CONF-8410185--1

DE85 002563

Development of Na^+-dependent hexose transport in cultured renal epithelial cells ($LLC\text{-}PK_1$)[1]

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[1] Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy under contract DE-AC05-84OR21400 with the Martin Marietta Energy Systems, Inc. E.R.W. is supported by Laboratory Graduate Participation Program with ORAU for USDOE. W.D.D. is supported by NIH Grant CA09336. K.A. present address: Max-Planck-Institut fur Systemphysiologie, Rheinlanddamm 201, D-4600 Dortmund 1, West Germany.

INTRODUCTION

The development in recent years of cell culture systems in which differentiated physiological functions arise *in vitro* has opened the possibility of investigating these processes in cloned cells. One such system, the LLC-PK_1 line derived from juvenile pig kidney by Hull et al. (11), when plated at low cell density at first grows vigorously and apparently transports hexoses only by the nonconcentrating, cytochalasin B-sensitive facilitated diffusion transporter. The cells grow in islands, and even at low-density are morphologically polarized with an apical surface (away from the substratum) studded with small microvilli. Contiguous cells are joined by tight junctions near the apical border. During this growth phase the cells have a substantial capacity for carrying out Na^+-dependent A-system amino acid transport as assayed by the uptake of α-aminoisobutyric acid (AIB) or methylaminoisobutyric acid (meAIB; Ref. 4, and see below). This is an activity of the basolateral membranes. At confluence the growth of the cells is stepped down but not stopped, A-system transport is also stepped down (4,16), and the activity of a Na^+-dependent hexose transporter, commonly assayed as the intracellular accumulation of the nonmetabolizable glucose analog α-methyl-D-glucopyranoside, (α-methylglucoside; α-meG) becomes observable. Over the next several weeks it attains high levels. This activity has the properties of a renal proximal tubule transporter not only in its dependence on Na^+ and its phlorizin sensitivity but also in its relatively weak interaaction with 3-O-methylglucose (2,18,22,27). The latter property distinguishes it from the similar intestinal transporter.

Our interest has been in the developmental aspects of this system. Although the increasing α-meG accumulating capacity has been described by several laboratories (2,16,18,21,22), we wished to determine more precisely what this meant in terms of transport parameters. Our second major concern has been with the cell biology of the system, specifically with the questions of whether the transport capacity developed simultaneously in all cells of the population or individual cells were recruited stochastically over a period of weeks into a "fully differentiated" subpopulation, and whether the cells with the maximum transport capacity were terminally differentiated or retained the ability to dedifferentiate and reinitiate growth. Before describing our approaches to these questions, we briefly describe the background to the work as it has been developed in our and other laboratories.

BACKGROUND

Concentrative α-meG uptake into LLC-PK$_1$ cells is absolutely Na^+-dependent (2,18,27). At a fixed Na^+ concentration and medium osmolality, increasing extracellular K^+ (which may be expected to depolarize the membrane) is partially inhibitory, a result consistent with the Na^+-electrochemical gradients' being the driving force (2,16). The Na^+:α-meG stoichiometry has been shown by independent methods to be 2:1 (19,20). The uptake of α-meG occurs at the apical (upper) surface (26). The diphenolic glucoside phlorizin is a powerful inhibitor (2,16,20,22,27). Trypsinization has been reported to abolish the uptake (26), but we find that trypsinization in the presence of EDTA will release the cells from their substrate and cell-cell attachments without loss of transport capacity.

After the cells form a monolayer on an impermeable substrate, dome or hemicyst formation is observed (22). Such doming appears to be caused by transmonolayer transport of salts and water in regions of low substrate adhesion (29,33). Doming disrupts the monolayer. If the cells are grown on porous substrates doming does not occur. In post confluent monolayers of cells grown in normal growth medium (5 mM glucose) and assayed in glucose-free buffer with 0.1 mM α-meG, α-meG uptake capacity develops over a period of 15-25 days and ultimately reaches very high levels, concentration ratios of the analog of more than 600 (cell:medium) having been reported (21) and confirmed in our laboratory.

Monolayers grown on porous supports can be mounted in Ussing chambers. In such preparations a transepithelial potential of 1-2 mV and resistances of $\sim$ 200 ohm-cm^2 have been recorded, as well as hexose-stimulated, phlorizin-sensitive, Na^+-borne short circuit currents (18).

For most of the experiments described here, we grew cells on collagen-coated Nucleopore filters (27) in Eagle's minimal essential medium supplemented with 10% fetal calf serum and 2 mM L-glutamine. The medium was changed every 2 days. Uptakes were measured by rinsing the filters in Hanks' balanced salt solutions (HBSS) and incubating them as desired in HBSS containing [^{14}C]-αmeG or other radioactive substrates as described in the figure legends. One filter is used for an experimental point, and each point was determined at least in triplicate.

In assaying α-meG uptake (Fig. 1) we find that the accumulation rate can, at all levels of accumulation capacity, be described by the first order relationship F-1

$$[S]_i = [S]_{i,\infty} \ (1 - \exp - kt)$$

where $[S]_i$ is the internal α-meG concentration at time t, $[S]_{i,\infty}$ is the final level, and $\underline{k}$ a rate constant with a value of 1.0 h^{-1}. This first order relationship is very convenient since any given time point in an experiment (usually, but not always, 60 min) can then be used to assess uptake rate.

We have observed that when cells are grown in the presence of Friend-cell inducer hexamethylene bisacetamide (HMBA) or the phosphodiesterase inhibitors dibutyryl cyclic AMP, theophylline, or methylisobutylxanthine (MIX), the development of α-meG accumulating capacity is markedly accelerated (Fig. 2). For this reason we commonly grow the cells in the most potent of these, MIX. Interestingly, the tumor promoter tetradecanoylphorbol acetate (TPA) inhibits development of α-meG uptake capacity and concomitantly blocks the normal stepdown of A-system transport (2,4). F-2

In these processes protein kinase C appears to play a role the nature of which is only beginning to be unraveled. This enzyme is a Ca^{++} and phospholipid-dependent kinase that is activated by diacylglycerols (reviewed in Nishizuka, 23). TPA can substitute for diacylglycerol in the activation (6). During the rapid growth phase of LLC-PK_1 cells, when the A-system is at a maximum and α-meG accumulation is not detectable, more than 90% of protein kinase C is found associated with the particulate (membranes plus organelles) fraction of the cells. In the postconfluent growth phase, when the A-system is stepped down to a low but stable level (see below) and α-meG accumulation capacity is rapidly increasing, the kinase is found in the soluble (cytosolic) fraction. Addition of TPA in this phase inhibits further development of α-meG uptake capacity but this

transport activity is not diminished over the next 1-2 days from its pre-TPA level. The effects on protein kinase C and A-system transport are more dramatic. The kinase activity in the cytosol diminishes rapidly (minutes) after TPA treatment to low or undetectable levels, and rises equally raipdly in the particulate fraction. Concomitantly A-system transport is stepped up as much as 4-fold, regaining or nearly regaining levels characteristic of rapidly growing cells. To explore further whether activation of the kinase is related to A-system activity, we added a permeating diacylglycerol, 1-octadecyl-2-acetyl-sn-glycerol, to postconfluent cells and observed again a prompt stimulation of the A-system (9). The details of these relationships are currently under investigation.

Finally, we have derived several subclones of LLC-PK_1. They have somewhat different morphologies and different sensitivities to the inducers (1), but all develop the α-meG accumulating capacity. Some of the experiments described below, as indicated in the figure legends, were done with subclone 4 which is the most responsive to MIX.

NATURE OF THE INCREASING ACCUMULATION CAPACITY

The accumulation of α-meG to any given level in a population of cells is due to the combined operation of a number of factors, including the number of functional transporters in the population, the distribution of these transporters among the individual cells, the Na^+ chemical gradient, the transmembrane potential, the pathways and activities of these pathways for efflux of glucoside, and possibly cell-cell coupling between accumulating and nonaccumulating cells. In the analysis of how these factors interact to yield the increasing transport capacity observable in the differentiating population we have explored each of these factors.

Cell-cell coupling: One of us (E.R.W.) visited the laboratory of Roobik Azarnia and Werner Loewenstein to measure coupling in LLC-PK_1 cells, particularly as it might be affected by tumor promoters. To our surprise, coupling was not demonstrable under any of the conditions examined, the conditions being those under which we routinely observe the development of glucoside-accumulation capacity. Electrical coupling between cells was less than 5% and carboxyfluorescein remained in the cell in which it was injected. The result was similar to that described in detail by Stefani and Cereijido (32) for MDCK cells, another renal epithelial line. With respect to LLC-PK_1, the consequence for our developmental analysis is that cell coupling may be neglected.

