J. Membrane Biol. 57, 13–23 (1980)

The Journal of
Membrane Biology

Permeation of Chinese Hamster Ovary Cells by Glycerol: Mechanism and Kinetics

Douglas C. Dooley
Cryobiology Laboratory, Blood Services Laboratories, American Red Cross, Bethesda, Maryland 20014

Summary. When monolayer cultures of Chinese hamster ovary cells were exposed to ^{3}H-glycerol ranging in concentration from 0.1 μM to 200 mM, glycerol influx was found to increase in direct proportion to the extracellular concentration of glycerol. Other experiments indicated that the same relationship existed at concentrations in excess of 1.0 M. Similarly, glycerol efflux was found to vary in direct proportion to the intracellular concentration of glycerol. In neither case could influx or efflux be saturated. Glycerol influx was not affected by depletion of ATP, alkylation by parachloromercuribenzene sulfonic acid, or exposure to persantine. Altering the pH or temperature also had little effect. Attempts at demonstrating countertransport of glycerol were negative. These data indicate that glycerol probably passes through the membrane by a nonmediated process. For cells in monolayer, the kinetics of influx and efflux are biphasic. Similar biphasic kinetics are observed with cells in suspension culture. A close fit to the data may be obtained by adding together two first-order curves. The pair of curves for influx are clearly different from the pair for efflux. The fit provided by the two pairs of first-order functions suggested that glycerol might diffuse into and out of two intracellular compartments. However, the experimental data do not agree with the predicted behavior of a two-compartment system. As a result, the exact nature of the diffusion limiting steps which are described by the first-order equations remains undefined.

The data of Collander (1949) led to the conclusion that the permeability of organic nonelectrolytes was determined by their size and oil-water partition coefficient. Stein (1967) interpreted Collander's data as evidence for the role of hydrogen bonds in cell permeability. According to this theory, membrane permeability falls as the hydrogen bonding capacity of the solute increases. Collander also concluded that the membrane possessed a "seive-like" character. This has led to calculations of pore size. For example, Giebel and Passow (1960) estimated that dicarboxylic acids diffuse through pores 7–9 Å in diameter. The unexpectedly high permeability of water through the membrane (Stein, 1967) has also been explained by the existence of pores which Macey, Karan and Farmer (1972) believe to be fairly specific for water. Christensen (1975), however, rejected the lipid solubility-pore model as an inadequate explanation of the behavior of biological membranes.

The mechanism of glycerol uptake has been examined in a number of systems. It is currently believed that at least two cell types possess transport systems for glycerol, *Escherichia coli* (Richey & Lin, 1972; Alemohammed & Knowles, 1974) and human erythrocytes (Carlsen & Wieth, 1976). Li and Lin (1975) presented preliminary evidence for mediated uptake by rat hepatoma cells. Bickis et al. (1967) estimated the permeation kinetics of glycerol using Novikoff ascites cells but did not investigate the mechanism of uptake.

In a previous study of glycerol uptake by Chinese hamster ovary (CHO) cells, it was concluded that these cells do not have a system for the active transport of glycerol (Dooley, 1980). However, it was not clear from that study whether glycerol entered the cells by a mediated or nonmediated process. This communication presents a series of experiments attempting to distinguish between those two possibilities. In addition, the kinetics of influx and efflux obtained during the course of this study are compared to models of solute diffusion analyzed by Kotyk and Janacek (1970).

0022-2631/80/0057-0013 $02.20

Materials and Methods

Tissue Culture

Chinese hamster ovary (CHO) cells were provided by Dr. T. Puck. Cultures were grown in RPMI 1640 (Microbiological Associates) supplemented with 10% fetal calf serum (North American Biologicals) and 50 units/ml penicillin G plus 50 mcg/ml streptomycin. Cells were grown in a 10% CO_2 atmosphere at 37 °C unless indicated otherwise. Direct culture methods indicated the cells were free of mycoplasma. To carry out permeation studies in monolayer, CHO stock cultures were trypsinized, and the cells plated in 60 mm Falcon petri dishes. The dishes were incubated at least 18 hr before use, at which time they were 40–90% confluent ($1.4–3.0 \times 10^6$ cells/dish). To conduct flux assays in suspension, stock cells were trypsinized and transferred to polycarbonate flasks containing standard 1640 growth medium. Flasks were incubated 16–20 hr at 37 °C without stirring. At the time of the assay, the cells were resuspended by gentle swirling and treated as described below.

Glycerol Influx in Monolayer

Overnight growth medium was removed and replaced with 1 or 2 ml of fresh growth medium containing 20 mM Hepes (pH 7.2) and the indicated concentration of glycerol plus 1 or 2 μCi/ml ^{3}H-glycerol (2-^{3}H-glycerol, 6–9 Ci/mmol, New England Nuclear) at 37 °C unless specified otherwise. At the desired time, the plate was flooded with ice cold phosphate buffered saline (PBS) supplemented with glycerol at the same concentration used in the assay unless specified otherwise. The plate was drained and washed four more times in the same fashion. The duration of exposure to ^{3}H-glycerol was measured from the time of addition of the labeled medium until the first addition of cold PBS. With two people working together, samples could be taken with as little as 2 sec exposure to the ^{3}H-glycerol. During the 13 sec required to carry out the cold wash, 2–3% of the intracellular label was lost to the extracellular washing medium. After completing the rinsing, the monolayer was lysed by the addition of 1.5 ml 0.1 M NaOH, 1.0 ml of which was mixed with 100 μl of 7% acetic acid and transferred to 10 ml of Hydrofluor (National Diagnostics) for scintillation counting.

Glycerol Efflux in Monolayer

Growth medium was removed from a single 60 mm dish and replaced with 2 ml medium containing the required concentration of glycerol plus 10 μCi/ml ^{3}H-glycerol. The plate was incubated at 37 °C for 30 min to allow intracellular equilibration of the isotope. To remove extracellular label, the monolayer was gently washed five times with warm PBS (a process which lowered the intracellular concentration of glycerol 26%, a fact taken into account in velocity calculations). The cells were then covered with 1.5 ml of fresh 37 °C medium containing 20 mM Hepes, pH 7.2. Glycerol efflux was monitored by periodically changing the medium on the plate and sampling that medium for radioactivity by mixing with Hydrofluor for scintillation counting. At the conclusion of sampling, the monolayer was lysed with 0.1 M NaOH and the radioactivity determined to measure residual intracellular tritium. The twenty successive medium changes removed less than 5% of the cells from the plate.

