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Glycerol permeability in two species of *Peromyscus*

Greg Evan Raecker
University of the Pacific

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GLYCEROL PERMEABILITY IN
TWO SPECIES OF PEROMYSCUS

A Thesis Presented to the
Graduate Faculty of the
University of the Pacific

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Greg Evan Raecker

May 1977

This thesis, written and submitted by

Greg Evan Raecker

is approved for recommendation to the Committee
on Graduate Studies, University of the Pacific.

Department Chairman or Dean:

F. R. Menden

Thesis Committee:

F. R. Menden

Chairman

Jane Faulkner

Lee Christianson

Dated May 3, 1977

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INTRODUCTION

A large number of permeability studies have been carried out on the erythrocytes of many different animal species for several penetrating nonelectrolytes. A study by Jacobs, Glassman, and Parpart (1935) of the osmotic properties of the erythrocyte determined the temperature coefficients (Q_{10}) of hemolysis in solutions of certain nonelectrolytes for 11 species of mammals. The data revealed distinct differences in erythrocyte permeability between species. The erythrocytes of some species (rat, mouse, rabbit, man, and guinea pig) displayed a high permeability to glycerol, with hemolysis times ranging from 10 seconds to several minutes, while erythrocytes of other mammals (cat, dog, horse, pig, sheep, and ox) showed times from 15 to 20 minutes. The temperature coefficients of the first group were low. The guinea pig, rabbit, and mouse showed intermediate values of 1.63, 1.46, and 1.63, respectively, while in man and the rat the temperature coefficients tended to approach unity. Those species belonging to the second group had high values, ranging from 3.48 for the cat to 3.93 for the ox.

In 1938, Jacobs, et al. discussed differences in the permeability of the erythrocytes of rat and mouse to erythritol, mannitol, glycerol, and thiourea. Rat

erythrocytes were more permeable to glycerol than to thiourea, whereas the reverse was true for mouse erythrocytes. Mouse erythrocytes also displayed a high degree of permeability to erythritol. However, this same substance penetrated rat cells much more slowly.

Small but recognizable permeability differences were soon demonstrated among a number of species within a single genus. Levine (1943) investigated species differences in rates of osmotic hemolysis within the genus Peromyscus. The four species studied were: P. eremicus, P. fraterculus, P. leucopus noveboracensis, P. gossypinus palmarius, and P. t. truei. Consistent differences in hemolysis times were demonstrated and it was possible to identify each species with certainty.

Later experiments (LeFevre, 1948) suggested that glycerol and glucose did not enter the human red cell by simple diffusion, but rather that these penetrants were transported into the erythrocyte by an active metabolic system in which at least one essential link involved a sulfhydryl group.

Jacobs, et al. (1950) examined the inter-class erythrocyte permeabilities of mammals, birds, reptiles, amphibians, and fish to isosmotic solutions of urea, thiourea, glycerol, and ethylene glycol. Their results, which were generally consistent within a given class, indicated an unusually high permeability for glycerol and ethylene glycol in bird erythrocytes, and for urea in

mammalian red cells. It was also found that the permeability of chicken red blood cells differed noticeably from the rather uniform pattern seen in the erythrocytes of such species as the pigeon, herring gull, English sparrow, pheasant, and turkey.

In 1961, Hunter studied the effect of butanol on the permeability of human, rabbit, sheep, and chicken erythrocytes to glycerol, monoacetin, thiourea, and ammonium chloride. This work suggested that when the movement across the cell membrane depended on simple diffusion, butanol increased the permeability, but the effect of butanol on facilitated diffusion was inhibitory.

A subsequent investigation by Hunter, George, and Ospina (1965) in which n-butyl alcohol and tannic acid were used, provided further help in distinguishing between simple and facilitated diffusion systems. This work suggested that a carrier system was present for glycerol in the erythrocytes of the rabbit, mouse, and man.

During his study of hexose transport in beef erythrocytes, Laris (1967) noted considerable variation in the capacity of cells from different cattle to accumulate fructose both in the presence and in the absence of a metabolic inhibitor. Upon closer examination, it was shown that beef erythrocytes could be divided into two distinct groups on the basis of differences in the fructose transport systems.