Efflux pathways: reversibility of the transporter: The steady-state accumulation level in a given population will ultimately be limited by efflux of α-meG; at steady state, efflux and influx are equal. The question arises, what pathways are available to α-meG for exit from the cell and to what extent is each utilized? Fig. 3 shows an experiment in which cells were pre-loaded to a concentration of α-meG 29-fold greater than that of the medium. The extracellular α-meG was washed away, the cells were returned to unlabeled growth medium with various additives, and the loss of α-meG from the population followed with time. The additives were: nothing, 2 μM cytochalasin B to inhibit hexose transport by Na^+-independent facilitated diffusion, 50 μM phlorizin to inhibit the Na^+-dependent transporter, or both. The efflux was first order in this and many similar experiments, including cases in which the intracellular concentration at the outset was greater than 10 mM. Cytochalasin B did not influence the efflux, an expected result in that it has been shown

F-3

previously that α-meG is not a substrate for the facilitated-diffusion transporter in other cell types (5,13,35). By contrast, cytochalasin B effectively blocks 3-O-methylglucose efflux from LLC-PK_1 cells (unpublished data), demonstrating that the transporter in these cells has the usual sensitivity to the drug. In other words, although a cytochalasin B-sensitive transporter is present in the cells it is not available to α-meG for efflux.

Phlorizin was also ineffective in slowing efflux (Fig. 3), suggesting that the reverse operation of the Na^+-dependent transporter was not an important component. To substantiate this conclusion we considered it important to demonstrate transport reversibility and its sensitivity to phlorizin, i.e., transinhibition. We considered it probable that efflux over the Na^+-dependent transporter might not have been detectable in Fig. 3 because the Na^+-electrochemical gradient was so unfavorable. Consequently, in the experiment shown in Fig. 4, at the same time that cells were being F-4
loaded with α-meG some of the cells were incubated in ouabain so that they were Na^+-loaded as well. This maneuver of course resulted in the ouabain-treated cells' taking up less α-meG during preloading (see figure legend), but since efflux was first-order in all cases the slopes of the efflux curves could be fairly compared independently of the initial loading. The ouabain-treated cells were not only Na^+-loaded but were also K^+-depleted, and the efflux assay was run in the continued presence of ouabain, blocking any electrogenic contribution of the Na,K-ATPase to the membrane potential. Hence it can be assumed, although it was not measured directly in this experiment, that the electrical component of the normal Na^+-electrochemical gradient was also run down. Under these conditions α-meG efflux was

enhanced 4-fold over the controls. In addition, this enhanced efflux was phlorizin sensitive. Added phlorizin, present only during the efflux measurements, reduced efflux from ouabain-treated cells to a level comparable to the untreated controls. From this we concluded that the Na^+-dependent transporter is both reversible and transinhibitable.

A further test, transstimulation, was applied to the reversibility of the Na^+-dependent transporter (Fig. 5). In this experiment all of the cells were Na^+-loaded by ouabain treatment at the same time that they were being α-meG loaded, and ouabain was again present during the efflux measurements. In addition, 10 mM nonradioactive α-meG was added to one group, and 10 mM α-meG + phlorizin to another. The external α-meG stimulated by more than 2-fold the efflux of radiolabeled α-meG from these cells, a clear example of classical transstimulation (7). Phlorizin not only abolished the effect, but as before (Fig. 4) inhibited the efflux from these ouabain-treated cells. F-5

These demonstrations of enhanced α-meG efflux from Na^+-loaded cells, its transinhibition by phlorizin, and its transstimulation by elevated external α-meG are qualitative in nature but clear evidence that the Na^+-dependent transporter is reversible. Under normal conditions, however, there is no observable phlorizin-sensitive component to efflux (Fig. 3, and see below). Even when α-meG has been concentrated 100-fold intracellularly (Table 1) phlorizin has no effect on subsequent efflux, nor can transstimulation by external α-meG be demonstrated in cells without Na^+-loading (unpublished). Hence, the Na^+-dependent pathway does not contribute significantly to escape of α-meG from the cells. The principal pathway appears to be simple, nonmediated passive diffusion. T-1

This set of experiments was undertaken to address a simple question: what role, if any, does a change in efflux parameters play in the increasing capacity of a population of LLC-PK_1 cells to accumulate α-meG? Two sets of observations prompted the investigations. Kimmich and Carter-Su (12) had shown that in isolated chick intestinal cells the ability to concentrate 3-O-methyl glucose by the Na^+-dependent mechanism could be substantially enhanced if the sugar's efflux by the Na^+-independent facilitated diffusion pathway were impeded by appropriate inhibitors. It has also been shown by a number of investigators that when several cultured cell types come into confluence the activity of the facilitated diffusion transporter is physiologically stepped down (10,14,30,34). Although α-meG is not known to be a substrate for this particular transporter, there remained the possibility that a physiological alteration, specifically a diminution in the α-meG efflux pathway might contribute to the increased accumulation capacity. To test the possibility we carried out efflux measurements like those in Fig. 3 on a series of samples showing increasing net uptake capacity (Table I). In cells that could concentrate α-meG in 60 min anywhere from 1.1 to over 100-fold, the efflux rate coefficient showed no significant change and was not significantly affected by phlorizin. The result enables us to eliminate this parameter as an important component in the development of α-meG accumulating ability.

<u>Na^+-electrochemical gradient</u>: The absolute dependence of α-meG accumulation on the Na^+-electrochemical gradient is well established. The associated developmental question is whether changes in this gradient over extended time in culture contribute to the changing accumulation capacity. Using functional tests for the gradient as a driving force, we have found a

consistently negative answer to this question.

The first of these functional tests was to estimate the membrane potential from the equilibrium distribution ratio of the lipophilic cation tetraphenylphosphonium (TPP^+; Refs. 4 and 15). According to this assay, the membrane potential in rapidly growing, nonaccumulating cells was about -100 mV. After 3 days in culture when the cells were nearing confluence the estimated potential decreased to about -75 mV and over the course of a week fell to -64 ± 8 mV. Since the transepithelial potential in LLC-PK_1 cells is of the order of only 1-2 mV (18) the membrane potential affecting the TPP^+ distribution ratio is presumably very nearly identical at both the apical and basolateral surfaces. Without independent confirmation, estimates of membrane potential from TPP^+ distribution must be interpreted very cautiously (15). The only conclusion we draw from these data is that, during the time α-meG accumulation capacity is increasing dramatically, the TPP^+ distribution suggests that the membrane potential is changing very little and the change, if any, is in the wrong direction to account for even a part of the increased α-meG accumulation.

The second functional test again relies on the virtual equivalence of the apical and basolateral membrane potentials as well as the assumption that cytosolic Na^+ is evenly distributed across the cell. In this case we compare, over days in culture, the Na^+-dependent uptakes of α-meG and alpha aminoisobutyric acid (essentially the same results are obtained with the more specific meAIB; Ref. 4). The results are shown in Fig. 6. During the F-6
first few days, while TPP^+ distribution ratio is falling, the uptake of AIB falls in parallel and the uptake of α-meG begins to rise. After the fourth day AIB uptake stabilizes at its stepped-down level and does not

change over the course of the next week. This suggests that the Na^+-electrochemical gradient is also unchanging during this time period unless there is a coincidental and precisely compensatory reciprocal change in the numbers of transporters and the driving gradient. The latter seems unlikely. During this week of stable AIB (or meAIB) uptake, the α-meG uptake capacity increases continuously to high levels. Together with the TPP^+ results, these functional assays indicate that the Na^+-electrochemical gradient is nearly constant during the period in which α-meG accumulating capacity is rapidly developing.