Glycerol Efflux in Suspension

Method I: ^{3}H-glycerol (1 mM, 10 μCi/ml final concentration) was added to cell suspensions (4 ml, 6×10^6/ml) for 40 min (37 °C) after which the cells were centrifuged for 8 min at $100 \times g$. The supernatant was removed, and the cell pellet suspended in 25 ml of glycerol free growth medium (27 °C) and sampling begun.

Method II: Cell suspensions were gently pelleted and the cells (6×10^7) suspended in 1.0 ml of 1640 RPMI containing ^{3}H-glycerol (1 mM, 4 μCi/ml) for 40 min at 37 °C. The 1-ml suspension was diluted directly into 300 ml of glycerol free medium (27 °C) and sampling initiated. The cell suspension was stirred continuously. Sampling was carried out by trapping 1×10^6 cells on nitrocellulose filters (0.45 μm, Schleicher and Shuell) and rinsing with 5 ml of 0 °C Hepes buffered (pH 7.0) isotonic saline. The filters were placed in Hydrofluor and counted after the filters had dissolved.

Chemicals

Persantine was obtained from Dr. W. Michaelis, Boehringer Ingelheim, Ridgefield, Connecticut. Parachloromercuribenzene sulfonic acid was purchased from Sigma.

Graphical Analysis

Biphasic semilogarithmic flux kinetics were analyzed according to Siegers et al. (1979). Using linear regression, a straight line was constructed through the late (monophasic) portion of the flux curve (2–14 min for influx, 9–20 min for efflux). The regression line was extrapolated back to zero time and the values so obtained were subtacted from the early curvilinear portion of the plot (0.10–0.45 min for influx, 0.16–2.0 min for efflux), the difference values generating a simple (straight line) exponential function. Linear regression was used to construct the best line through the resultant early points. Half time values were calculated from the slopes of the regression lines.

When the efflux of intracellular tritium became so slow as to become undetectable, a small amount of isotope was consistently found to remain cell-associated. This fraction was excluded from calculations when the full time course of efflux was plotted. For monolayer cultures, this poorly permeable fraction amounted to 4% of the total intracellular label, while for suspension cultures, it constituted 20% of the total.

Results

Concentration Dependence

When CHO cells are exposed to ^{3}H-glycerol, there is a net flow of label into the cells until the glycerol concentration inside the cells equals that of the surrounding medium (Dooley, 1980). This is illustrated in Fig. 1 where it is seen that the cells require approximately 20 min at 37 °C to reach equilibrium in 13.5 μM glycerol. If G_T is the amount of intracellular ^{3}H-glycerol/dish at time T and if G_∞ is the amount of intracellular ^{3}H-gylcerol/dish at equilibrium, then $[1 - G_T/G_\infty]$ appears to fall off exponentially as a func-

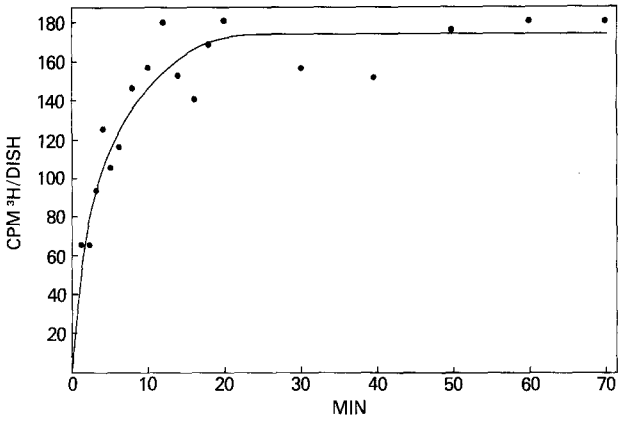

Fig. 1. Influx of ^{3}H-glycerol into CHO monolayer cultures. CHO cells growing at 37 °C in 60-mm dishes were exposed to 2.0 ml of growth medium containing 13.5 μM glycerol and 1 μCi/ml ^{3}H-glycerol. Plates were harvested at the indicated times and analyzed for the amount of intracellular label as described in Materials and Methods

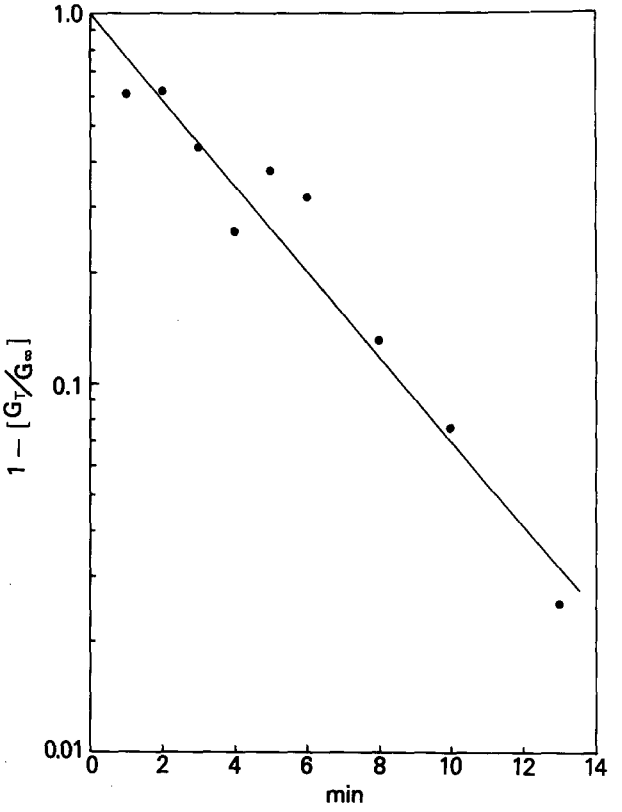

Fig. 2. Exponential approach to equilibrium during influx of 13.5 μM ^{3}H-glycerol. G_T = CPM intracellular ^{3}H/culture dish at time T. G_∞ = CPM intracellular ^{3}H/culture dish at equilibrium. Data from Fig. 1

tion of time (Fig. 2; Dooley, 1980). These are the kinetics expected of nonmediated diffusion (the rate of uptake varying with the concentration gradient across the membrane). However, Stein (1967) showed that Fick's law of diffusion was kinetically indistinguishable from mediated transport when the substrate concentration was considerably lower than the K_m of the transport system. Therefore, the uptake kinetics were examined in more detail in order to differentiate between the diffusion of glycerol and its mediated transport across the membrane.

