from the American Type Culture Collection (ATCC), which had in turn obtained them from Dr. Hull. Though the two lines originated from the same source, a number of laboratories have reported anomalous behavior of the cells obtained from ATCC. It has been reported that some LLC-PK_1 cells obtained from ATCC do not express Na^+-dependent hexose uptake (private communication). To further point up the variability of the LLC-PK_1 cell lines used in various laboratories, Dr. M. Sanders has found that LLC-PK_1 cells used in his laboratory appear to have lost the ability to accumulate hexoses after a number of years of subculture (private communication). These observations, coupled with the fact that the three clones derived from the LLC-PK_1 cell line being studied in this laboratory display differing characteristics, suggests that the lines being used in various laboratories are not necessarily comparable. The internal consistency of the results obtained by Rabito and by me, i.e. the K_d for Na^+-dependent phlorizin binding is very similar to the

measured $K_{I,0.5}$ for phlorizin inhibition of α-meG uptake in each of the two studies, strongly supports the assertion that the differences in binding characteristics are due to inherent differences in the cell lines being used in the two studies.

Having established the characteristics of Na^+-dependent phlorizin binding to intact Cl 4 cells, the development of this binding activity during growth in culture was monitored and compared to the development of α-meG accumulating capacity. The K_d for Na^+-dependent phlorizin binding to Cl 4 cells did not vary significantly during development of hexose concentrating capacity. There was a close correlation between the level of Na^+-dependent hexose transport and the total number of Na^+-dependent phlorizin binding sites. This is the result predicted by the model for development of Na^+-dependent hexose transport presented in Chapter IV. A linear relationship between α-meG concentrating capacity and total number of Na^+-dependent phlorizin binding sites was found for Cl 4 cells grown in complete medium containing 200 μM MIX or in complete medium containing 2 mM HMBA. It is concluded that these two compounds, though probably initiating development of the transport function through different mechanisms (see Chapter IV), ultimately bring about the same cellular changes.

In addition these results demonstrate that the development of the hexose transport function in Cl 4 cells is brought about through accumulation of active Na^+-dependent hexose transporters in the membranes of the cells in the population rather than through modulation of other parameters such as driving force for transport. In the

previous chapter it was concluded that development of the hexose transport function was not due to the development of an increasing number of transporters per cell. Since data presented in this chapter indicate that the number of Na^+-dependent hexose transporters in the cell population is increasing with time, it is concluded that this is due to a progressive increase in the number of cells in the population expressing Na^+-dependent hexose transport. Thus these results strongly support the proposal that development of hexose accumulating capacity in a cell population is due to the continuing recruitment of cells into the fraction of the population which is capable of concentrative hexose uptake.

CHAPTER VI

IRREVERSIBLE INACTIVATION OF Na^+-DEPENDENT HEXOSE TRANSPORT BY A PHOTOAFFINITY LABEL FOR THE Na^+-DEPENDENT D-GLUCOSE TRANSPORTER

Introduction

To this point the analysis of development of the concentrative hexose transport function has relied on measurement of functional activity, i.e. measurement of α-meG uptake or Na^+-dependent phlorizin binding. These analyses have yielded a great deal of information concerning the mechanism(s) by which Cl 4 cells and LLC-PK_1 cells acquire Na^+-dependent hexose uptake. Further studies of this developmental process would be greatly aided by the ability to identify and examine at a molecular level the Na^+-dependent hexose transporter during acquisition of transport activity.

A number of laboratories have attempted to identify and purify the Na^+-dependent hexose transporter from renal or intestinal brushborder membranes derived from a variety of sources. The techniques most commonly used are: (1) negative purification of the transporter by selective removal of other proteins from the brushborder membrane (Klip et al., 1979); (2) semiselective labelling of the transporter with $HgCl_2$ (Klip et al., 1980); and (3) solubilization and reconstitution of the transporter into liposomes (Fairclough et al., 1979; Koepsell et al., 1980; Lin et al., 1981). To date these procedures have been only partially successful in identifying the transporter. This is probably

due to two complications. First, the surface density of the transporter in the brushborder membrane is extremely low (Silverman and Black, 1975; Toggenburger et al., 1978). Second, the transporter has been reported to have a relatively poor stability after detergent solubilization (S. Takesue and Y. Takesue, unpublished data, 1979). The problem of a low surface density of the transporter in the starting material is accentuated in Cl 4 cells for two reasons. First, in vivo the transporter is probably maximally expressed, whereas in Cl 4 cells maximal expression occurs only after a long period in culture and the time of interest for analysis is when the transport function is developing, i.e. when the transporter is less than maximally expressed. Second, in contrast to in vivo material, purification of the brushborder membrane from Cl 4 cells has not been well characterized. Therefore, the complications that hamper identification of the transporter in brushborder membrane fragments are even more of a hindrance to identification of the transporter in Cl 4 cells.

To initiate a project with a reasonable probability of identifying the transporter in Cl 4 cells, a search was made for a very specific label for the transporter. Hosang et al. (1981) have described the use of 4-azido-phlorizin as a specific photo-affinity label for the Na^+-dependent hexose transporter of the rabbit intestinal brushborder membrane. This compound interacts with the Na^+-dependent hexose transporter with characteristics that are very similar to those of phlorizin, i.e. Na^+-dependence of binding, inhibition of binding by appropriate sugars and phlorizin but not by para-phlorizin. A quantity

of this compound was obtained from E. M. Gibbs and D. Diedrich (University of Kentucky) and the usefulness of this compound as a specific label of the transporter in the Cl 4 cell line was analyzed.