In a recent study, Hunter (1976) added six small

mammals to the list of species in which a number of different nonelectrolytes penetrate the erythrocyte by facilitated diffusion. One of these six was the deer mouse (P. maniculatus), and it, like the other five species examined, was shown to have a carrier which is shared by glycerol and ethylene glycol. Hunter also obtained data which hinted at the possibility of permeability differences among separate populations of deer mice (Hunter, personal communication). Such findings prompted the present study of the permeability of red cells to glycerol in two different populations of the pinyon mouse (P. truei). In addition to this population comparison, the glycerol permeabilities of two closely related species, P. truei and P. californicus, were investigated.

MATERIALS AND METHODS

All mice used in this study were captured at Camp Chesebrough, Boy Scouts of America (Township 8S, Range 3W, Sections 1 and 12) in the Santa Cruz Mountains, Santa Cruz County, California. This region can be characterized as a Redwood - Mixed Evergreen forest (Munz, 1959). At higher elevations the predominant plant species are: Tanbark-Oak (Lithocarpus densiflora), Madrone (Arbutus Menziesii), Douglas Fir (Pseudotsuga Menziesii), Giant Chiquapin (Castanopsis chrysophylla), California Bay (Umbellularia californica), Big Leaf Maple (Acer macrophyllum), Oak (Quercus sp.), Mountain Dogwood (Cornus Nuttallii), Blue Brush (Ceanothus thyrsiflorus), and California Lilac (Ceanothus Parryi). The following species are common at lower elevations: Redwood (Sequoia sempervirens), Douglas Fir, Tanbark Oak, Redwood Sorrel (Oxalis Oregana), and Sword Fern (Polystichum munitum).

Specimens were collected in Sherman live traps baited with rolled oats along a road at an elevation of approximately 2380 feet, and along Oil Creek at approximately 1240 feet elevation. These two trapping sites were separated by a distance of about one-half mile. P. truei and P. californicus were captured concurrently at each of these sites. All mice were transported to the

University of the Pacific campus in Stockton, California, where they were maintained in cages with a constant supply of Purina lab chow and water until selected for experimental use. A total of 24 P. truei and 12 P. californicus were used in this study.

Blood was obtained by decapitation, with heparin as an anticoagulant. The cells were washed three times in a 1% sodium chloride solution buffered to pH 7.5 (6.05 g Tris plus 3.45 ml concentrated hydrochloric acid per liter). Following each centrifugation, the supernatant and buffy layer were removed by aspiration. After the third centrifugation, 0.5 ml of the packed red cells were suspended in 5.0 ml of 200 or 400 mM glycerol. Fifteen minutes were allowed for this cell suspension to reach equilibrium.

Volume changes of the cells were measured in a densimeter (Mawe, 1956). A temperature of 25° C was maintained by circulating water from a constant temperature bath through a jacket which surrounded the chamber of the densimeter. A beam of light passed through the water jacket and densimeter chamber, falling upon a photoelectric cell cathode located opposite the light source. This photocell was connected to a D.C. amplifier and, in turn, to an Esterline Angus pen recorder. The shrinking or swelling of the suspended erythrocytes results, respectively, in a decrease or increase in the level of

light transmission into D.C. current. This current is then amplified by the D.C. amplifier, and transmitted to the pen recorder which records on moving paper the variations in voltage, which are proportional to variations in current. The sensitivity of the system can be altered by changes in the concentration of the erythrocyte solution, by changing the settings of the D.C. amplifier, or by varying the light intensity.

An aliquot (0.25 ml) of the erythrocyte suspension was added to the chamber of the densimeter, in which had previously been placed 10 ml of a 1% or 1.5% sodium chloride solution containing increasing amounts of 200 or 400 mM glycerol. The external glycerol concentrations of the 200 mM system were: 4.6, 9.5, 14.3, 18.7, and 23.1 mM. For the 400 mM system, the concentrations were: 9.2, 28.7, 48.2, 67.7, and 87.2 mM.

The data obtained from measuring the shrinking of the cells were analyzed by the method of Sen and Widdas (1962), which is based on an equation described earlier by Widdas (1954). That equation is as follows:

$$\frac{dS}{dt} = K \left(\frac{C}{C + \phi} - \frac{S/V}{S/V + \phi} \right) \quad (1)$$

in which S = amount of penetrant in the cells, t = time, C = external concentration of penetrant, V = volume of cell water, ϕ = value of half-saturation constant of the carrier, and K = maximum transport rate.