If it were possible to saturate the velocity of glycerol uptake, one would have presumptive evidence for mediated transport. This would require measuring influx as a function of glycerol concentration. However, the absolute rate of uptake cannot be measured directly since the *measured* rate is the net difference between the *absolute* rates of inflow and outflow. However, the measured uptake rate approaches the absolute rate as the time of exposure to labeled glycerol becomes progressively briefer. Therefore, the velocity of uptake (nmol/min/10^6 cells) was measured after a 60-sec exposure to ^{3}H-glycerol. The concentrations ranged from 10 to 200 mM. Figure 3 reveals that the initial velocity of uptake is directly proportional to the extracellular concentration of glycerol. It also appears that the mechanism of uptake is not saturated by concentrations as high as 200 mM, the results expected of diffusion.

The concentration dependence of glycerol efflux was examined next. Cells were loaded with different concentrations of ^{3}H-glycerol, washed free of extracellular isotope, and placed in medium at 37 °C to allow the intracellular ^{3}H-glycerol to escape from the cells. The velocity calculations were based upon 60-sec incubations, an interval which should provide exit rates in the absence of significant reentry of the isotope. Reentry was made unlikely by the fact that the volume of the surrounding medium was about 1 000 times larger than the total intracelluar water volume. There also seemed to be no problem with formation of boundary layers around the cells. For example, the same efflux kinetics were observed when the medium over the cells was changed once every 10 sec or once every 4 min. Even when extracellular diffusion was slowed sixfold by increasing the viscosity of the medium with 9% hydroxyethyl starch, there was no effect upon the rate of efflux (Dooley, 1980). Figure 4 summarizes the results, and it appears that efflux is directly proportional to the intracellular concentration of glycerol up to a concentration of 8 mM.

If CHO cells had a facilitated diffusion mechanism with a low K_m, its presence might well be obscured by the simultaneous simple diffusion of glycerol, especially at higher concentrations. However, this is made unlikely by Fig. 5 which shows that the same proportionality between flux and glycerol concentra-

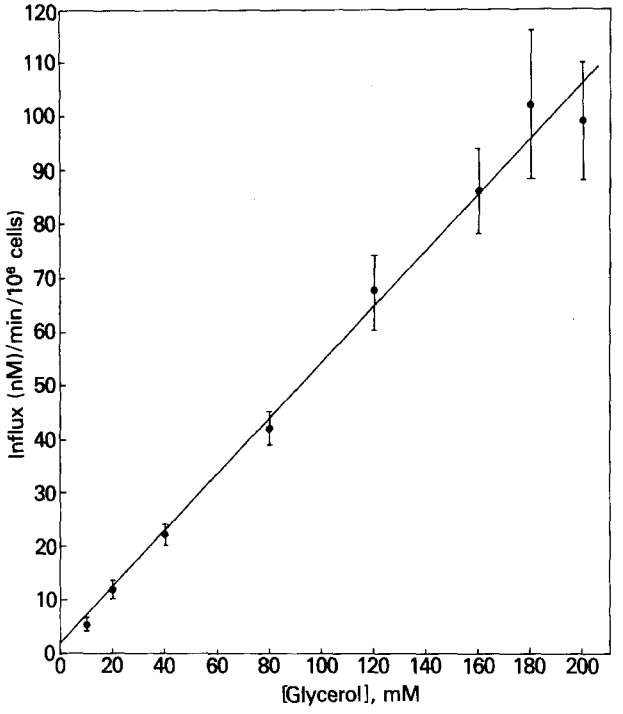

Fig. 3. The influx of glycerol as a function of extracellular glycerol concentration. Culture dishes containing a known number of cells were exposed for 60 sec to 37 °C medium containing ^{3}H-glycerol of known specific activity. The monolayers were washed with PBS plus isosmotic glycerol and the number of nM of glycerol taken up per min per 10^6 cells calculated from the amount of intracellular label. The calculations included a correction for the amount of intracellular isotope lost during the rinse. Bars indicate standard deviation. The line was drawn by linear regression, with $r=0.995$

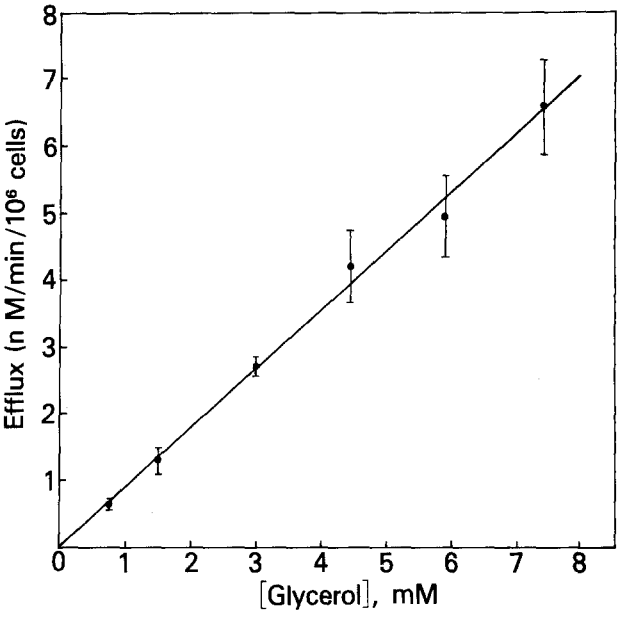

Fig. 4. The efflux of glycerol as a function of intracellular glycerol concentration. Cultures were equilibrated with the indicated concentrations of ^{3}H-glycerol, washed free of extracellular label with 37 °C PBS and placed in glycerol free medium at 37 °C. After 60 sec, the medium was harvested to determine the amount of tritium which had escaped from the cells. Velocity calculations took into account the fact that the pre-zero time 37 °C rinse caused the intracellular concentration to drop 26%. Bars indicate standard deviation. The line was drawn by linear regression, with $r=0.997$

tion extends down to or below the micromolar range for both influx and efflux.

In the flux experiments shown in Figs. 3–5, glycerol was present on only one side of the membrane. As a result, the velocity measurements were obtained under somewhat hypertonic (influx) or hypotonic (efflux) conditions. From Figs. 3 and 5, it appears that extracellular osmolalities as high as 500 mOsm (200 mM glycerol) had little appreciable effect upon the velocity of uptake. In contrast, it was found that when cells containing more than 30 mOsm of glycerol were transferred to glycerol-free medium, there was a disproportionate increase in the rate of outflow, presumably because of transient swelling and membrane leakage (data not shown). Therefore, the efflux data of Figs. 4 and 5 were limited to concentrations less than or equal to 10 mM. In a separate series of experiments, glycerol flux was conducted under isosmotic conditions (equilibrium exchange), experiments permitting the use of much higher glycerol concentrations. Under these conditions, even 1.3 M glycerol could not saturate the mechanism(s) of influx and efflux (Dooley, 1980).