Materials and Methods

Cell Culture and Measurement of Uptake

These procedures were performed essentially as described in Chapter II except that in the measurement of α-meG uptake BES-buffered HBSS, pH 6.5, (B-HBSS) was substituted for HEPES-buffered HBSS, pH 7.4. The binding of phlorizin and 4-azido-phlorizin to the Na^+-dependent hexose transporter are pH sensitive, binding being greater at lower pH, and thus the lower pH buffer was used both for the binding reaction and measurement of uptake. Also, α-meG uptakes were measured after 15 min instead of 60 min to obtain initial rate measurements.

Measurement of Photolysis of 4-Azido-Phlorizin

One mM 4-azido-phlorizin was prepared as a stock solution in 95% EtOH. This was diluted with B-HBSS to the appropriate concentration. For measurement of photolysis of the compound by blacklight, 1 ml 50 μM 4-azido-phlorizin in B-HBSS was placed in a plastic petri dish. The dish was then placed under two desklamps containing two blacklight bulbs (General Electric, 15 W) each. The distance from the surface of the fluid to the blacklight bulbs was approximately 8 cm. The lights were then turned on and the dishes, with tops on, exposed to the light for various lengths of time. The absorbance of the 4-azido-phlorizin

solution at 251 nm was measured before and after light exposure using a Cary 14 recording spectrophotometer (Monrovia, CA).

Treatment of Cl 4 Cells with 4-Azido-Phlorizin

Cl 4 cells grown on collagen-coated Nucleopore filters were rinsed three times with 1 ml B-HBSS or Na^+-free B-HBSS, choline being substituted in equimolar concentration for Na^+. Then 1 ml B-HBSS or Na^+-free B-HBSS containing 10 μM 4-azido-phlorizin was added to each dish and the dishes were incubated at 37°C for 5 minutes to permit binding of the compound to the transporter. Dishes were removed and placed under the blacklight for various lengths of time. After exposure the dishes were rinsed three times with ice-cold B-HBSS. B-HBSS was added and the dishes incubated at 37°C for 5 min. This fluid was aspirated, 0.9 ml B-HBSS was added and the dishes were assayed for α-meG uptake as described previously. Control dishes were carried through the same procedures using B-HBSS without 4-azido-phlorizin. When the dishes were to be exposed to a number of treatments with 4-azido-phlorizin the above procedure was repeated, the dishes being rinsed with B-HBSS or Na^+-free B-HBSS and fresh 4-azido-phlorizin in the appropriate buffer being added after each treatment. In the final procedure dishes were exposed to 4-azido-phlorizin five times, each exposure lasting 10 minutes under the blacklight. Where indicated, other compounds were present during all exposures to light.

Materials

The photoaffinity lable, 4-azido-phlorizin, was a kind gift of D. Diedrich and E. M. Gibbs, University of Kentucky.

Results

Reversible Inhibition of α-meG Uptake by 4-Azido-Phlorizin

Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown in complete medium containing 200 μM MIX for 6 days. At this time the cells exhibited a high level of α-meG uptake. In the dark, cultures were incubated with [^{14}C]-α-meG and various concentrations, 0.1-10 μM, of 4-azido-phlorizin in the presence or absence of Na^+. The level of α-meG uptake was measured after 15 minutes (Fig. VI-1). In the absence of Na^+, α-meG uptake was unaffected by 4-azido-phlorizin (data not shown). However in the presence of Na^+ there was a strong inhibition of α-meG uptake by 4-azido-phlorizin. At 10 μM 4-azido-phlorizin there was a 90% inhibition of Na^+-dependent α-meG uptake. The $K_{I,0.5}$ for inhibition of α-meG uptake by 4-azido-phlorizin was approximately 1 μM. Under this treatment protocol the inhibition of α-meG uptake was fully reversible. Transport activity could be fully restored by washing the cultures with buffer after exposure to 4-azido-phlorizin and prior to measurement of α-meG uptake.

Irreversible Inhibition of α-meG Uptake by 4-Azido-Phlorizin

Since 4-azido-phlorizin can reversibly inhibit Na^+-dependent α-meG uptake, the ability of the compound to irreversibly inhibit uptake following exposure to light was examined. Before this study was possible it was necessary to determine the optimum conditions for photolysis of 4-azido-phlorizin. This was investigated by monitoring the absorbance