α-meG accumulation and specific phlorizin binding: Several laboratories have shown that the diphenolic glucoside phlorizin is a potent inhibitor of α-meG uptake in LLC-PK_1 cells and apical vesicles prepared from these cells (2,3,16,20,22,27). [^{3}H]-Phlorizin binds to these cell systems in a complex manner, showing both a high-affinity and an essentially nonsaturating low-affinity component. The high-affinity component is Na^+-dependent and can be easily assessed by subtracting out the binding observed in Na^+-free medium. The Na^+-specific binding component has a k_d equal to the k_i for inhibition of α-meG uptake, both of them (in our laboratory) equal to .08 ± .04 μM (3). There is no significant fluctuation in these binding and inhibitory constants throughout development of α-meG accumulating capacity. We have used this specific binding as a measure of the number of transporters in the population (31) as development proceeds. Fig. 7 shows F-7 the result of one such experiment. Uptake capacity of α-meG was assayed as the 60 min accumulation of the intracellular hexose. Specific phlorizin binding was derived from the usual extrapolation to maximum binding on

Scatchard plots of the Na^+-dependent component (3). Three points emerge from the data. First, no Na^+-dependent phlorizin binding is observed in growing cells that are incapable of accumulating α-meG. Second, to which the first is in fact a corollary, α-meG accumulation is linearly related to phlorizin-binding capacity of the population. Third this linear relationship holds whether the population has been allowed to develop spontaneously or whether it has been induced by either methylisobutylxanthine or hexamethylene bisacetamide.

From the linearity of the relationship it appears that the overriding if not the only significant factor in the development of α-meG accumulating capacity is the number of transporters in the population. This conclusion is consistent with the earlier ones: changes in cell coupling, or in efflux rate coefficient, or in the Na^+-electrochemical gradient, play little role in the observed increase in uptake capacity. The conclusion also simplifies the directions for future research in the development process, but leaves unanswered a basic cell biological question.

Distribution of transporters among cells in the population: The experiments described above were all done with unfractionated populations of cells. The cell biological question is: how does the accumulation capacity develop in individual cells? There would seem to be two extreme possibilities. On the one hand, the number of transporters on all cells might more or less in synchrony develop slowly over a period of two to three weeks. At the opposite extreme, each cell may develop its full complement of transporters over a time period that is relatively short, and the long time course for differentiation of the population would then

represent the slow recruitment of transport-competent cells into the differentiated subpopulation. Intermediate cases may of course be equally well postulated.

Our approach to this question has been to separate transporting from nontransporting cells on density gradients. Part of the reason for choosing this avenue was that we wished to recover viable cells for replating experiments to test for terminal differentiation. The principle is straightforward: cells that take up significant quantities of α-meG can be expected to swell osmotically and the swelling should reduce their density in comparison to cells that do not take up the hexose. The problem has been to establish the conditions under which these expectations can be realized.

One of our first controls was to make single cell suspensions by mild trypsinization with EDTA, from populations of young, transport-incompetent cells and more "mature" transport-competent cells. These were centrifuged in Percoll-HBSS gradients without added α-meG, and both populations were recovered as single peaks at the same density of $\rho = 1.050 \pm 0.01$ gms/cc. We then suspended transport-competent cells in two Percoll-HBSS gradients, differing only in that one contained 1 mM α-meG (Fig. 8; F-8 for experimental details see figure legend). Although the cells in the α-meG-containing gradient took up millimolar quantities of the hexose, they sedimented at the identical density as the control cells. Clearly the cells were capable of volume regulation in response to potential osmotic water shifts accompanying the hexose accumulation. We therefore tried, without success, a number of agents known to interfere in various ways with volume regulation in other cell systems. These agents included furosemide,

quinine, quinidine, and barium ion. In all cases, uptake of substantial quantities of α-meG was done isovolumetrically or, to be more exact, isopycnically.

Finally, considering that the counter-swelling regulation was most probably effected by loss of KCl from the cells, we chose a protocol that would reduce the possibility of this mechanism's effective operation. Trypsin-EDTA-detached cells were suspended in a K^+-free and Cl^--free Na-gluconate medium also containing 1.3 mM Ca^{++}, 1.3 mM Mg^{++}, and buffered to pH 7.2 with 20 mM Hepes. In this medium the cells lose in 30 min about half of their cell K^+. They shrink to about 60% of their normal size and remain shrunken. Their density on Percoll:Na-gluconate gradients is concomitantly increased to about 1.087 as expected for a 45% water loss. Of course these cells, like the ouabain-treated cells in Figs. 4 and 5, are markedly inhibited in the α-meG uptake. The addition of low concentrations of permeant anions, as counterions to the Na^+ taken up with the α-meG, did not help; neither 10 mM Cl^- nor 10 mM Cl^- with 2 mM HCO_3^- to accelerate Cl^- uptake had any effect on α-meG transport. Clearly it was necessary to allow the cells to extrude Na^+ without re-accumulating K^+. This was accomplished by adding to the K^+-free gluconate medium 4 mM NH_4^+ in the form of $(NH_4)_2SO_4$, since NH_4^+ is effective as a K^+ congener in stimulating the Na,K-ATPase (24,25). Under these conditions the cells transport α-meG nearly as well as the controls in HBSS (Table 2). That the mechanism of NH_4^+-stimulated α-meG uptake is operating through the Na,K-ATPase was demonstrated by its ouabain sensitivity. T-2

This medium was then used as the basis of Percoll gradients (Fig. 9). As before, the control cells shrink to a mean density of 1.087. This F-9

shrinkage shows that despite the operation of an NH_4^+-stimulated cation pump the cells do not retain or regain their normal volume and thus are not accumulating NH_4^+-salts. In similar gradients to which α-meG has been added, uptake of hexose is substantial, reaching in the example shown an intracellular concentration of about 40 mM at the peak. This corresponds to about a 12-percent increase in the osmotic content of the shrunken cells and osmotically obligated water uptake reduces the peak density by the expected amount. In the presence of phlorizin the α-meG uptake is prevented and the density shift does not occur.

The small peak at the top of all three gradients is commonly although not invariably observed. It seems to be little affected by α-meG. We believe it may represent damaged or dead cells in the population and we neglect it in our analysis.

Using this approach, we find the following course of events as the population develops. Initially, the cells carry out little or no transport, and both the controls and α-meG-exposed cells sediment at the high density. When α-meG accumulation first becomes evident (day 4), the α-meG-exposed cells are shifted only very slightly toward a lighter density with a corresponding peak of [^{14}C]-α-meG radioactivity (not shown). At later times data like those of Fig. 9 are obtained. The peak of radioactivity, identifying the transporting cells, is nearly symmetrical around the lighter density but with a skew toward the denser side. There remains a distinct shoulder of cells remaining at the control density, which shoulder becomes smaller as the α-meG accumulation capacity of the total population increases. The results suggest that a middle course between the two extremes hypothesized above is being followed. In the

early phases of accumulation, accumulation is insufficient to bring about a major density shift. In the later phases, not all cells in the population participate. Although our analysis is not yet complete it appears that a stochastic model (17), with cell-by-cell recruitment into the differentiated population, best accounts for the observations like those of Fig. 9. But the early (4-day) result suggests that the development may, in each cell, occur sufficiently slowly so that although the initial accumulation capacity involves enough cells to be clearly observable on the gradients by the [^{14}C]-α-meG uptake, it is insufficient to move this subpopulation well away from the controls. At later times, as in the figure, the presence of the skew in the radioactivity curve similarly suggests that a fraction of the transporting cells have not achieved the mean accumulating capacity of this subpopulation. At very late times the cells on the denser side of the transporting population move away from the corresponding controls suggesting that virtually all cells in the population are now able to carry out accumulative uptake. Nevertheless, subcultures may be establishd from such mature populations. Whether this means that a few undifferentiated and growth-competent cells remain in the mature cultures, or whether cells may de-differentiate and resume growth, is not yet established.

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FIGURE LEGENDS

Fig. 1 Uptake of α-meG by LLC-PK_1 cells that had been cultured for the number of days indicated in growth medium supplemented with 1 mM MIX. Error bars are 1 SD of triplicate samples. The curves are fit to the first order uptake equation given in the text with a rate constant $\underline{k}$ = 1.0 h^{-1}. Values for $([S]_i/[S]_o)_{t,\infty}$ are: 10.4 on day 2, 60.2 on day 5, and 112 on day 8. From Cook et al.(1982) with permission.

Fig. 2 Effects of phosphodiesterase inhibitors, without and with added TPA, on the development of α-meG concentrating capacity. Cells were seeded onto collagen-coated filters 2 days before the onset of the treatments on day 0. Ordinate: 60-min uptake of α-meG from a medium concentration of 0.1 mM assayed on the days indicated. An uptake of 60 nmol/mg protein corresponds to an intracellular concentration of 10 mM, or a 100-fold cell:medium concentration ratio. Abscissa: days of treatment. Treatments were, in order: open triangles, 1 mM MIX; open squares, 1 mM theophylline; open circles, untreated controls; closed triangls, 1 mM MIX + 0.1 μM TPA; closed squares, 1 mM theophylline + 0.1 μM TPA. Cells cultured in 0.1 μM TPA alone were not viable. From Amsler and Cook (1982), with permission.