While the preceding data are consistent with first-order diffusion of glycerol across the membrane, mediated transport cannot be eliminated on this basis alone. Thus, the mechanism of permeation was further characterized. Countertransport is considered to be a diagnostic property of facilitated diffusion (Stein, 1967; Kotyk & Janacek, 1970; Christensen, 1975). For example, CHO cells have a facilitated diffusion system for uridine and, as expected, carry out countertransport of uridine (Plagemann, Marx & Wohlhueter, 1978). The possibility that CHO cells might show the same phenomenon with glycerol was investigated. Cells containing either 0 or 10 mM glycerol were exposed to 1, 50, or 1000 μM ^{3}H-glycerol and the influx of label followed. It was apparent that preloading the interior of the cell with glycerol did not accelerate the rate of uptake (data not shown). Thus, at least within this concentration range, no evidence for countertransport could be obtained.

Transport systems may be rather sensitive to changes in temperatures. For example, the Q_{10} for 2-deoxyglucose uptake by Novikoff rat hepatoma cells is 2.5 (Renner, Plagemann & Bernlohr, 1972) while val-

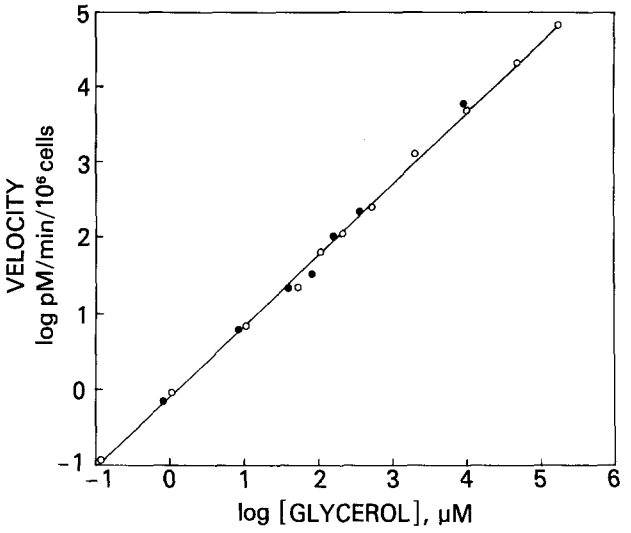

Fig. 5. Glycerol influx and efflux at low and high glycerol concentrations. Influx and efflux experiments were carried out as described in Figs. 3 and 4. Influx data (○) are plotted as a function of extracellular glycerol concentration, while efflux data (●) are plotted against the intracellular glycerol concentration at zero time. Influx was measured after 60 sec uptake, while efflux was measured after 30 sec outflow

Table 1. Effect of inhibitors on glycerol and uridine uptake by CHO monolayer cultures

	CPM ^{3}H/dish	
	Uridine	Glycerol
KCN + NaIodoacetate[a]	–	857
Control	–	748
PCMBS[b]	210	760
Control	10,860	550
Persantine[c]	155	365
Control	5,450	278

[a] Cells were treated with or without (controls) 5 mM KCN + 5 mM NaIodoacetate for 10 min prior to addition of ^{3}H-glycerol to a final concentration of 1 mM (2.5 μCi/ml). After 30 sec incubation (37 °C) uptake was terminated by washing the monolayer in 0° PBS. Intracellular isotope was determined as described in Materials and Methods.

[b] Cells were exposed to serum free RPMI 1640 containing or lacking (controls) 50 μM PCMBS for 10 min at 37 °C. Cells were then exposed to 1 mM ^{3}H-glycerol (2.5 μCi/ml) or 1 μM ^{3}H-uridine (2 μCi/ml) in serum-free 1640 with or without PCMBS for 60 sec at 24 °C. Uptake was terminated as above.

[c] Cells were exposed to 1 μM (2 μCi/ml) ^{3}H-uridine or 1 μM (2.5 μCi/ml) ^{3}H-glycerol in the presence or absence (controls) of 20 μM Persantine in standard growth medium at 37 °C. After 30 sec, uptake was terminated as above.

ues of 3.7, 3.5, and 2.9 were found for the nonmediated uptake of L-glucose, cytosine, and prednisolone (Graff, Wohlhueter & Plagemann, 1977). The temperature dependence of glycerol permeation was found by measuring the initial velocity of uptake of 100 μM glycerol at 2 and 37 °C. In seven trials, the velocity of uptake fell an average of 3.5 (±1.3) fold over 35 °, the equivalent of a Q_{10} of 1.41 ± 0.15, close to the expected value for diffusion through a lipid bilayer (Christensen, 1975).

Inhibitors

A number of agents have well defined inhibitory activity towards transport systems, and their effects upon glycerol flux were examined. When cells were depleted of ATP by a 10 min exposure to 5 mM KCN plus 5 mM Na-iodoacetate (Wohlhueter et al., 1976) the initial velocity of uptake of 1 mM glycerol was virtually the same as that in untreated control cells (Table 1). It appears that cells do not require ATP to take up glycerol from the surrounding medium. Parachloromercuribenzene sulfonic acid (PCMBS) is an alkylating agent known to inhibit nucleoside transport in CHO cells (Plagemann et al., 1978). A 10-min preincubation of 50 μM PCMBS inhibited the rate of uridine uptake approximately 50-fold, while having little or no effect on glycerol flux (Table 1).

Persantine (dipyridamole) inhibits uptake of monosaccharides by human erythrocytes. It also slows the influx of 2-deoxyglucose and uridine into rat hepatoma cells (Renner et al., 1972; Plagemann et al., 1978). However, Persantine does not influence glycerol uptake by CHO cells: a concentration sufficient to depress uridine influx 35-fold had no effect on glycerol permeation (Table 1).

The glycerol facilitated diffusion system of human erythrocytes is quite sensitive to pH changes, membrane permeability decreasing 30-fold when the pH is lowered from 6.5 to 5.5 (Carlsen & Wieth, 1976). Quite different results are obtained with CHO cells. In five experiments, glycerol influx at pH 5.9 measured after 60 sec of uptake was 70% (±15%) that of the pH 7.4 control. Influx under alkaline conditions was 108% (±7%) that of the pH 7.4 cultures.

Although the possibility cannot be completely excluded, on the basis of the preceding data it appears unlikely that CHO cells possess a transport system for glycerol. Presuming, therefore, that glycerol enters the cell by nonmediated diffusion, experiments were conducted to further characterize that process.