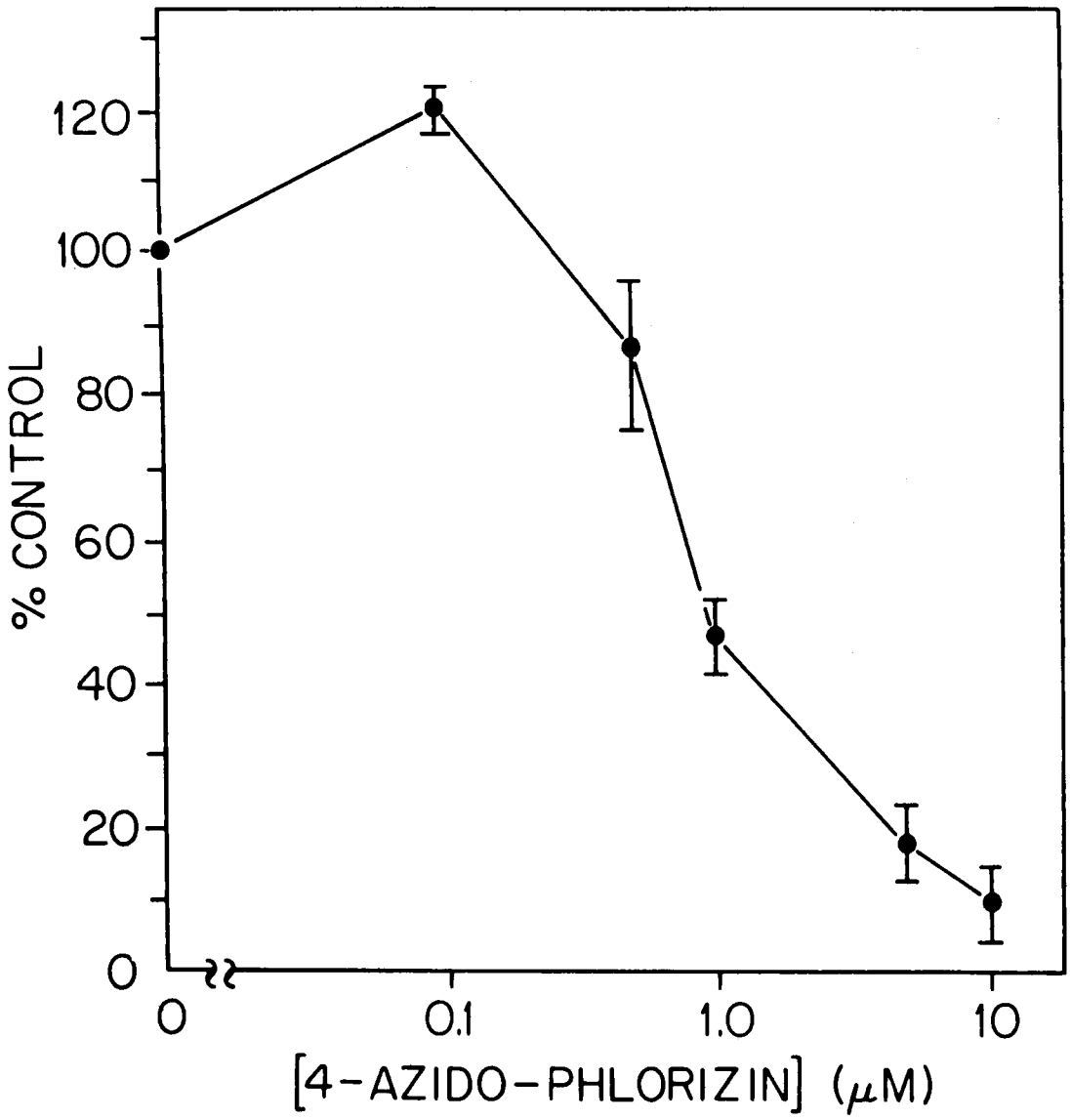


Fig. VI-1. Reversible inhibition of α-meG uptake in Cl 4 cells by 4-azido-phlorizin. Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown in complete medium plus 200 μM MIX for 6 days. Uptake of α-meG after 15 minutes was measured in the dark in B-HBSS or Na^+-free B-HBSS containing various concentrations of 4-azido-phlorizin. Uptake in control cultures was 17.23 ± 0.90 nmoles/mg protein-15 minutes.

of 4-azido-phlorizin at 251 nm before and after exposure to various light sources. The phlorizin molecule has a characteristic absorbance maximum at 251 nm. Loss of absorbance at this wavelength is indicative of a breakdown of the compound. In parallel experiments the effect of these light sources on α-meG uptake was also monitored. It was found that common blacklights were capable of causing extensive photolysis of 4-azido-phlorizin but were only minimally inhibitory to α-meG uptake (unpublished observation).

The kinetics of photolysis of 4-azido-phlorizin with a blacklight source were more carefully analyzed. Photolysis was assessed by monitoring the loss of absorbance at 251 nm after exposure of the compound to light for various lengths of time. Two ml B-HBSS containing 50 μM 4-azido-phlorizin in a plastic petri dish was exposed to blacklight, approximately 8 cm between the bulbs and the surface of the fluid. The level of absorbance was monitored and the change in specific A_{251} due to absorbance by 4-azido-phlorizin was computed as a percentage of the original absorbance (Fig. VI-2). During exposure to blacklight there was a progressive increase in the nonspecific absorbance at 251 nm. To determine the change in the specific absorption by the compound it was necessary to subtract this nonspecific component from the total absorbance at 251 nm for each length of exposure to blacklight. The data are presented on a semi-logarithmic plot. The level of absorbance slowly declined to 87% of control during the first 5 minutes of exposure to blacklight. Further exposure to blacklight produced a logarithmic decline in the level of specific absorbance at 251 nm. After 27 minutes