Fig. 3 Efflux of α-meG from LLC-PK_1 cells (clone 4) and its insensitivity to phlorizin and cytochalasin B. Cells cultured 5 days in 0.2 mM MIX were preloaded for 60 min in 0.1 mM [^{14}C]α-meG, reaching an internal concentration of 2.9 mm ($[S]_i/[S]_o$ = 29). They were subsequently washed, and incubated at 37°C in α-meG-free medium containing no additives (closed circles), 50 μM phlorizin (closed squares), 2 μM cytochalasin B (closed triangles), or both (open

circles).

Fig. 4 Augmented efflux and its phlorizin sensitivity in Na^+-loaded LLC-PK_1 cells (clone 4). Cells cultured 5 days in 0.2 mM MIX were preloaded for 40 min in 0.1 mM [^{14}C]α-meG in the presence or absence of 0.8×10^{-4} M ouabain. In controls without ouabain (circles), α-meG reached an internal concentration of 4.3 mM ($[S]_i/[S]_o$ = 43), which was subsequently released into the medium with an efflux rate constant of 0.65 h^{-1}. In Na^+-loaded cells treated with ouabain only (squares), α-meG reached an internal concentration of 1.7 mM ($[S]_i/[S]_o$ = 17) and was subsequently released into the medium with a rate constant of 2.3 h^{-1}. In cells Na^+-loaded by treatment with ouabain and subsequently incubated in ouabain plus 50 μM phlorizin (triangles), α-meG reached an internal concentration of 2.0 mM ($[S]_i/[S]_o$ = 20) and was subsequently released into the medium with a rate constant of 0.44 h^{-1}.

Fig. 5 Transstimulation of α-meG efflux from Na^+-loaded LLC-PK_1 cells (clone 4). Cells cultured 5 days in 0.2 mM MIX were preloaded for 40 min in 0.1 mM [^{14}C]α-meG in the presence of 0.8×10^{-4} M ouabain; -meG reached an internal concentration of 2.0 mM ($[S]_i/[S]_o$ = 20). Cells were washed, and incubated at 37°C for efflux measurements in HBSS plus ouabain only (circles), ouabain plus 10 mM nonradioactive α-meG (triangles), or ouabain plus 10 mM nonradioactive α-meG plus 50 μM phlorizin (squares).

Fig. 6 Changes in the 60-min uptakes of α-AIB and α-meG in LLC-PK_1 cells as a function of time in culture. Cells were seeded onto collagen-coated filters 2 days before the onset of the experiment, and on day 0 the filters

were transformed to growth medium supplemented with 0.2 mM MIX. The data are replotted from refs. 2 and 4.

Fig. 7 Linear correspondence of α-meG accumulation and specific phlorizin binding to LLC-PK_1 cells (clone 4). Circles represent untreated cells; squares, cells cultured in 0.2 mM MIX; diamonds, cells cultured in 2 mM HMBA. The values of N for [^{3}H]-phlorizin binding were obtained by extrapolation to maximum binding on Scatchard plots of the Na^+-dependent component (MIX and HMBA) or from measurements of Na^+-dependent binding at saturating (500 μM) concentrations of [^{3}H]-phlorizin (untreated cells).

Fig. 8. Detached cells were rinsed in HBSS, suspended in HBSS ± 1 mM α-meG for 60 min at 37°C, then pelleted and overlayed on a 42% Percoll gradient. The gradient was preformed by centrifugation at 20,000 g for 30 min. The gradient containing cells was then spun at 2000 rpm for 15 min. Densities (right ordinate) were determined by refractometry. Open cicles: cells in Percoll-HBSS; Closed circles: cells in Percoll-HBSS plus 1 mM α-meG. Percoll does not interfere with transport.

Fig. 9. Detached cells were rinsed in Na-gluconate medium (see text) and suspended in 45% Percoll containing no additives, 1 mM α-meG or 1 mM α-meG + 10 μM phlorizin in Na-gluconate medium for 10 min. The gradients were spun at 20,000 g for 45 min and fractionated.

Table 1

Comparison of rate constants for efflux of α-meG from LLC-PK_1 cultures at different levels of concentrating activity in the absence and presence of phlorizin.

Days in MIX	$[S]_i/[S]_o$	$k_{eff}(h^{-1})$	
		Control	+Phz
3	1.1	-	0.53 ± 0.04
3	9.2	0.58 ± 0.05	-
4	26.4	0.49 ± 0.06	0.48 ± 0.02
5	48.2	0.46 ± 0.02	0.47 ± 0.01
6	54.9	0.74 ± 0.12	0.41 ± 0.02
7	108.2	0.48 ± 0.05	0.40 ± 0.05

Cells grown in 0.2 mM MIX for the number of days indicated were loaded with 0.1 mM [^{14}C]α-meG for 60 minutes. Filters were rinsed with cold HBSS. Control cultures were placed in 37°C HBSS for efflux measurements. Other cultures were placed in warm HBSS containing 50 μM Phz.

$[S]_i/[S]_o$ is the concentration ratio of α-meG inside the cells with respect to the external medium.

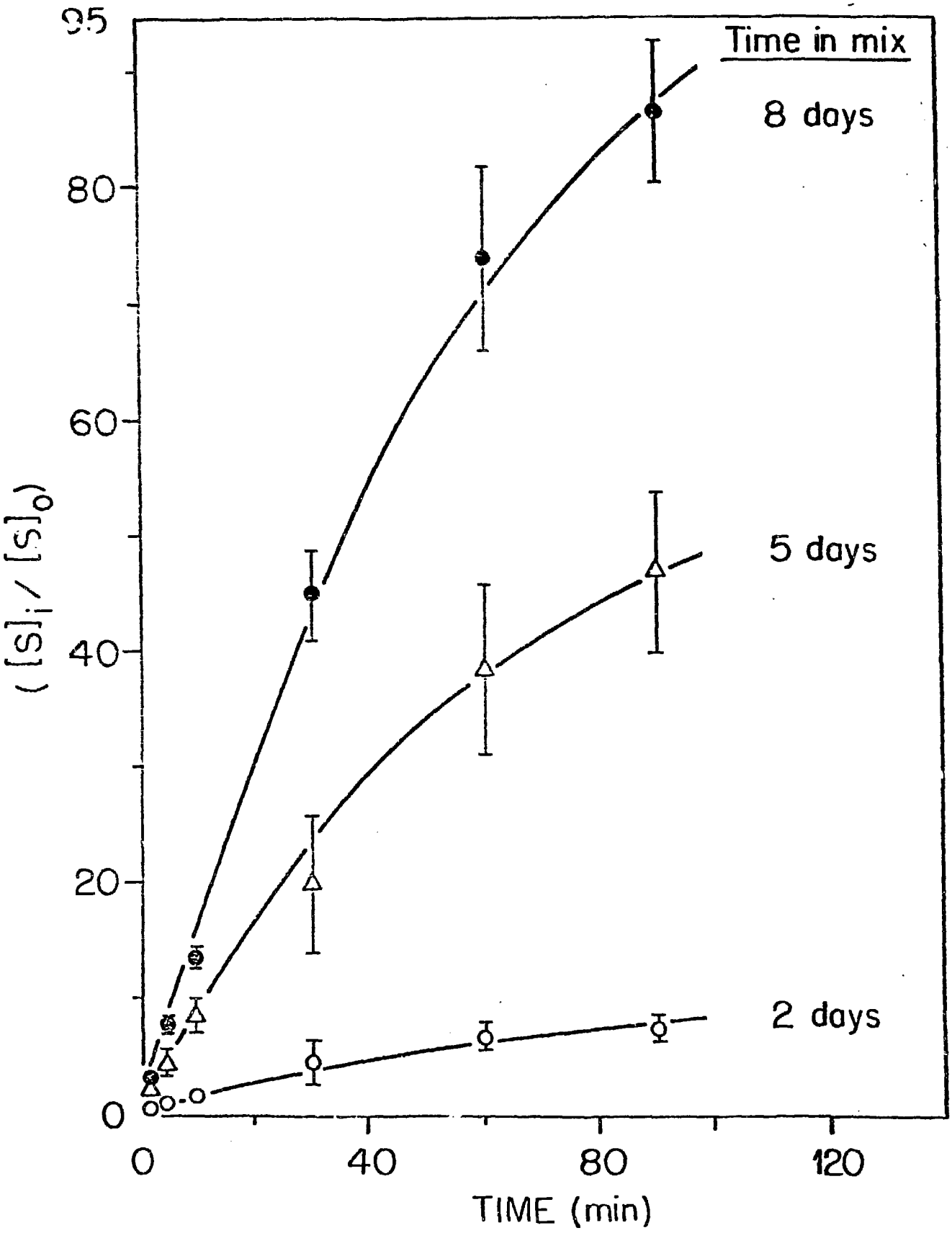
k_{eff} is rate constant for efflux ± 95% confidence interval.