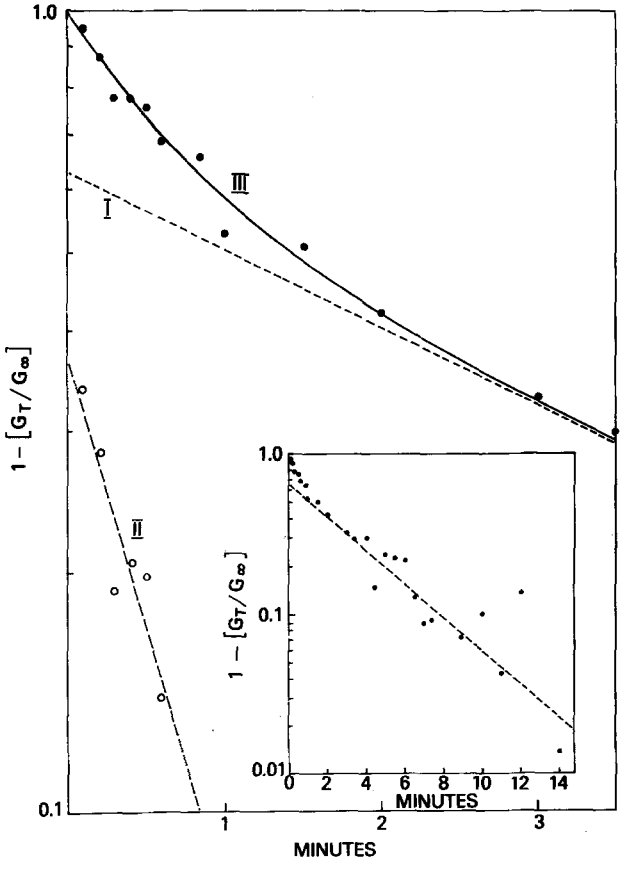

Fig. 6. Biphasic kinetics of glycerol influx in monolayer. Monolayer cultures were exposed to 1.5 ml of medium at 37 °C containing 1 mM ^{3}H-glycerol (1 μCi/ml). The experiment was carried out as described in Fig. 1, but with a higher sampling frequency. A straight line was constructed through the 2–14 min time interval by linear regression (inset) and extrapolated to zero time (curve *I*). The difference between the experimental data (•) and curve *I* were plotted (○) and curve *II* drawn by linear regression. Simple addition of *I* and *II* gives the solid line (curve *III*). $T_{\frac{1}{2}}$ values are 3.13 and 0.45 min for curves *I* and *II*, respectively. The ordinate is the same as defined in Fig. 2

Table 2. First order components of biphasic flux curves from monolayer cultures

	$T_{\frac{1}{2}}$ (min) ± SD		*Y*-Intercept ± SD	
	Influx[a]	Efflux[b]	Influx[a]	Efflux[b]
Curve *I*	2.85 ± 0.38	5.21 ± 0.23	0.655 ± 0.114	0.088 ± 0.009
Curve *II*	0.59 ± 0.21	0.95 ± 0.13	0.308 ± 0.113	0.850 ± 0.34

[a] Data based on eight experiments using glycerol concentrations ranging from 1 μM to 1.5 M as described in Fig. 6.
[b] Data based on five experiments using 1 mM glycerol as described in Fig. 7.

Kinetics of Influx

In carrying out influx experiments as described in Fig. 2, it was found that $[1 - G_T/G_\infty]$ did not always extrapolate to a value of 1.0 at zero time. This prompted a closer analysis of influx with special emphasis on the first 60 sec of exposure to the isotope. The inset of Fig. 6 displays the full time course of 1 mM ^{3}H-glycerol influx. The superior resolution afforded by the increased sampling frequency during the earliest stages of uptake shows that influx is actually a biphasic process. Linear regression was used to construct a straight line (line *I*) through the 2–14 min time interval. A discrepancy between that line and the data points collected over the first 60 sec is readily apparent. Values on line *I* were subtracted from the data points covering the first 36 sec of uptake, the differences plotted (open circles), and line *II* constructed by linear regression. The addition of functions *I* and *II* yields line *III*, a curvilinear function which agrees reasonably well with the observed data. It therefore appears that the uptake of 1 mM glycerol is a biphasic process which can be described by the summation of two first-order processes.

Since influx assays were performed with stationary culture dishes, it seemed possible that the biphasic kinetics might be attributable to "unstirred layers". To test this hypothesis, the dishes were placed on a rotary platform so that the medium could be constantly swirled over the monolayers during influx. It was found that under these conditions, glycerol uptake was still biphasic.

The generality of these observations were tested by examining extracellular glycerol concentrations ranging from 1 μM to 1.5 M. In some cases, cells were pre-equilibrated with isosmotic glycerol for the purpose of either avoiding osmotic stress or observing equilibrium exchange. In each instance, the influx curve was biphasic, and closely resembled Fig. 6. Table 2 summarizes eight independent influx experiments: the faster component had a $T_{\frac{1}{2}}$ of 0.59 ± 0.21 minutes and a *Y*-intercept of 0.308 ± 0.113. The slower component had a $T_{\frac{1}{2}}$ of 2.85 ± 0.38 min and a *Y*-intercept of 0.655 ± 0.114. The significance of these two components is explored below.

Kinetics of Efflux

There are at least two factors which limit the precision of the influx data. First, the assay requires as many as 35 replicate culture dishes, and variation in the number of cells residing on each dish necessarily contributes to experimental error. Second, data points lying on or scattered above the plateau value (Fig. 1) cannot be included in the semilogarithmic plot (Fig. 2) because their values are zero or negative. As

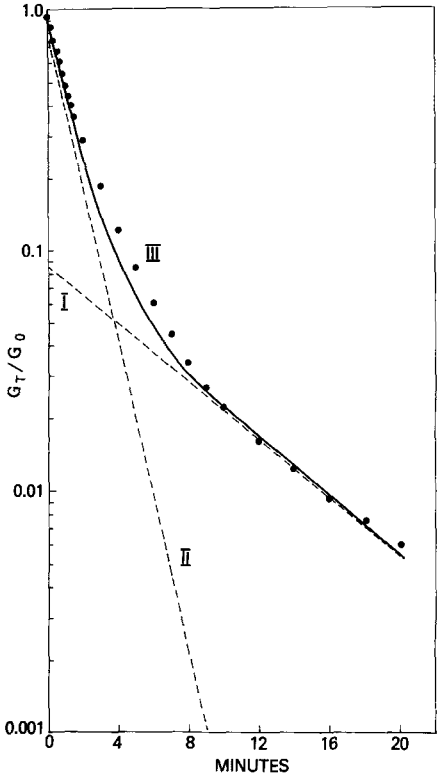

Fig. 7. Biphasic kinetics of glycerol efflux in monolayer. Cells containing 1 mM ^{3}H-glycerol (10μCi/ml) were rapidly washed and transferred to fresh glycerol free medium at 37 °C. The rate of efflux was determined by sampling the medium for the appearance of label. A straight line was constructed through the 9–20 min time interval by linear regression (curve *I*). The values of curve *I* were subtracted from the experimental data between 0.16 and 2.0 min, and the differences used to construct curve *II* by regression. Curve *III* (solid line) represents the sum of *I* and *II*. The $T_{\frac{1}{2}}$ values are 5.14 and 0.93 min for curves *I* and *II*, respectively. G_T = CPM ^{3}H-glycerol/dish at time *T*. G_o = CPM ^{3}H-glycerol/dish at zero time

