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Measurement of Membrane Potentials (Ψ) of Erythrocytes and White Adipocytes by the Accumulation of Triphenylmethylphosphonium Cation

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Summary. The accumulation of the lipophilic cation, triphenylmethylphosphonium, has been employed to determine the resting membrane potential in human erythrocytes, turkey erythrocytes, and rat white adipocytes. The triphenylmethylphosphonium cation equilibrates rapidly in human erythrocytes in the presence of low concentrations of the hydrophobic anion, tetraphenylborate. Tetraphenylborate does not accelerate the uptake of triphenylmethylphosphonium ion by adipocytes. The cell associated *vs.* extracellular distribution of the triphenylmethylphosphonium ion is proportional to changes in membrane potential. The distribution of this ion reflects the membrane potential determining concentration of the ion with dominant permeability in a "Nernst" fashion. The resting membrane potentials for the human erythrocyte, turkey erythrocyte, and rat white adipocyte were found to be -8.4 ± 1.3, -16.8 ± 1.1, and -58.3 ± 5.0 mV, respectively, values which compare favorably with values obtained by other methods. In addition, changes in membrane potential can be assessed by following triphenylmethylphosphonium uptake without determining the intracellular water space. The method has been successfully applied to a study of hormonally induced changes in membrane potential of rat white adipocytes.

The regulation of a number of biological processes by agents acting at the cell surface are associated with changes in ion transport and membrane potential. In order to elucidate the mechanism of this membrane regulation it is necessary to determine accurately steady state and resting membrane potentials (ψ)[1].

Direct electrophysiological techniques (i.e., microelectrodes) have been used to measure ψ in a variety of cell systems. However, this technique is not applicable to all systems of interest. Small cells, e.g., human erythrocytes, are difficult to impale with microelectrodes (Lassen, 1972). Furthermore, such impalements may result in severe ionic leaks at the electrode membrane junction which can make the accuracy of the potentials determined by this method uncertain (Lassen et al., 1971). Problems also exist with larger cells, such as white adipocytes which have large water immiscible inclusions. In this case, the intracellular water space is small and impalement by microelectrodes is therefore interfered with by clogging of the electrode tip with fat globules (*unpublished observations*).

As summarized in the Goldman-Katz equation (Katz, 1966), ψ is determined by the distribution and permeabilities of several ions. In small cells where ψ cannot be determined with microelectrodes, it may be determined from equilibrium ion distribution measurements (Fünder & Wieth, 1966; Hoffman & Lassen, 1971; Freedman & Hoffman, 1979*a*, *b*). The ψ of human erythrocytes is established by the Cl^- Donnan equilibrium (Warburg, 1922; Harris & Maizels, 1952; Fünder & Wieth, 1966). It has been determined routinely by estimating the internal and external concentrations of Cl^- and employing the Nernst equation

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[1] *Abbreviations used:* $TPMP^+$, triphenylmethylphosphonium cation; [^{3}H]-$TPMP^+$, $TPMP^+$([^{3}H]-CH_3); TPB^-, tetraphenylborate anion; BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; ψ, membrane potential; $[I]_o$ and $[I]_i$, concentrations of solutes or ions extracellularly and intracellularly, respectively; pH_o, extracellular pH; ATPase, adenosine triphosphatase.

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(Hoffman & Laris, 1974). There are, however, three major drawbacks inherent in ion distribution determinations of ψ. (i) It is not a simple task to determine which ion, if only one ion, determines and/or reflects the ψ in certain cells. (ii) The rate of equilibration of the ion whose distribution is being measured may be so slow that it may not be suitable for measurements of dynamic changes in ψ. (iii) In cells such as white adipocytes, where it is difficult to estimate accurately the intracellular water space, determinations of intracellular ion concentrations are very difficult (Perry & Hales, 1969; Minemura, Lacy & Crofford, 1970; Zierler, 1972).