Table 2

60-Min α-meG uptake in HBSS and K^+-free Na-gluconate media with various additives.

Medium	α-meG Uptake (nmol/mg protein - 60 min)
HBSS	66 ± 8
Na-gluc	17.5 ± 6
Na-gluc + 0.1 mM ouabain	10 ± 1.5
Na-gluc + 2 mM $(NH_4)_2 SO_4$	57 ± 6
Na-gluc + 2 mM $(NH_4)_2SO_4$ + 0.1 mM ouabain	13.5 ± 1

Cells (clone 4) were cultured on collagen-coated filters 6 days in 0.2 mM MIX, and pre-incubated 30 min in HBSS or K^+-free Na-gluconate medium before assay in the same medium plus 0.1 mM [^{14}C]α-meG.


Time in mix
8 days
5 days
2 days
95
80
60
40
20
0
0
40
80
120
TIME (min)
([S]i / [S]o)

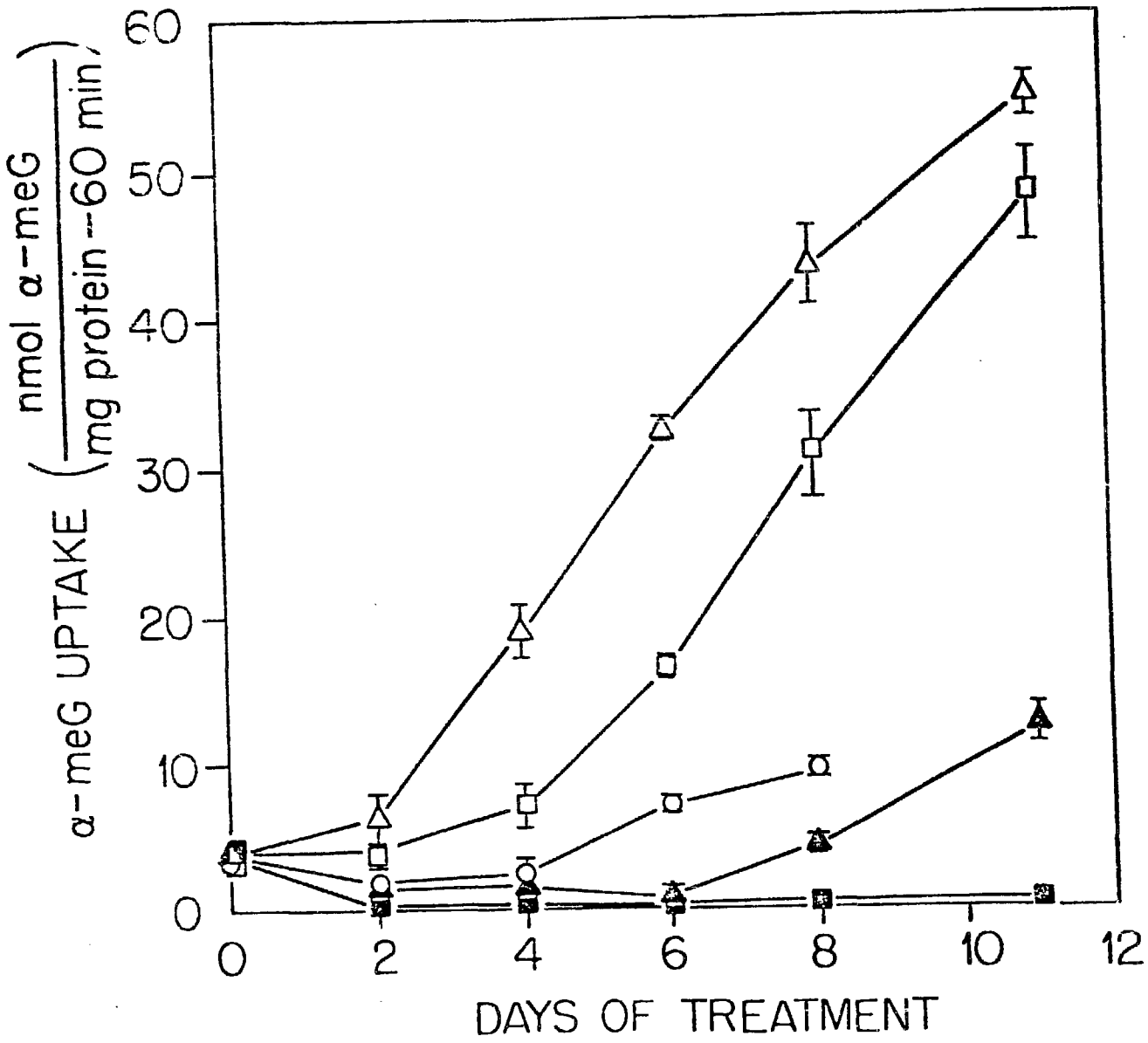

α-meG UPTAKE (nmol α-meG / mg protein--60 min)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
DAYS OF TREATMENT