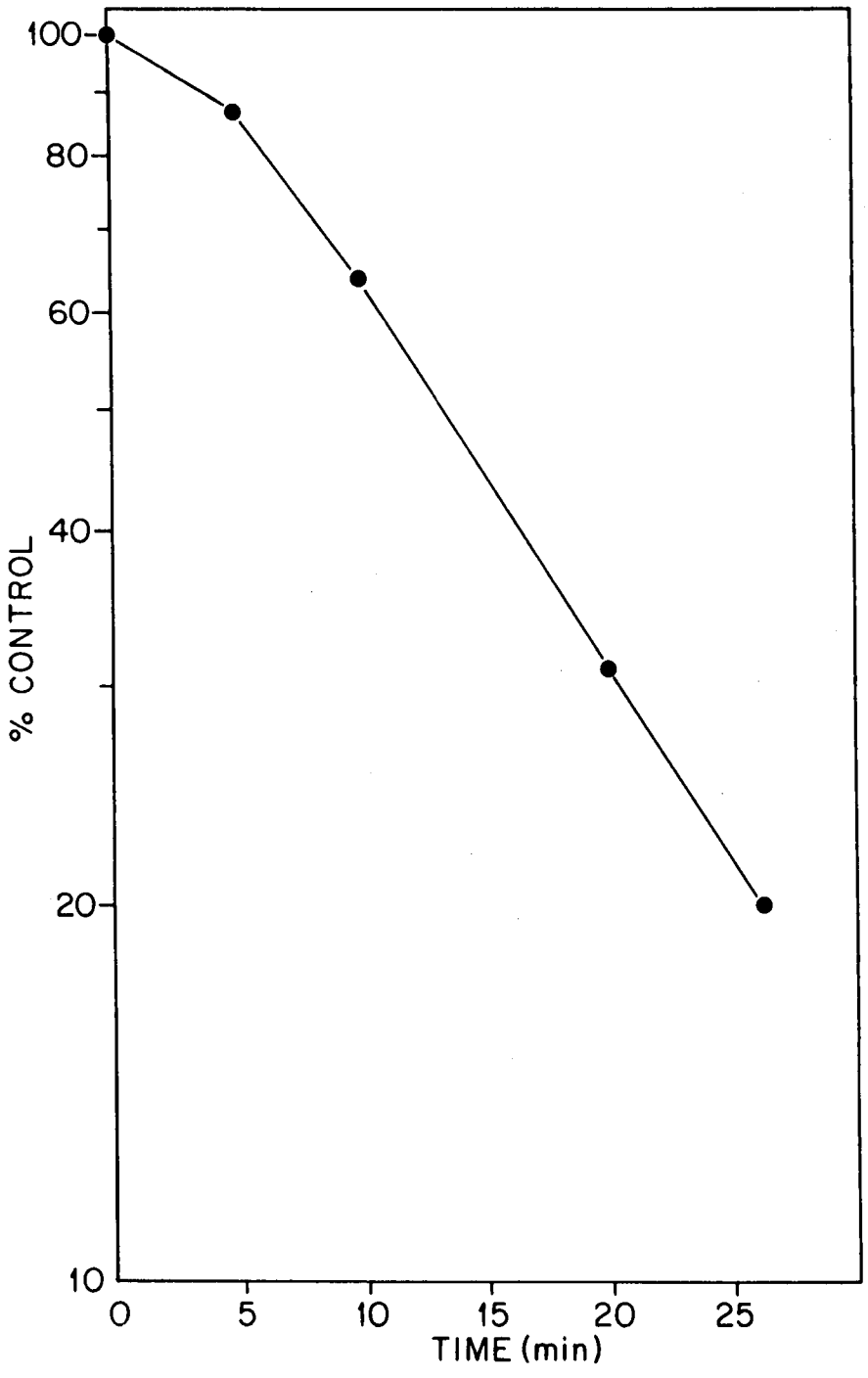


Fig. VI-2. Photolysis of 4-azido-phlorizin by blacklight. Two ml 50 μM 4-azido-phlorizin was placed in a plastic petri dish and exposed to blacklight for various lengths of time. The $A_{251\ nm}$ was monitored before and after exposure and the loss of absorbance by the compound with increasing exposure to light was calculated.

exposure the level of A_{251} had decreased to less than 20% of the original value. The half-time for photolysis of the compound was between 13 and 14 minutes of exposure.

The effect of exposure of cells to blacklight with or without 10 μM 4-azido-phlorizin in the incubation buffer was then assessed by monitoring the rate of α-meG uptake (Fig. VI-3). Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown in the presence of 200 μM MIX for 8 days. The cells were then rinsed with B-HBSS and B-HBSS with or without 10 μM 4-azido-phlorizin was added. The cultures were exposed to blacklight for various lengths of time. In the absence of 4-azido-phlorizin there was no significant change in the level of α-meG uptake even after 40 minutes exposure to blacklight. In the presence of 4-azido-phlorizin there was a progressive decrease in the level of α-meG uptake which reached a minimum of between 76% and 83% of control after 10 minutes exposure. The decreased level of uptake remained constant even when the exposure time was extended up to 40 minutes. In the following experiments each exposure to light and 4-azido-phlorizin was carried out for 10 minutes.

In order to maximize the level of inactivation obtained with 4-azido-phlorizin the cells were subjected to repeated cycles of exposure to 4-azido-phlorizin and light. After each exposure the cultures were rinsed with B-HBSS and fresh B-HBSS with or without 4-azido-phlorizin was added (Fig. VI-4). In the absence of 4-azido-phlorizin repeated exposure of Cl 4 cells to blacklight caused a slight decrease in the level of uptake. In the presence of 4-azido-phlorizin