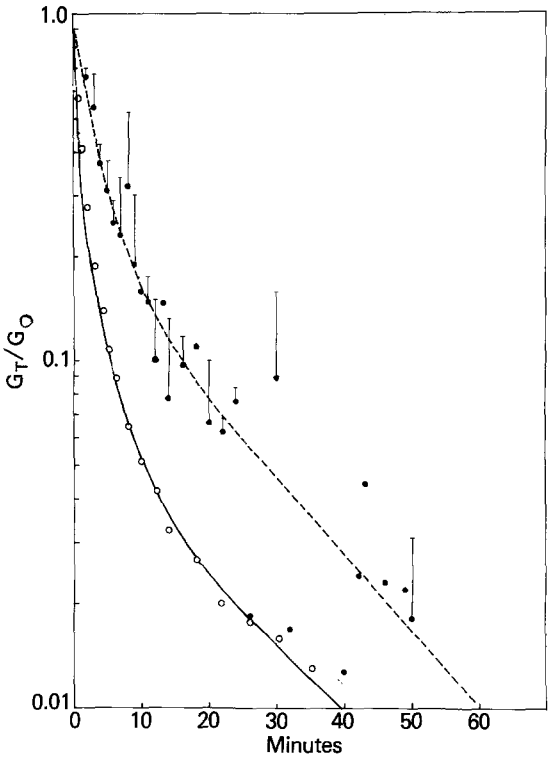

Fig. 8. Efflux of glycerol from cells in suspension or in monolayer. *Suspension:* Cells were equilibrated with 1 mM ^{3}H-glycerol, and then transferred to glycerol-free medium (27 °C) using Method *I* (two experiments) or Method *II* (three experiments). See Materials and Methods. The efflux of glycerol into the suspending medium was monitored by filtering aliquots through nitrocellulose discs to determine the amount of ^{3}H remaining within the cells. Methods *I* and *II* yielded equivalent results. Each point (●---●) represents the average of 2–5 separate trials. Standard deviation bars are included for points averaging three or more trials. *Monolayer:* Cells were loaded with 1 mM ^{3}H-glycerol, washed and placed in 27 °C glycerol-free medium. The course of efflux was followed as in Fig. 7 (○—○). For the suspension assays, G_T = CPM intracellular ^{3}H-glycerol per unit sample at time *T* and G_o = CPM intracellular ^{3}H-glycerol per unit sample at zero time. For the monolayer assay, G_T and G_o are the same as defined in Fig. 7

a result, Fig. 2 may underestimate the uptake rate during the final approach to equilibrium. However, both of these problems are avoided in studies of efflux. This is because the entire time course of efflux is obtained from a single culture dish and the fact that none of the data are excluded from the semilogarithmic plot. As a result, the kinetics of efflux may be followed for a longer period of time and with greater accuracy.

Figure 7 shows the efflux of 1 mM ^{3}H-glycerol into glycerol-free medium. The time course of glycerol efflux is clearly biphasic. As described above for influx, two exponential functions (*I* and *II*, Fig. 7) were extracted, the sum of which (line *III*, Fig. 7) agrees closely with the experimental data. Five similar experiments are summarized in Table 2.

The one way entry and exit rates may be obtained by examining the initial slope of curve *III* in Figs. 6 and 7. It is apparent that the slopes are virtually identical for the first 30–60 sec ($T_{\frac{1}{2}}$ = 1.15 and 0.95 min for influx and efflux, respectively). That is, influx of 1 mM glycerol initially proceeds at the same velocity as does the efflux of 1 mM glycerol. However, after the first minute of flux, the uptake and exit curves diverge. For example, inspecting curve *III* in Fig. 6, one sees that during the influx of glycerol, the intracellular concentration reaches 90% of its equilibrium value after 8 min. In contrast, efflux re-

quires only 4 min to reach 90% completion (curve *III*, Fig. 7). These differences are reflected in the different $T_{\frac{1}{2}}$ and *Y*-intercept values for components *I* and *II* for influx and efflux (Table 2).

All of the permeation experiments carried out thus far utilized monolayer cell cultures. It therefore was of interest to see how cell attachment influenced the kinetics of glycerol flux. Towards that end, cells in suspension were loaded with 1 mM ^{3}H-glycerol and then transferred to glycerol-free medium (27 °C) either by direct dilution or after centrifugation and removal of the radioactive extracellular medium. Figure 8 displays the data from five independent efflux experiments, and it appears that the exit of glycerol from suspended cells is once again biphasic. However, the rate of outflow seemed less than predicted for the temperature used (27 °C). Accordingly, the time course of efflux was examined at 27 °C in monolayer. Figure 8 includes these data, and it appears that glycerol efflux is indeed slower in suspension than monolayer. A similar observation was made by Pofit and Strauss (1977) who noted that rabbit macrophages transported both adenosine and lysine more slowly in suspension than in monolayer.

Preliminary data indicate that CHO cells are not unique in presenting biphasic flux curves. When purified populations of human granulocytes are added to tissue culture dishes, they attach to form monolayers. The kinetics of glycerol permeation can then be determined in the same way as for CHO cells. It was found that granulocytes are approximately three times more permeable than the CHO cells but still exhibit the same biphasic pattern for influx and efflux. Further experiments will be necessary to determine the generality of this observation.

Discussion

Preliminary experiments examining the uptake of glycerol by CHO cells indicated that at equilibrium, the intracellular concentration of glycerol was equal to that of the surrounding medium (Dooley, 1980). Thus, active transport of glycerol seemed unlikely. The purpose of the present paper was to determine whether the permeation of glycerol was due to a nonconcentrative facilitated diffusion mechanism or if instead glycerol passed through the membrane by nonmediated diffusion.