If the values of ϕ and concentrations are small, substitutions and simplification can be made in the above equation to give the following:

$$t = \frac{(S_i + \phi)(C + \phi)}{\phi K} \quad (2)$$

where S_i = initial amount of penetrant in the cells. This equation suggests that there should be a linear relationship between the exit times and external concentration of penetrant, and that when the exit time equals zero, the external concentration is equal to $-\phi$, the half-saturation constant.

Miller (1965) proposed a method for the determination of the half-saturation and maximum transport rate constants which is similar to that of Widdas, but does not require small values for either ϕ or the concentrations of penetrant. If concentrations of penetrant and ϕ are not small, he suggests that exit times should be multiplied by the following factor:

$$\underline{A} = \frac{E(E + \bar{C} - C_0)}{(E - C)(E + \bar{C})} \quad (3)$$

in which isotonic units are used and E = external concentration of the non-penetrating species, \bar{C} = external penetrant concentration, and C_0 = internal penetrant concentration at $t = 0$.

A plot of $\underline{A}t$ (ordinate) against C (abscissa) should

give a straight line, and when \underline{At} equals zero, C should equal $-\phi$, the half-saturation constant.

In addition, Miller has shown that:

$$K = \frac{\phi + C_0}{t (E + C_0)} \quad (4)$$

(Miller's symbols have been altered to conform with those used by Sen and Widdas.) Thus, it is possible to determine both the half-saturation constant and maximum transport rate from a plot of exit times versus external concentrations of penetrant.

A record was obtained as the glycerol moved out of the cells. A tangent was drawn to the initial, steep portion of each shrinking curve, and the time was noted when this tangent intersected a horizontal line drawn through the equilibrium volume. These times were then plotted against external concentrations of penetrant. Using the method of least squares, a straight line was drawn through these points, and the intercepts and their 95% confidence limits were calculated by methods outlined by Sokal and Rohlf (1969). A reasonable approximation of the range of possible K 's was obtained by the substitution of these values for the variables t and ϕ in equation (4). The slopes of the regression lines were compared by the method described by Simpson, Roe, and Lewontin (1960).

RESULTS

Figure 1 shows a series of typical shrinking curves. The initial portion of these curves is linear. As described previously, times were measured from the curves, multiplied by Miller's A , and plotted against external concentration. Using the method of least squares, a straight line was drawn through these points, and values of ϕ and K were calculated. These data, the 95% confidence limits of ϕ 's, and the approximated ranges of all possible K 's are presented in Table 1. The tests used to compare the slopes of the regression lines showed that the difference between the 200 and 400 mM glycerol regressions in P. truei was not significant. However, the difference between these same two regressions in P. californicus was shown to be significant at the 0.001 level.

Figures 2 and 3 display statistically the data obtained from the 200 and 400 mM glycerol systems for P. truei and P. californicus, respectively. Figure 2 includes regression lines for the 200 and 400 mM glycerol systems, as well as for the combination of all (i.e. 200 and 400 mM) P. truei data. However, Figure 3 shows only a regression line for the 400 mM glycerol system in P. californicus. This discrepancy, and the absence in Table 1 of values for ϕ and K for 200 mM glycerol in this same

species, are explained in the Discussion.

A graph depicting the data obtained from the comparison of two populations of P. truei is not included. These results are also discussed in the next section.

TABLE 1. Values for half-saturation (ϕ) in millimoles and maximum transport rate (K) in isotones per minute for Peromyscus truei and P. californicus as calculated by the Miller method. The upper and lower 95% confidence limits of ϕ are indicated directly below the single value given for half-saturation. Similarly, the range of possible K's is indicated below the single calculated value for maximum transport.

TABLE 1

Experimental System	ϕ	K
<u>P. truei</u> /200 mM glycerol	71.4 10.9 to 384.3	2.02 1.34 to 5.31
<u>P. truei</u> /400 mM glycerol	29.5 -14.0 to 74.9	6.95 5.15 to 9.72
<u>P. truei</u> /combined data (200 + 400 mM)	40.7	*
<u>P. truei</u> /400 mM glycerol upper trapping site	29.8 -20.5 to 84.3	6.70 4.40 to 11.4
<u>P. truei</u> /400 mM glycerol lower trapping site	28.9 -11.2 to 72.2	7.11 4.90 to 11.4
<u>P. californicus</u> /400 mM glycerol	32.6 7.8 to 59.7	3.86 3.10 to 4.87

FIGURE 1. Shrinking of Peromyscus truei erythrocytes equilibrated with 400 mM glycerol in 1% NaCl when added to 1.5% NaCl with increasing concentrations of glycerol outside the cells as indicated on each curve.