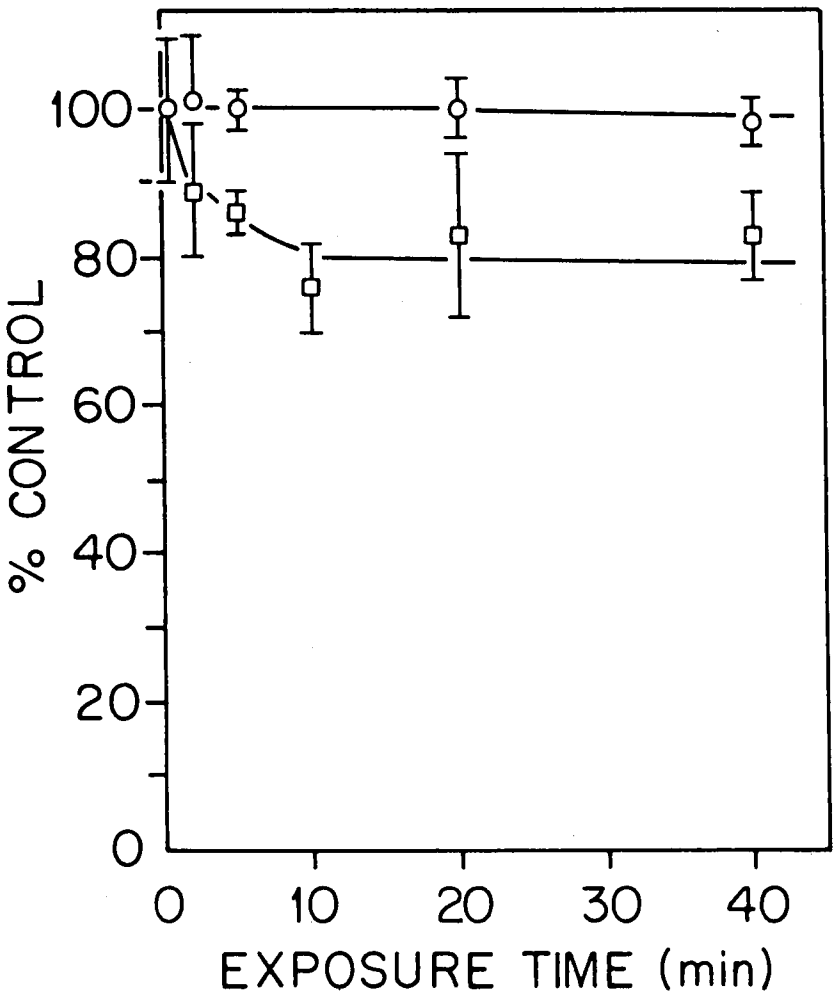


Fig. VI-3. Irreversible inactivation of α-meG uptake by Cl 4 cells exposed to 10 μM 4-azido-phlorizin and blacklight. Uptake in control cultures was 11.47 ± 1.13 nmoles/mg protein-15 minutes.

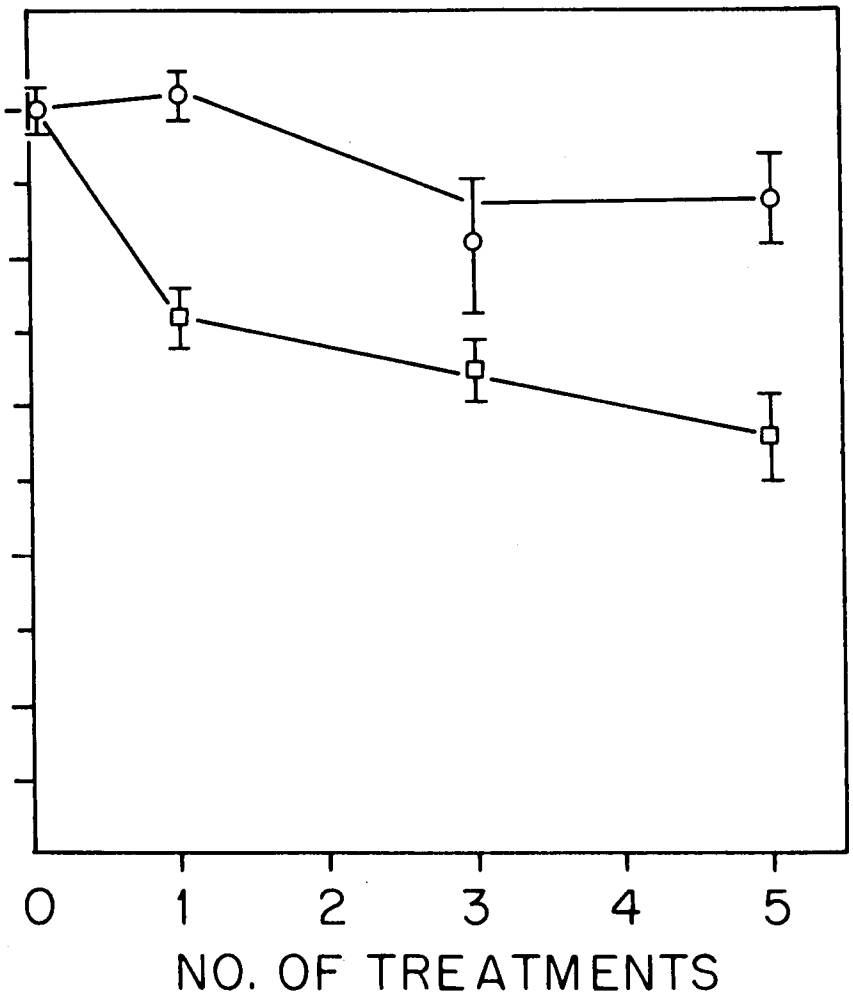


Fig. VI-4. Irreversible inactivation of α-meG uptake in Cl 4 cells by repeated exposure of cultures to 4-azido-phlorizin and blacklight. Cl 4 cells were seeded on collagen-coated Nucleopore filters and grown in complete medium containing 200 μM MIX for 6 days. Cultures were then treated with B-HBSS without (circles) or with (squares) 10 μM 4-azido-phlorizin as described.

there was a substantial decrease in the level of α-meG uptake although the amount of decline per treatment became less. After five cycles of exposure of the cells to 4-azido-phlorizin and light there was a 44% inhibition of α-meG uptake, while the same number of exposures in the absence of 4-azido-phlorizin produced only a 12% decrease in uptake. In the following experiments exposure to 4-azido-phlorizin and light was performed five times to insure maximum inhibition of transport.