Determining the permeation kinetics of glycerol required prolonged exposure of the cells to isotope. Conversion of the labeled molecules to other metabolites was a possible complication. For example. when cells in monolayer were loaded with ^{3}H-glycerol and then used in efflux experiments, 96% of the label readily escaped from the cells while the remaining 4% seemed to be permanently cell-associated. However, the reduced permeability of the tritium labeled molecules was apparently not metabolic conversion, but seemed instead to be due to sequestration of ^{3}H-glycerol in one or more poorly permeable intracellular compartments. Consistent with this explanation is the fact that the poorly permeable fraction constituted 4% of the total intracellular isotope at all starting concentrations between 1 μM and 1.50 M. Furthermore, since the shape of the biphasic efflux curve was independent of the starting intracellular glycerol concentration, it seems unlikely that a significant fraction of the label exiting from the cells represented metabolic products of ^{3}H-glycerol. Finally, there was no indication that influx measurements were distorted by incorporation of ^{3}H-glycerol since the plateau value did not increase with time (Fig. 1).

With regard to efflux in suspension, approximately 20% of the label was converted to the "poorly permeable" state. This result was found with 1 and 200 mM glycerol. Once again, intracellular trapping seems the most likely explanation for the long term cell association of the isotope. It is not clear why five times more glycerol is sequestered in suspension than in monolayer.

An examination of the concentration dependence of glycerol flux (Fig. 5) showed that the velocity of uptake was directly proportional to the extracellular glycerol concentration. The same direct proportionality was found between efflux and the intracellular glycerol concentration. In fact, entry and exit velocities measured during the first 60 sec of flux fall on the same line (Fig. 5). Thus, for a given concentration, the initial rate of migration through the membrane must be the same in both directions. This conclusion is supported by the full time course of glycerol permeation (Figs. 6 and 7) where it was noted that the slope of curve *III* was initially the same for influx and efflux. These data are consistent with the inability of the cells to accumulate glycerol against a concentration gradient.

It should be pointed out that while efflux and influx are initially identical, the full time courses are quite different. This explains why exit and entrance rates are equivalent in Fig. 5, whereas the exponential components for influx and efflux are different in Table 2.

As a first approximation, the inability to saturate the glycerol permeation mechanism suggests that glycerol crosses the membrane by diffusion (Stein, 1967). However, Christensen (1975) has stated that such a

conclusion might be unwarranted since similar kinetics could be produced by a facilitated transport system with a very high K_m. For example, the K_m for glycerol transport in human erythrocytes, is approximately 0.5 M (Carlsen & Wieth, 1976). In order to avoid misinterpretations, very high concentrations should be used to approach and preferably surpass the K_m. Christensen noted, however, that the high concentrations required might create an environment so unphysiological that data become unreliable as a result. While Christensen may be correct, it is still noteworthy that the velocity of uptake determined by the rapid sampling technique (as seen in Fig. 6) was directly proportional to glycerol concentration from 1 μM to 1.5 M. Thus, no evidence for saturability could be found.

That glycerol probably does traverse the membrane by nonmediated diffusion is supported by other experiments. For example, migration through the membrane is relatively insensitive to changes in temperature and pH. Countertransport is considered a strong indicator of mediated uptake (Stein, 1967), and no evidence for this process could be found. In addition, reagents such as Persantine and PCMBS, known to interfere with facilitated diffusion, had no effect upon glycerol flux. Competition experiments would have been useful in characterizing glycerol permeation. However, this approach could not be utilized because competitive analogs would have been required at extremely high concentrations, given the previously mentioned inability to saturate the flux at concentrations of glycerol as high as 1.5 M.

The most likely conclusion from the foregoing data is that glycerol enters into and escapes from the cells by nonmediated diffusion. If true, how does one explain the biphasic kinetics of permeation? Conceivably, biphasic kinetics in monolayer could be attributed to the involvement of the extracellular space lying between the cell and its substratum. Such a space could act as a second compartment with its own permeability characteristics. However, that possibility seems unlikely since the kinetics are biphasic in suspension. Furthermore, experiments testing the influence of sampling frequencies, medium viscosity, and cell agitation all indicated that the presence of unstirred layers is not a likely explanation. Finally, the fact that the same kinetics are observed at concentrations ranging from 1 μM to 1.5 M indicates that the process generating biphasic kinetics is nonsaturable, and probably eliminates glycerol-derived metabolites as contributing factors. It therefore seemed appropriate to ask whether the permeation kinetics could be explained in terms of simple first-order processes.

Biphasic Kinetics

Figures 6 and 7 each display a pair of exponential functions (*I* and *II*) the sum of which (*III*) closely approximates the experimental data. This suggests that a CHO cell resembles a two-compartment system. The question immediately arises as to whether the compartments are in parallel or in series. Before answering this question, it is necessary to briefly consider the flux kinetics for a single compartment. In the case of efflux, assume a compartment contains ^{3}H-glycerol (G_i, CPM/ml) and that the glycerol diffuses into a vastly larger reservoir such that reentry of the label is negligible. In this case, the rate of change of G_i is given by

$$\frac{dG_i}{dt} = -kG_i \tag{1}$$

or

$$\frac{G_{i,t}}{G_{i,t=0}} = e^{-kt}. \tag{2}$$

For influx, consider the entry of extracellular ^{3}H-glycerol (G_o, CPM/ml) from a reservoir such that G_o is virtually constant. The rate of inflow is the net difference between the one-way rates of entrance and exit which in turn is proportional to the concentration difference across the membrane. Therefore,

$$\frac{dG_i}{dt} = k\,(G_o - G_i) \tag{3}$$

or

$$1 - \frac{G_{i,t}}{G_o} = e^{-kt}$$

and since at equilibrium, $G_o = G_{i,t=\infty}$

then

$$1 - \frac{G_{i,t}}{G_{i,t=\infty}} = e^{-kt}. \tag{4}$$

Equation (4) was used to plot the influx data (Figs. 2 and 6) and Eq. (2) was used for the efflux data (Figs. 7 and 8).

The rate constant reflects the permeability properties of the membrane, and it is not unreasonable to assume that in the case of diffusion, the rate constant is the same in both directions across the membrane.

Therefore, if k were the same in Eqs. (2) and (4), the kinetics of influx and efflux must be identical for a diffusion-limited single compartment system.