FIGURE 1

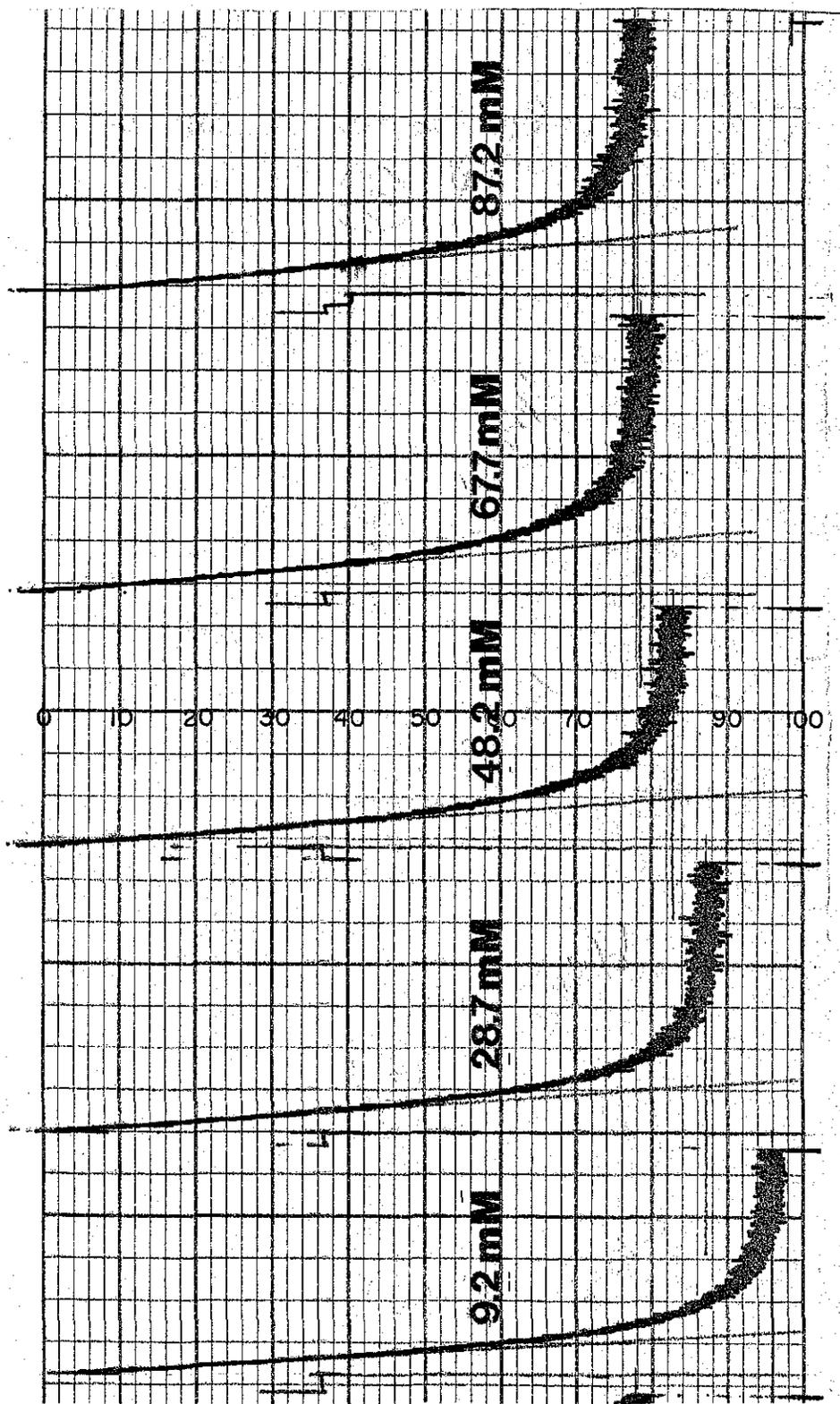


FIGURE 2. Exit times of 200 and 400 mM glycerol in the erythrocytes of Peromyscus truei. Vertical lines show observed ranges. Shaded rectangles mark standard deviations of the 400 mM data. Unshaded rectangles mark standard deviations of the 200 mM data. The means are indicated by a horizontal line, and the number of time measurements at each external concentration is shown in parentheses. The data displayed for the lowest external penetrant concentration of the 400 mM system (i.e. 9.2 mM) have been shifted slightly to the left. Regression lines a, b, and c are those calculated from the 200 mM, 400 mM, and combined data, respectively, and in that order fit the following equations: $\bar{A}t = 0.0161C + 1.15$, $\bar{A}t = 0.0263C + 0.777$, and $\bar{A}t = 0.0236C + 0.961$.