Specificity of Irreversible Inactivation of α-meG Uptake

The above protocol was used to study the specificity of the inactivation of α-meG uptake by 4-azido-phlorizin. Specificity of the inactivation process was determined by examining the Na^+-dependence, D-glucose sensitivity, and phlorizin sensitivity of the inactivation process. In addition, the effect of the treatment on meAIB uptake was also measured (Table VI-1). When Na^+ was omitted from the incubation medium during exposure of the cells to light and 4-azido-phlorizin and the level of α-meG uptake was then assayed in a Na^+-containing buffer, there was no inhibition of uptake when compared to control cultures exposed to light without 4-azido-phlorizin being present. The inactivation of the transport function could also be blocked by exposing the cultures to light and 4-azido-phlorizin in the presence of either 20 μM phlorizin or 100 mM D-glucose. Thus the inactivation of α-meG uptake by 4-azido-phlorizin is dependent on the presence of Na^+ and can be blocked by compounds known to interact with the Na^+-dependent hexose

Table VI-1. Specificity of irreversible inactivation of α-meG uptake in Cl 4 cells by 10 μM 4-azido-phlorizin.

Treatment	% Control Uptake (α-meG or meAIB)
+ 10 μM 4-Azido-Phlorizin	57 ± 4
+ 10 μM 4-Azido-Phlorizin + 20 μM Phlorizin	95 ± 5
+ 10 μM 4-Azido-Phlorizin in Na^+-Free B-HBSS	97 ± 4
+ 10 μM 4-Azido-Phlorizin + 100 mM D-Glucose	78 ± 8
+ 10 μM 4-Azido-Phlorizin (meAIB Uptake)	92 ± 3

Note: Additions or deletions as indicated are performed for all exposures to 4-azido-phlorizin and blacklight. After exposures cultures are rinsed with B-HBSS and measurement of uptake in 15 min (α-meG or meAIB) was performed as described.

transporter. In addition, exposure of cells to light in the presence of 4-azido-phlorizin had little effect on meAIB uptake.

Discussion

The characteristics of 4-azido-phlorizin interaction with Cl 4 cells have been analyzed in order to determine the usefulness of this compound as a photoaffinity label for the Na^+-dependent hexose transporter expressed by these cells. In the dark 4-azido-phlorizin inhibits α-meG uptake by Cl 4 cells in a specific, dose-dependent, and reversible manner (Fig. VI-1, p. 104). The $K_{I,0.5}$ for this inhibition is approximately 1 μM. The $K_{I,0.5}$ measured for 4-azido-phlorizin is five to ten times higher than that measured for phlorizin, $K_{I,0.5} \cong 0.2$ μM. This is consistent with the finding that 4-azido-phlorizin also has a lower affinity than phlorizin for the Na^+-dependent D-glucose transporter of the rabbit intestinal brushborder membrane and rat intestinal brushborder membrane (E. M. Gibbs and D. Diedrich, personal communication). This has been attributed to disruption of hydrophobic interactions between some site on the membrane and the phenolic ring structures of the phlorizin molecule. The high affinity of phlorizin for the Na^+-dependent D-glucose transporter is in part due to these hydrophobic interactions (Bode et al., 1972). Therefore, disruption of these interactions by introduction of an azido group into one of the phenolic rings might be expected to lower the affinity of the compound for the transporter.

The inhibition of α-meG uptake by 4-azido-phlorizin was found to be a Na^+-dependent process. In the absence of Na^+ exposure of Cl 4 cells to 4-azido-phlorizin and blacklight produced no inhibition of uptake. In the presence of Na^+ the inhibition of α-meG uptake was also found to be blocked by inclusion of D-glucose in the incubation buffer during exposure of the cells to 4-azido-phlorizin and light. Further, addition of phlorizin to the incubation medium along with 4-azido-phlorizin also blocked the inhibitory action of 4-azido-phlorizin. The specificity of the inactivation process was demonstrated by the fact that meAIB uptake was unaffected by treatment with 4-azido-phlorizin. This indicates that the inhibition of α-meG uptake is not due to some general interaction of the compound with the cell membrane, e.g. to cause an alteration in the driving force for uptake. Therefore it is concluded that 4-azido--phlorizin can serve as a specific and irreversible label for the Na^+-dependent hexose transporter of Cl 4 cells.