Fig 2

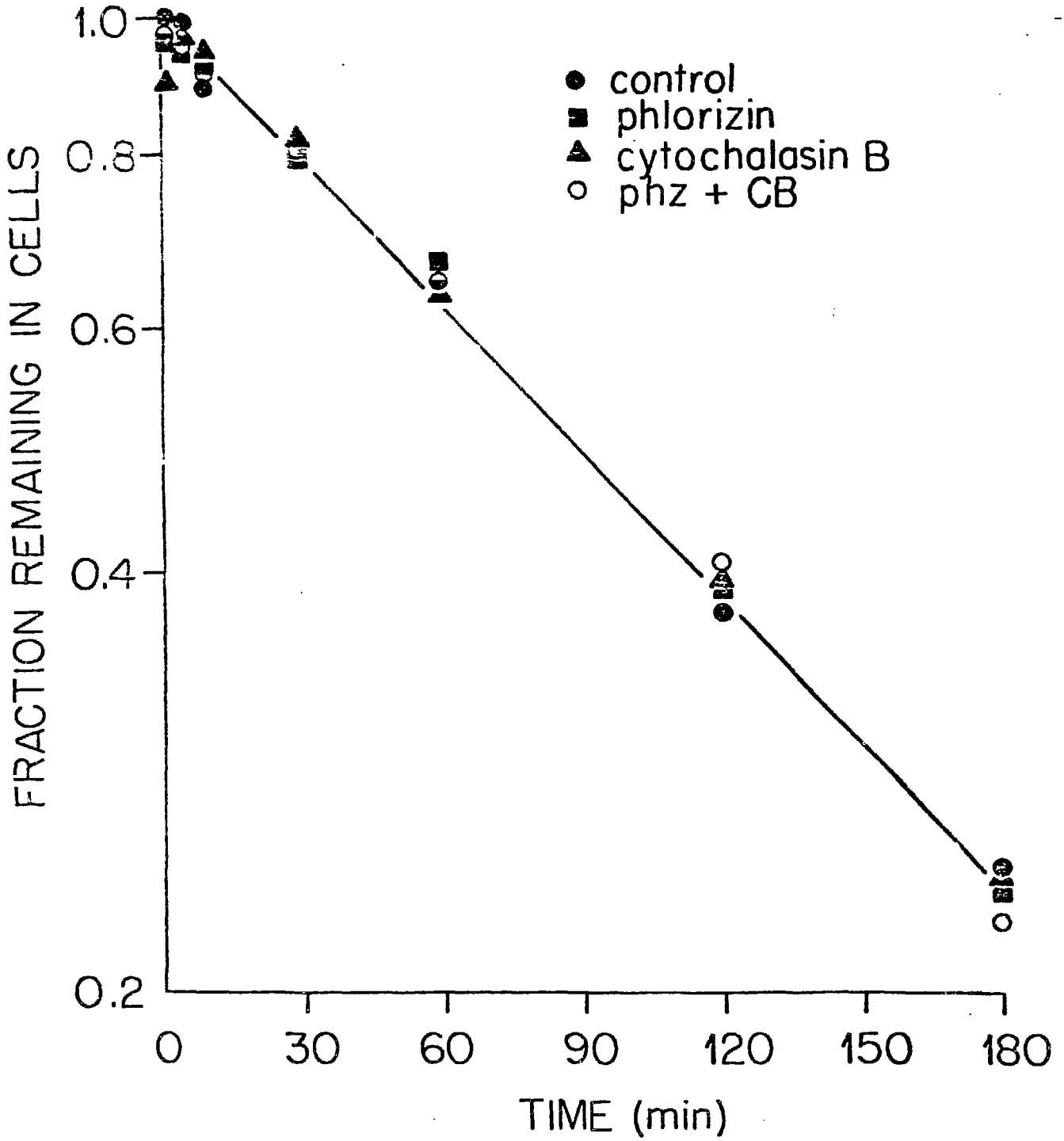
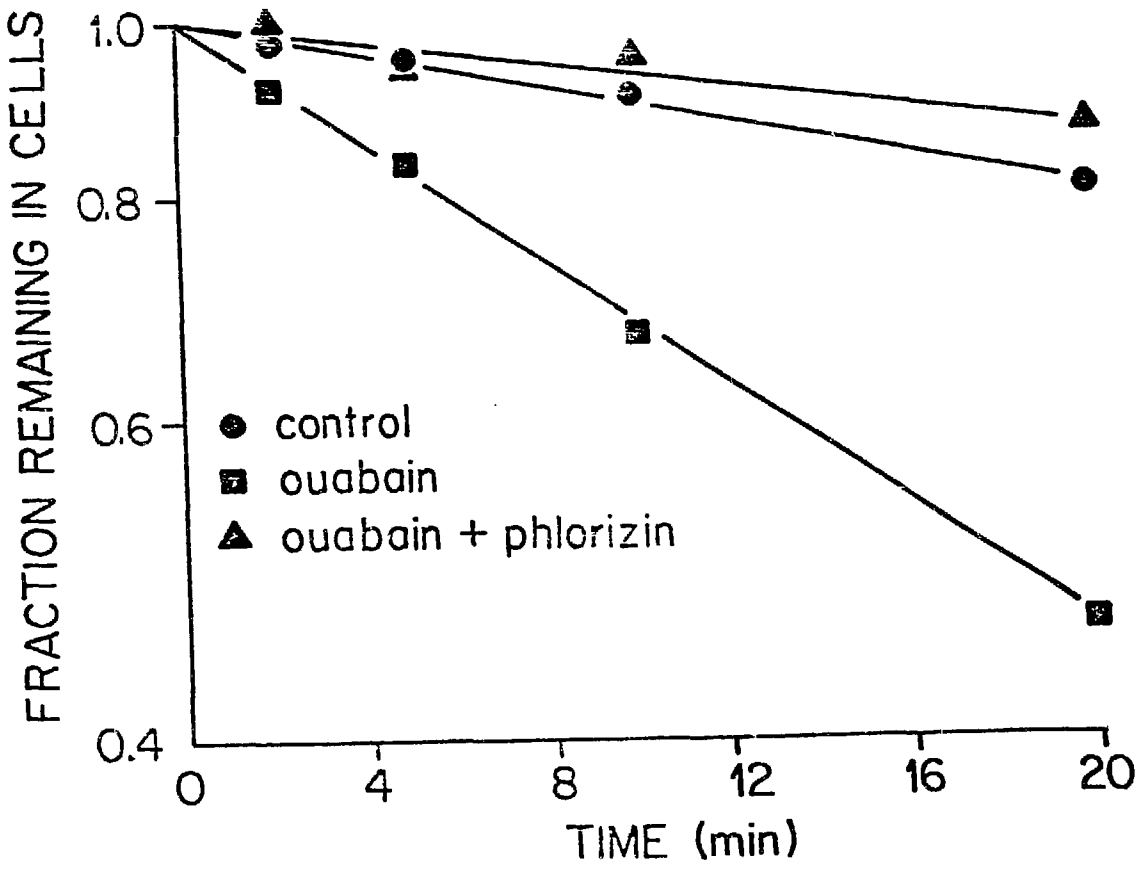
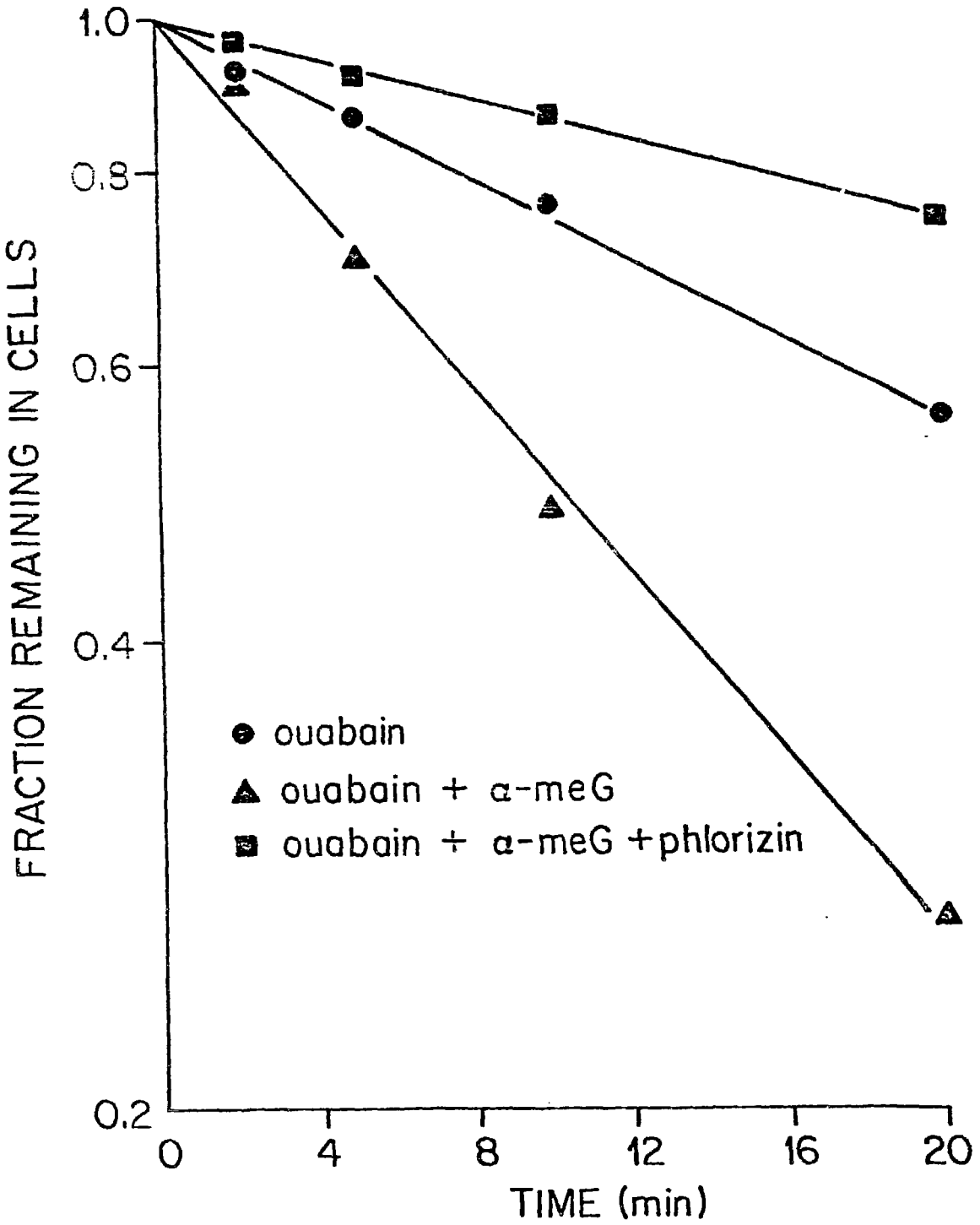

1.0
0.8
0.6
0.4
0.2
FRACTION REMAINING IN CELLS
control
phlorizin
cytochalasin B
phz + CB
0
30
60
90
120
150
180
TIME (min)