FIGURE 2

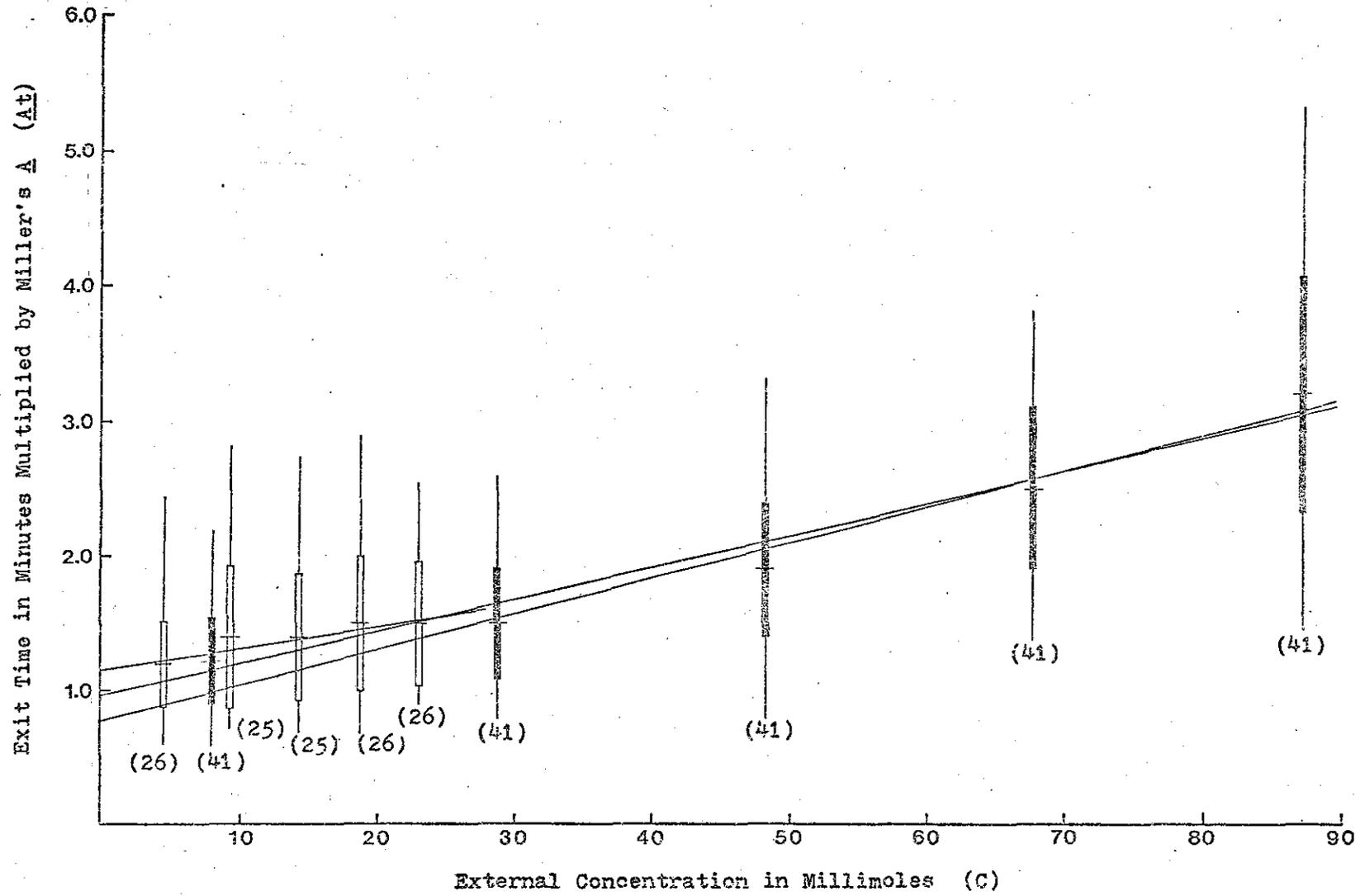