Calculation of the extent of labeling that could be obtained by treatment of whole Cl 4 cells with 4-azido-phlorizin under optimal reaction conditions using a [^{3}H]4-azido-phlorizin, also provided to us by E. M. Gibbs and D. Diedrich, indicates that only 1 cpm specifically incorporated radioactivity could be expected from analysis of slices from a polyacrilamide gel electrophoresis of whole cell protein. This is due to the previously stated fact that the Na^+-dependent hexose transporter will comprise only a very small portion of the total cell protein, even at maximal expression of the transport function. The limitations in the amount of protein that can be analyzed by PAGE

analysis exacerbate this problem. Therefore, identification of the Na^+-dependent hexose transporter of Cl 4 cells using this label will still require substantial purification of the component. Development of a relatively purified apical membrane preparation for Cl 4 cells will partially solve this problem. In addition, the procedure of negative purification of the transporter that has been used previously on isolated intestinal brushborder membrane fragments (Klip et al., 1979) may be applicable to this system.

CHAPTER VII

SUMMARY

LLC-PK_1 cells, and the clones derived from this line, express a Na^+-dependent hexose transport function which bears many similarities to the transport function observed in isolated brushborder membrane fragments derived from the renal proximal tubule. The appearance of this transport function can be modulated by the addition of HMBA, phosphodiesterase inhibitors, and TPA to the culture medium. The appearance of this transport function is correlated with a cessation of cell growth and an increase in intracellular cAMP levels.

Na^+-dependent amino acid transport is regulated by the cells in a reciprocal manner from the regulation of hexose transport. Addition of TPA to cells caused an acceleration in the rate of amino acid transport in cells which have acquired a low rate of transport due to quiescence. The effect of TPA on amino acid transport rate is sensitive to cycloheximide and actinomycin D, suggesting that protein synthesis and RNA synthesis are required for this action. The TPA stimulation of amino acid transport is not due to an increase in the driving force for transport through an increase in the electrogenicity of Na^+-K^+-ATPase.

Using a mathematical model for the appearance of the hexose transport function in a cell population it was determined that the development is probably due to the independent development of full transport capacity in individual cells. The increasing level of transport is due to an increasing proportion of the population

possessing the Na^+-dependent hexose transport function. An involvement of the Na^+-electrochemical gradient in the regulation of the transport function is argued against by the coordinate decline in Na^+-dependent amino acid transport since both transport functions are driven by the same Na^+-electrochemical gradient.

Direct demonstration of an increasing number of Na^+-dependent hexose transporters in the cell population during development of hexose accumulating capacity was obtained by measuring Na^+-dependent phlorizin binding during development of the hexose transport function. There is a linear correlation between the level of hexose accumulating capacity exhibited by a cell population and the total number of Na^+-dependent phlorizin binding sites. The K_d for Na^+-dependent phlorizin binding does not vary in a consistent manner indicating that the changing level of hexose accumulating capacity is not due to changing transporter characteristics.

In order to analyze the development of Na^+-dependent hexose transport at a molecular level the use of a photoaffinity label for the transporter, 4-azido-phlorizin, was assessed. In the dark 4-azido-phlorizin reversibly inhibits Na^+-dependent hexose uptake ($K_{I,0.5} \cong$ 1 μM). Exposure of cells to blacklight in the presence of this compound causes an irreversible inactivation of hexose accumulating capacity. This irreversible inactivation is dependent on the presence of Na^+ in the incubation buffer and can be inhibited by the simultaneous inclusion of phlorizin or D-glucose during exposure. Na^+-dependent amino acid transport is not affected by this treatment indicating that the

inactivation is a specific process. Thus this compound can serve as a photoaffinity label for the Na^+-dependent hexose transporter of Cl 4 cells.

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APPENDIX

The electrical model for development of Na^+-dependent hexose transport in a cell population is presented in Fig. A-1. The model describes the kinetics of hexose uptake as the charging of a capacitor at a given voltage through a resistor:

$$Q_t = Q_{max}(1-e^{-t/RC}) \qquad (1)$$

$$Q_{max} = CV$$

where Q_t is the charge on the capacitor at time $\underline{t}$, R the resistance of the circuit through which the charge is being delivered, C is the charge capacity, and V the motive force.

In the cells, the motive force is the Na^+-electrochemical gradient and the capacity is the maximum uptake of substrate into the accumulating cells at a given Na^+ gradient. The resistance is more conveniently considered as its reciprocal, the conductance g ($g = 1/C$). Uptake in the population is then:

$$([S]_i/[S]_o)_t = f([S]_i/[S]_o)^*_{max}\ (1 - e^{-gt/C}) \qquad (2)$$

$([S]_i/[S]_o)_t$ is the measured parameter, the concentration of the substrate α-methylglucopyranoside in the cells relative to the concentration in the medium at any time t. $[S]_i$ in this term is the concentration in the whole culture population. The term f is the fraction of the total population volume in which concentrative accumulation is taking place. If the cells are uncoupled, f is the fractional number of cells that are differentiated. If the cells are