Fig 8


FRACTION REMAINING IN CELLS
1.0
0.8
0.6
0.4
0
4
8
12
16
20
TIME (min)
control
ouabain
ouabain + phlorizin


1.0
0.8
0.6
0.4
0.2
FRACTION REMAINING IN CELLS
ouabain
ouabain + α-meG
ouabain + α-meG +phlorizin
0
4
8
12
16
20
TIME (min)

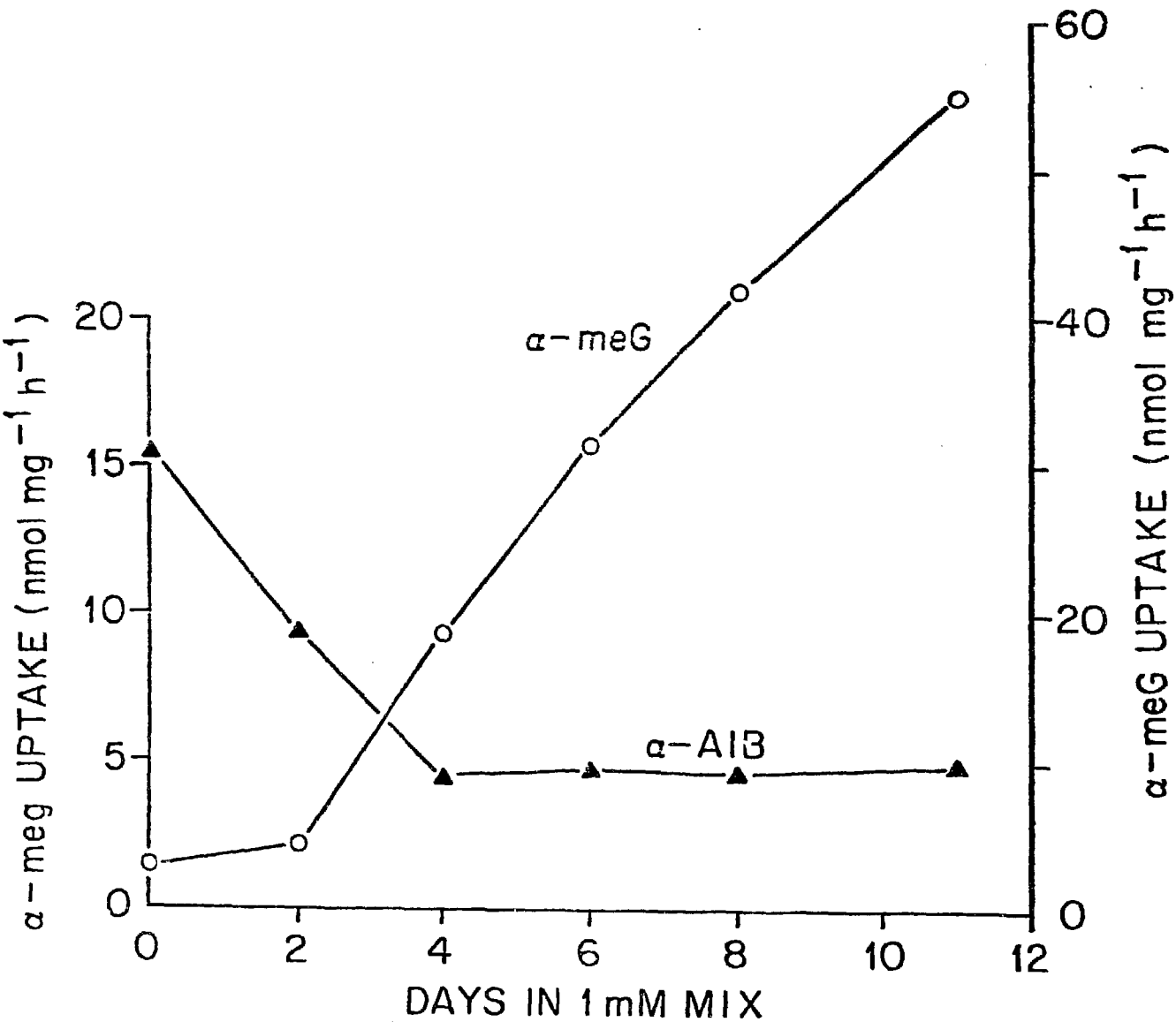
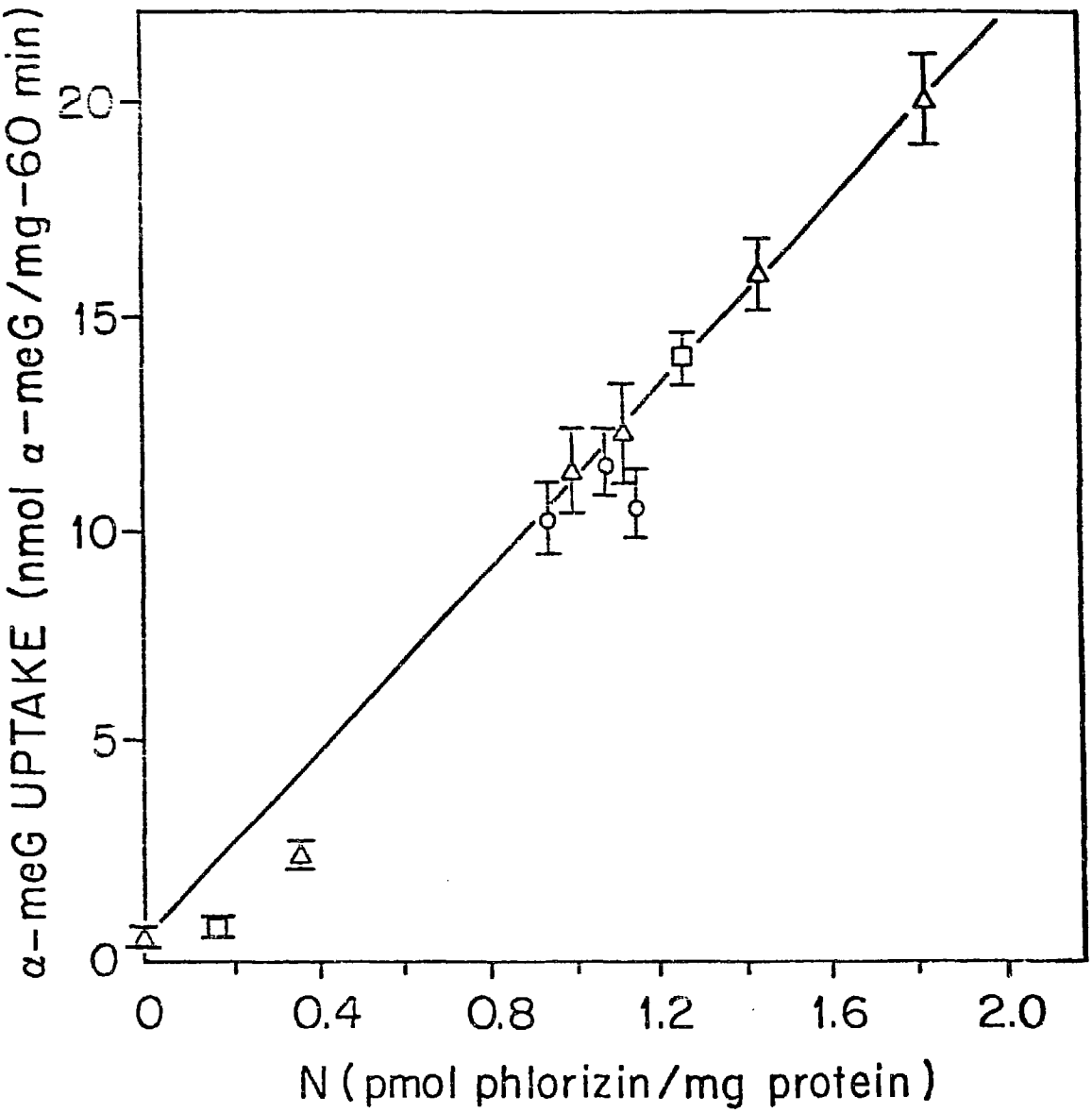

α-meG
α-AIB
α-meg UPTAKE (nmol mg⁻¹ h⁻¹)
α-meG UPTAKE (nmol mg⁻¹ h⁻¹)
DAYS IN 1 mM MIX
0
5
10
15
20
0
20
40
60
0
2
4
6
8
10
12