Consider now two separate compartments each of which communicates with a reservoir, i.e., two chambers in parallel. Each chamber is characterized by its own first-order rate constant for glycerol diffusion. Since each compartment acts independently of the other, the kinetics of diffusion into both compartments are obtained by adding together the influx equations for the individual compartments (Eq. (4)). Similarly, the kinetics of simultaneous efflux from both compartments are described by adding together the efflux equations for the two separate compartments (Eq. (2)). In both cases, biphasic kinetics are obtained. In this model, the rate constants for glycerol diffusion are the same in both directions across the membrane. As a result, the kinetics of influx and efflux are the same for each individual compartment (*see* above). It therefore follows that the biphasic kinetics of influx into both chambers must be the same as the biphasic kinetics of efflux out of both chambers. Reference to Figs. 6 and 7 and Table 2 shows immediately that this model is not applicable because the kinetics of influx and efflux are actually different. One may therefore conclude that CHO cells do not contain two separate and independent glycerol pools.

This, however, still leaves open the possibility that a different arrangement of the two compartments might generate the required biphasic kinetics. Kotyk and Janacek (1970) analyzed the efflux kinetics of an isotope from two compartments, into a reservoir when one compartment was contained entirely within the other (e.g., nuclear compartment and cytoplasmic compartment). They derived an expression which is shown below in highly simplified form

$$\frac{G_{i,t}}{G_{i,t_o}} = Me^{r_1 t} + Ne^{r_2 t} \tag{5}$$

where r_1 and r_2 represent different expressions, each containing four rate constants. The rate constants also appear in coefficients M and N. The equation describing influx is more complicated as it requires five rate constants. Nevertheless, the exponents r_1 and r_2 have the same values for influx and efflux (Dr. Charles Glinka, *personal communication*). In other words, when two compartments are arranged in series, the two exponential functions should have the same slopes for influx and efflux. Reference to Table 2 shows that the slopes actually differ by a factor of approximately two. Therefore, this model is also inadequate at explaining the experimental results.

To summarize, the observed kinetics of glycerol influx and efflux are described by different pairs of first-order equations. It can be shown that the data are not consistent with a two-compartment model with chambers in series or in parallel. It therefore remains uncertain what subcellular structures or processes the descriptive first-order equations are based upon. Presumably, diffusion across the plasma membrane is one of the rate limiting steps for both influx and efflux. If this were the principle event measured at the beginning of glycerol flux, it would explain why the *initial* rates of inflow and outflow were the same. Clearly though, there is not an exponential function common to both influx and efflux (Table 2) which might represent diffusion through the plasma membrane. A possible reason for this is provided by the analysis of Kotyk and Janacek (1970). They showed that in complex rate expressions (abbreviated in Eq. (5)) the exponential terms r_1 and r_2 can include rate constants for several rate limiting steps. Therefore, each derived function (*I* or *II*) may not represent a unique step or compartment in glycerol permeation.

In conclusion, glycerol permeation of CHO cells is not a mediated process. Instead, glycerol appears to enter into and exit from the cells by diffusion. The kinetics of influx and efflux are clearly different, though both are biphasic. Two different pairs of first-order functions describe the kinetics of inflow and outflow. The precise nature of the rate limiting steps in glycerol diffusion remains uncertain.

This paper is Contribution No. 456 from the American Red Cross Blood Services-Bethesda. This work was supported in part by NIH Grant No. GM 17959 and BRSG No. 2S07RR05737.

References

Alemohammad, M.M., Knowles, C.J. 1974. Osmotically induced volume and turbidity change of *Escherichia coli* due to salts, sucrose, and glycerol with particular reference to the rapid permeation of glycerol into the cell. *J. Gen. Microbiol.* **82**:125

Bickis, I.J., Kazaks, K., Finn, J.J., Henderson, I.W.D. 1967. Permeation kinetics of glycerol and dimethyl sulfoxide in Novikoff hepatoma ascites cells. *Cryobiology* **4**:1

Carlsen, A., Wieth, J.O. 1976. Glycerol transport in human red cells. *Acta Physiol. Scand.* **97**:501

Christensen, H.N. 1975. Biological Transport. W.A. Benjamin, Reading (Mass.)

Collander, R. 1949. The permeability of plant protoplasts to small molecules. *Physiol. Plant.* **2**:300

Dooley, D.C. 1980. Glycerolization of Chinese hamster cells in monolayer cultures. *Cryobiology* **17**:338

Fabry, M.E., Eisenstadt, M. 1978. Water exchange across red cell membranes. II. Measurement by nuclear magnetic resonance T_1; T_2, and T_{12} hybrid relaxation. The effects of Osmolarity, cell volume, and medium. *J. Membrane Biol.* **42**:375

Giebel, F., Passow, H. 1960. Die Permeabilitat der erythrocyten membran für organische Anionen. *Arch. Geo. Physiol.* **271**:378

Graff, J.C., Wohlhueter, R.M., Plagemann, P.G.W. 1977. Effect of temperature and of crytochalasin B and persantin on the nonmediat permeation of nonelectrolytes into cultured Novikoff rat hepatoma cells. *J. Biol. Chem.* **252**:4185

Kotyk, A., Janacek, K. 1970. Cell membrane transport. Plenum Press, New York.

Li, C.C., Lin, E.C.C. 1975. Uptake of glycerol by tumor cells and its control by glucose. *Biochem. Biophys. Res. Commun.* **67**:677

Macey, R.I., Karan, D.M., Farmer, R.E.L. 1972. Properties of water channels in human red cells. *In:* Biomembranes. Vol 3. F. Krentzer and J.F.G. Slegard, editors. Plenum Press, New York

Plagemann, P.G., Marx, R., Wohlhueter, R.M. 1978. Uridine transport in Novikoff rat hepatoma cells. *J. Cell. Physiol.* **97**:49

Pofit, J.F., Strauss, P.R. 1977. Membrane transport by macrophages in suspension and adherent to glass. *J. Cell. Physiol.* **92**:249

Renner, E.D., Plagemann, P.G., Bernlohr, R.W. 1972. Permeation of glucose by simple and facilitated diffusion by Novikoff rat hepatoma cells. *J. Biol. Chem.* **247**:5765

Richey, D.P., Lin, E.C.C. 1972. Importance of facilitated diffusion for effective utilization of glycerol by *Escherichia coli*. *J. Bacteriol.* **112**:784

Siegers, M.P., Feinendegen, L.E., Lahiri, S., Cronkite, E.A. 1979. Proliferation kinetics of early hemopoietic precursor cells with self-sustaining capacity in the mouse, studied with 125-I labeled iodo-deoxyuridine. *Exp. Hematol.* **7**:469

Stein, W.D. 1967. The Movement of Molecules across Cell Membranes. Academic Press, New York

Wohlhueter, R.M., Marx, R., Graff, J.C., Plagemann, P.G. 1976. Application of rapid kinetic techniques to the transport of thymidine and 3-O-methylglucose into mammalian cells in suspension culture. *J. Cell. Physiol.* **59**:605

Received 11 February 1980; revised 12 May 1980