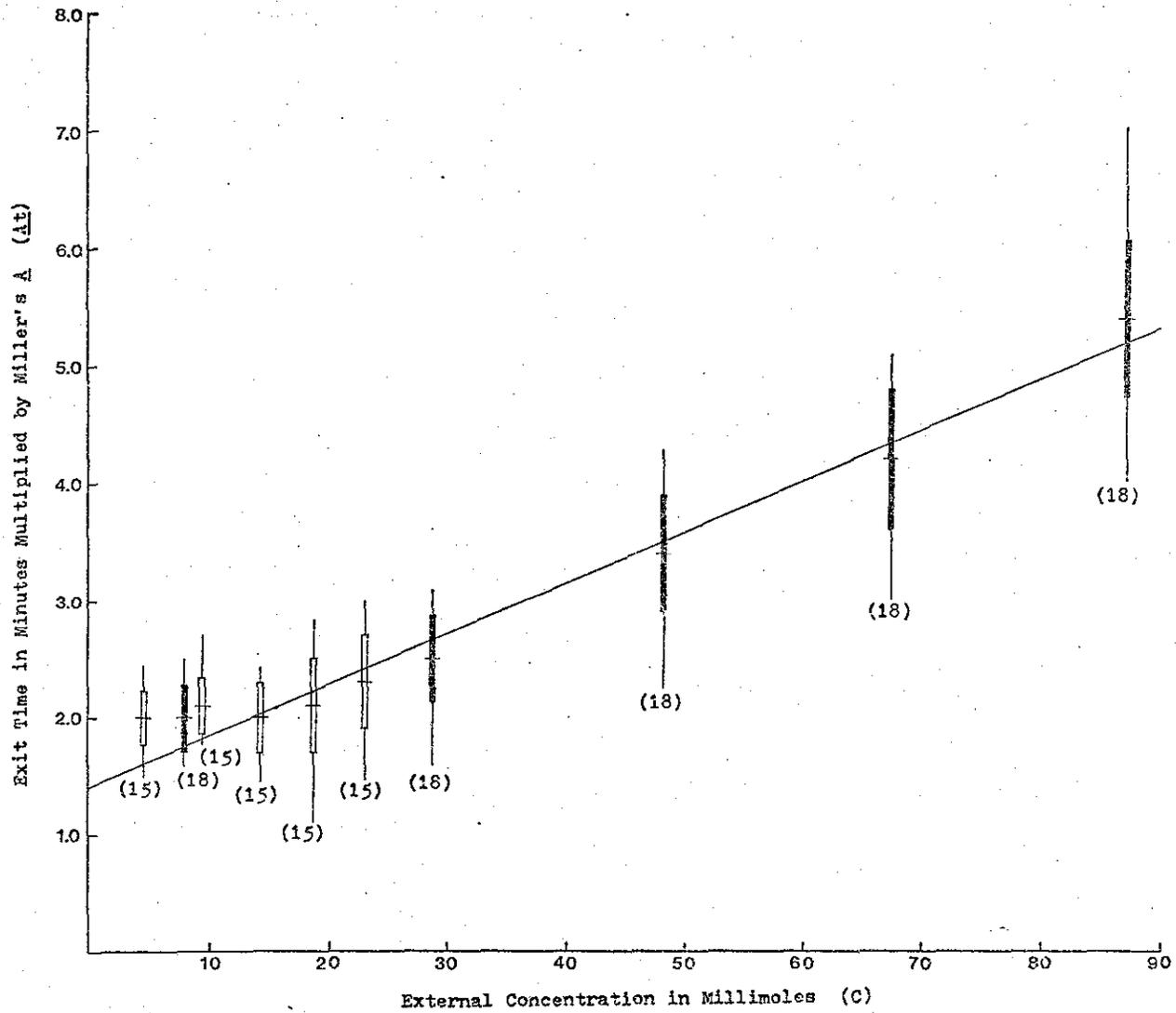