Fig. 6


α-meG UPTAKE (nmol α-meG/mg-60 min)
0
5
10
15
20
0
0.4
0.8
1.2
1.6
2.0
N (pmol phlorizin/mg protein)

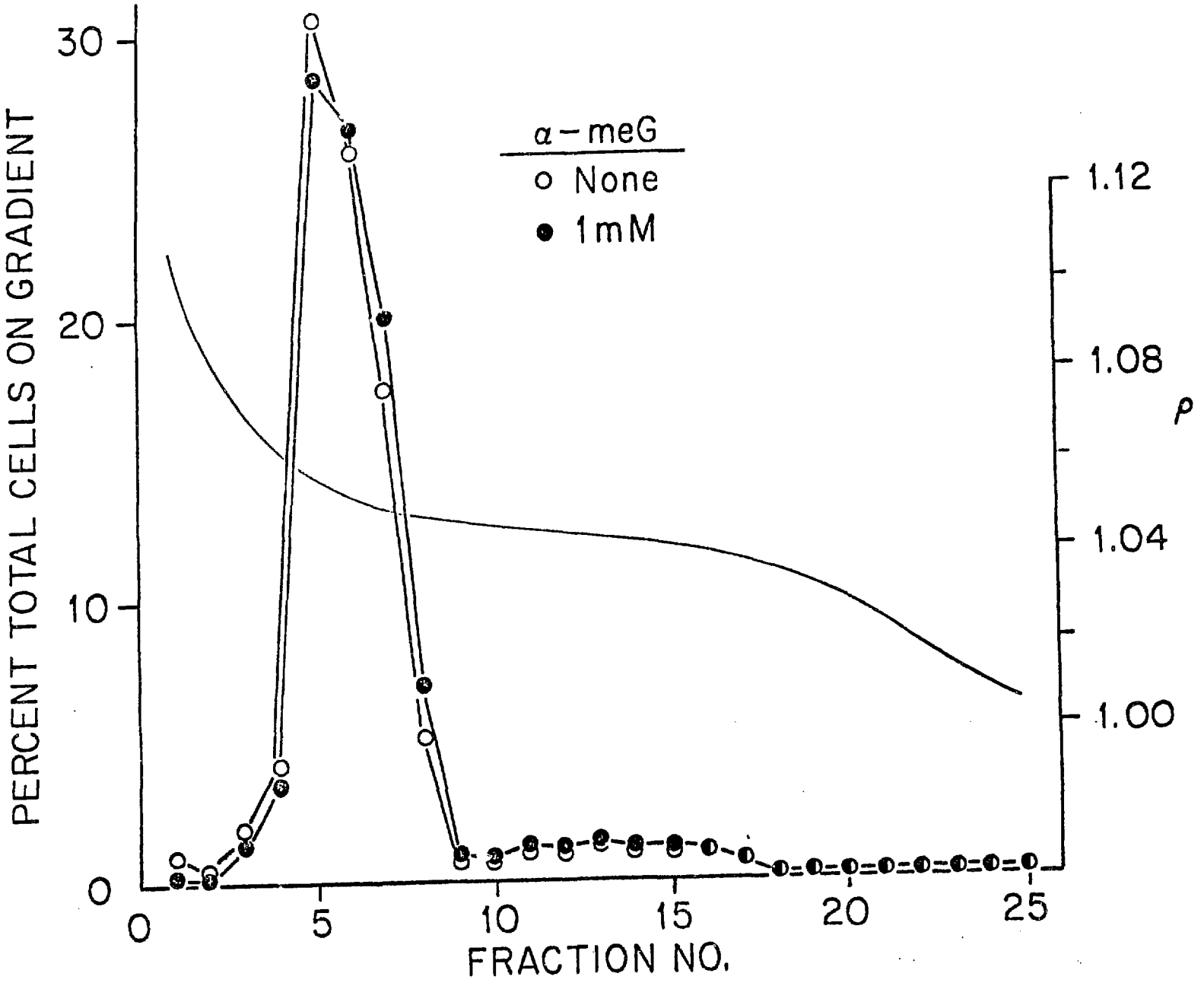
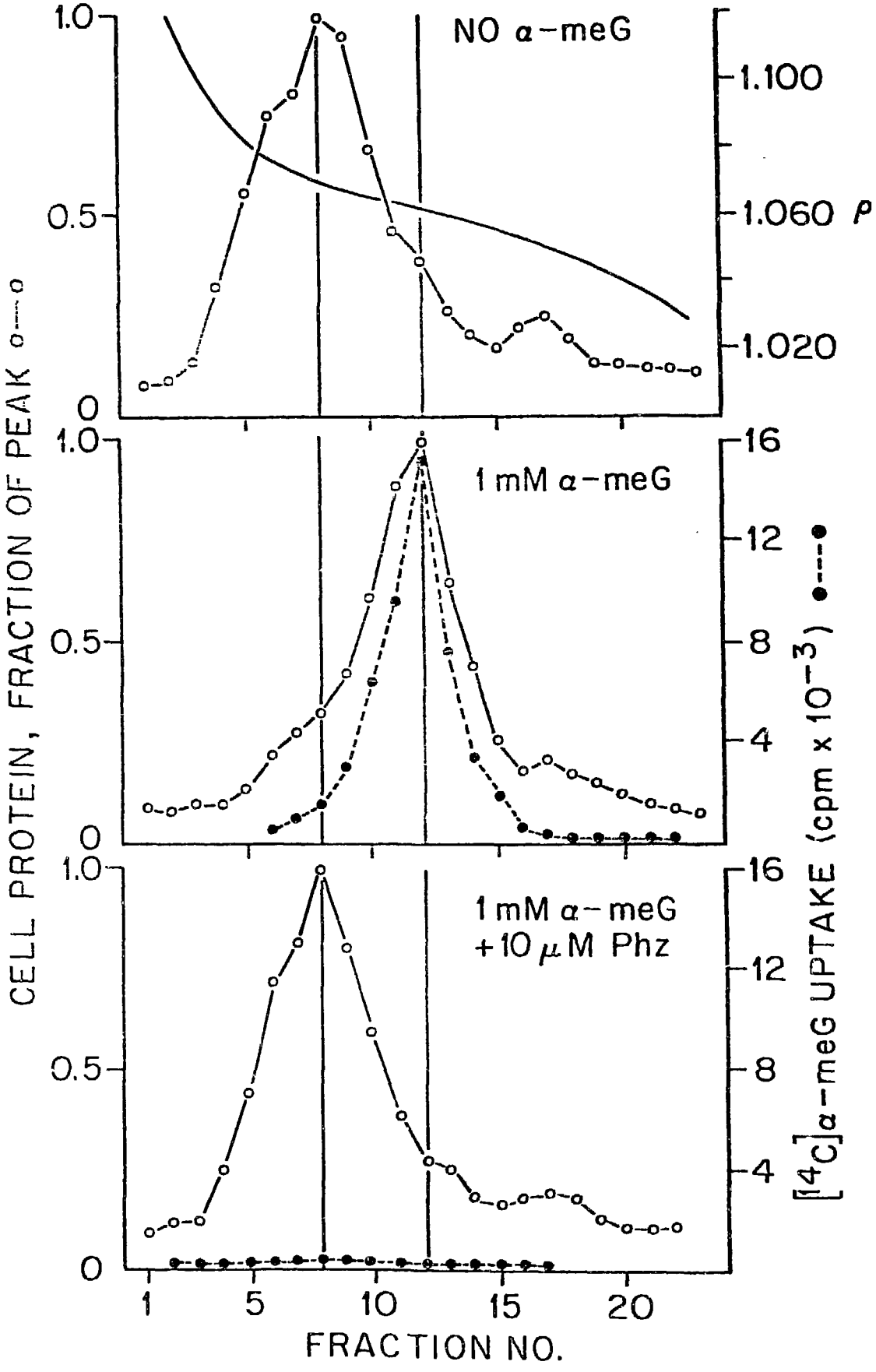

PERCENT TOTAL CELLS ON GRADIENT
30
20
10
0
0
5
10
15
20
25
FRACTION NO.
α-meG
None
1mM
1.12
1.08
1.04
1.00
ρ

Fig. 8


NO α-meG
1 mM α-meG
1 mM α-meG
+10 μM Phz
1.0
0.5
0
1.100
1.060 P
1.020
16
12
8
4
1
5
10
15
20
FRACTION NO.
CELL PROTEIN, FRACTION OF PEAK o—o
[14C]α-meG UPTAKE (cpm x 10−3) •—•