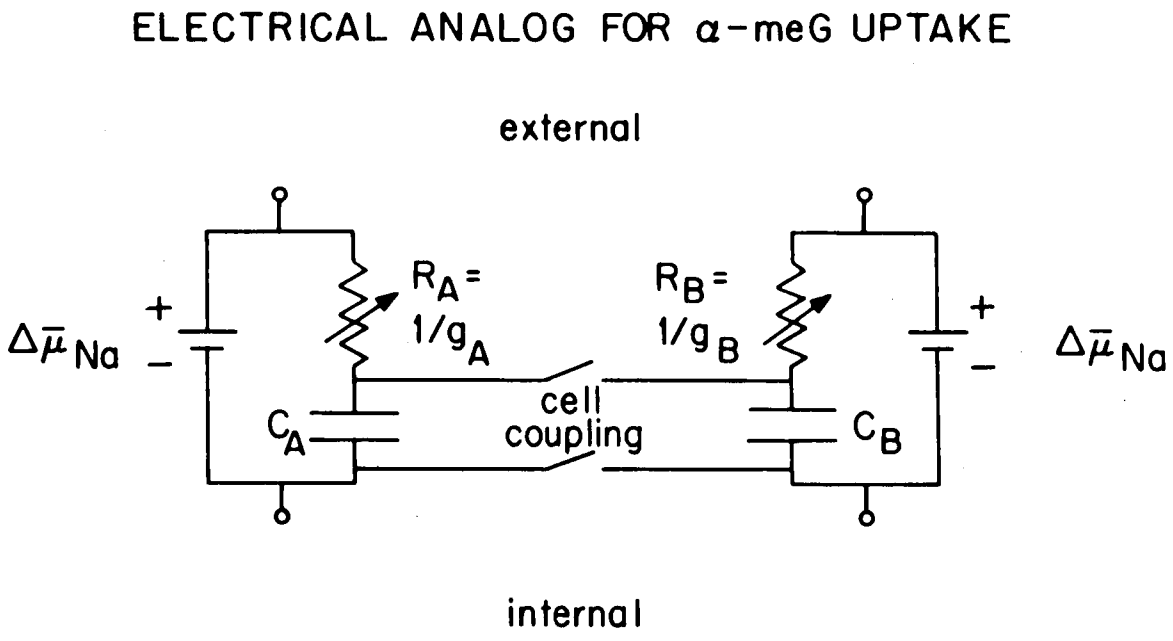

Fig. A-1. Electrical analog for concentrative hexose uptake in Cl 4 cells. The driving force for uptake is the Na^+-electrochemical gradient. The resistance is the inverse of the conductance, the latter being a permeability coefficient expressing the proportionality between the rate of uptake and the driving force; in the model the conductance is considered to be a function of the number of transporters per unit of accumulating space. The capacitance expresses the proportionality between the steady-state accumulation level and the driving force. If the switches in the coupling circuit are closed (i.e., if gap junctions are opened between cells), capacitor B can be charged through resistor A even if resistor B remains infinite (i.e., even if cell B has no functional transporters).

coupled, f is the sum of the number of cells that are carrying out transport plus the number of nontransporting cells to which they are functionally coupled.

$([S]_i/[S]_o)^*_{max}$ is the maximum accumulation capacity in those cells in which concentrative uptake is taking place. The * distinguishes this term from the similar term on the left hand side equation, which is a measurement made on the entire population. Thermodynamically:

$$([S]_i/[S]_o)^*_{max} \leq \left[\frac{[Na^+]_o}{[Na^+]_i} \exp\left(\frac{-ZF\Delta\psi}{RT}\right) \right]^n \qquad (3)$$

where $\Delta\psi$ is the membrane potential (inside negative) and n the stoichiometry of Na^+ to α-methylglucopyranoside on the transporter.

The conductance g is defined:

$$g = \frac{d}{dt} ([S]_i/[S]_o)^*/(\Delta\bar{\mu}_{Na^+})^n \qquad (4)$$

This again refers to the rate of uptake into the accumulating space (*), as a function of the Na^+-electrochemical gradient. In all experiments used in the analysis, $[S]_o$ is constant at 100 μM, which is well below the transport K_M; saturation of the transporter is thus neglected. If the $\Delta\bar{\mu}_{Na^+}$ remains constant, g is primarily a function of the number of transporters per unit accumulating volume. During an uptake assay, it is assumed that g remains constant, which is tantamount to assuming that at low $[S]_o$ the quantity of cotransported Na^+ is insufficient to perturb $\Delta\bar{\mu}_{Na^+}$ (Villereal and Cook, 1978).

The capacity C is $([S]_i/[S]_o)^*_{max}/(\Delta\bar{\mu}_{Na^+})^n$, and is the maximum accumulation in the accumulating cells at a given Na^+-electrochemical gradient. In the time constant for uptake, g/C, the Na^+ gradient terms cancel. Thus the time constant is a function of the number of transporters and of the accumulation capacity, but not of the driving force.

In the application of equation (2) to the uptake data, the further assumption is made that Na^+-independent fluxes are negligible. The potentially most important such flux is the escape of α-methylglucopyranoside via the facilitated-diffusion transporter. As noted above, it is found that concentration of the hexose within LLC-PK_1 cells is not affected by the addition of cytochalasin B, and hence the cytochalasin B-sensitive pathway is neglected.

A number of distinct alternative explanations for development of concentrative hexose uptake in a cell population were examined using the model. These alternatives are:

1. If an increasing fraction of cells are being recruited into the differentiated population, then the fit of the data on any one day should be able to fit the data of any other day by changing f according to the given ratio of uptake rates.

2. A change in the electrochemical gradient changes $([S]_i/[S]_o)^*_{max}$ (eq. 3); i.e. the capacity.

3. A change in the number of transporters per cell, assuming the cells to be uncoupled, can be modeled as a change in the conductance, g.

4. Coupling of accumulating to nonaccumulating cells increases the fractional volume of the culture in which accumulation is occurring (↑f) but decreases the number of transporters per unit volume (↓g).

This analysis of course considers only whether modulation of a single parameter can describe the results. A fortuitous modulation of several parameters at once will also give equally good fits. The simplest interpretation remains that the increasing uptake capacities observed on successive days represents the recruitment of new fully differentiated, uncoupled cells into the hexose-accumulating population.

VITA

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