FIGURE 3. Exit times of 200 and 400 mM glycerol in the erythrocytes of Peromyscus californicus. Vertical lines show observed ranges. Shaded rectangles mark standard deviations of the 400 mM data. Unshaded rectangles mark standard deviations of the 200 mM data. The means are indicated by a horizontal line, and the number of time measurements at each external concentration is shown in parentheses. The data displayed for the lowest external penetrant concentration of the 400 mM system (i.e. 9.2 mM) have been shifted slightly to the left. The single regression line is that calculated for the 400 mM system, and fits the following equation: $t = 0.0433C + 1.41$.

FIGURE 3



DISCUSSION

There is much evidence suggesting that in the erythrocytes of various species, a number of different nonelectrolytes cross the membrane by facilitated diffusion. The present work adds to the list two small mammals in which there is a carrier for glycerol.

The linearity of the initial portion of the typical shrinking curves shown in Figure 1 can be explained on the basis of a nearly complete saturation of the penetrant carriers located along the inside of the cell membrane, and a low saturation of those on the outside. This low outside saturation is maintained because the external concentration is not significantly changed by glycerol lost from the cells. As long as the inside of the membrane is nearly saturated, the shrinking process proceeds at a constant and nearly maximal rate. When the inside glycerol concentration falls, the linear part of the record is not maintained, and it curves toward the equilibrium volume.

The 200 mM glycerol system was employed at the onset of this study. However, as can be seen from Figures 2 and 3, only slight variations were found to exist between mean values of Δt at each of the five external penetrant concentrations. In the case of the P. truei data, the ranges of Δt for the five concentrations were quite similar

(see Figure 2). Regression analysis of the 200 mM glycerol system in the erythrocytes of P. californicus yielded nonlinear results ($r = 0.197$), thereby making calculations of ϕ and K meaningless. In search of results with possibly more reliability, subsequent experiments were carried out using 400 mM glycerol. From Figures 2 and 3, steeper slopes can be seen for the 400 mM regression lines. Highly significant correlation coefficients were computed for the 400 mM systems in P. truei ($r = 0.786$) and P. californicus ($r = 0.916$), and the corresponding regression lines were drawn with a high degree of confidence. For these reasons, species and population comparisons were made using primarily the 400 mM data.

It can be seen from Table 1 that while P. truei and P. californicus have similar values for half-saturation, the maximum transport rates of these two related species are apparently different. The ϕ values appear to be in the same range as that computed by Hunter (1976) for glycerol in the deer mouse (P. maniculatus). However, the K's for these two species are larger than the corresponding value of 1.7 calculated for the deer mouse (Hunter, 1976). Such results agree with those of Widdas' 1955 study on the transport of glucose and sorbose by red cells of fetal animals of various species. He found a similarity in affinities for a given penetrant in different species, but differences in maximum transfer rates. Also, the present data seem to support Hunter's (unpublished) suggestion that

for a single penetrant and carrier there is, among different species, less variation in half-saturation values than in maximum transport rates. In contrast, LeFevre (1962) obtained results which showed that for a number of different penetrants sharing the same carrier in a single species, the K values were nearly invariant, whereas those for ϕ covered a wide range.

As mentioned previously, data obtained from the 400 mM glycerol systems in two separate populations of P. truei have not been displayed graphically. It can be seen from Table 1 that with respect to half-saturation and maximum transport rate these two systems are essentially identical, and the regression line for each would be very similar to b of Figure 2. Although no differences in values of ϕ and K were seen between the populations examined in this study, one should not rule out the possibility of such a situation existing in other species.

SUMMARY

1. A photo-electric method was used to measure the rate of exit of glycerol from the erythrocytes of Peromyscus truei and P. californicus.

2. Initial results obtained from experiments using 200 mM glycerol were inconclusive. Therefore, the 400 mM glycerol system with its higher penetrant concentrations was utilized.

3. P. truei and P. californicus were shown to have similar values for half-saturation, however the maximum transport rates of these two species appeared to be different.

4. A comparison of two separate populations of P. truei showed them to be essentially identical with respect to half-saturation and maximum transport rate.

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