Macey, Adorant and Orme (1978) have reported on the determination of erythrocyte ψ from H^+ distribution in unbuffered solutions. The method overcomes some of the problems associated with other ion distribution methods and has a firmer theoretical basis than that of the ψ-dependent fluorescent probe techniques. However, the method is only applicable to cells whose H^+ distribution is in rapid equilibrium. Furthermore, the use of an unbuffered medium with pH-sensitive cells limits the usefulness of this technique when measuring biological responses.

Because the ion distribution methods are not generally applicable to determinations of dynamic changes in ψ, hydrophobic ions have been employed, as these generally equilibrate across membranes quite rapidly (Skulachev, 1971). Fluorescent membrane probes, whose optical properties correspond to changes in ψ, have come into wide use (for review, *see* Waggoner, 1976). However, the mechanism(s) by which these probes sense the reported ψ's are only qualitatively understood (Russell, Beeler & Martonosi, 1979*a*, *b*). The complexity of this method has recently been reviewed (Waggoner, 1979).

In the present study, by using the lipophilic cation triphenylmethylphosphonium (TPMP$^+$), we have determined ψ's of human erythrocytes, turkey erythrocytes, and rat white adipocytes. We have demonstrated that, for human erythrocytes, [^{3}H]-TPMP$^+$ equilibrates rapidly when accelerated by TPB$^-$ and accurately reflects changes in ψ. The cell associated *vs.* extracellular distribution of [^{3}H]-TPMP$^+$, in the presence of low concentrations of the hydrophobic counterion, tetraphenylborate (TPB$^-$), was shown to be proportional to changes in ψ for human erythrocytes and rat white adipocytes. More explicitly, we show that the distribution of [^{3}H]-TPMP$^+$ reflects the ψ determining concentration of the ion with dominant permeability in a "Nernst" fashion. Furthermore, resting ψ's estimated by this method compare favorably with values obtained by other methods. More importantly, we find that rapid changes ($\geqq 1$ min) in the ψ of human erythrocytes can be directly monitored by following TPMP$^+$ uptake without determining the intracellular water space.

Experimental

I. *Theory*

TPMP$^+$ has been shown to be a freely permeant cation across cell membranes (Skulachev, 1971), and its equilibrium distribution has been demonstrated to be dependent on the resting ψ in a variety of systems (Bakeeva et al., 1970; Grinius et al., 1970; Hirata, Altendorf & Harold, 1973; Lombardi et al., 1974; Altendorf, Hirata & Harold, 1975; Heinz, Geck & Peitreyk, 1975; Schuldiner & Kaback, 1975; Azzone et al., 1976; Miller & Budd, 1976; Grollman et al., 1977; Azzone et al., 1978; Deutsch & Küla, 1978; Korchak & Weismann, 1978; Deutsch et al., 1979*b*; Lichtshtein et al., 1979*a*; Ramos et al., 1979; Deutsch et al., 1979*a*). Therefore, by estimating the transmembrane concentration gradient of TPMP$^+$, ψ can be calculated simply from the Nernst relation:

$$\psi = \frac{RT}{zF} \ln [\text{TPMP}^+]_o / [\text{TPMP}^+]_i, \tag{1}$$

where $[\text{TPMP}^+]_o$ and $[\text{TPMP}^+]_i$ are the concentrations of hydrophobic cation extracellularly and intracellularly, respectively, R is the gas constant, z is the ionic valency, F is the Faraday constant, and T is the temperature in °K. This formula may not be readily applicable in certain cell types for two reasons: (i) Although TPMP$^+$ binding to cell membranes may be negligible in cells having high ψ (inside negative) (Komar & Tanner, 1976), membrane-associated TPMP$^+$ may become very significant in cells with low ψ such as human erythrocytes (-9 mV; Fünder & Wieth, 1966). The amount of TPMP$^+$ associated with membranes can be estimated under certain conditions (i.e., $\psi = 0$ mV) (Lichtshtein, Kaback & Blume, 1979*b*). However, the membrane-bound TPMP$^+$ has been shown to change with ψ in some systems (Bakker, Rottenberg & Caplan, 1976). Therefore, the corrections (Deutsch et al., 1979*b*) made by subtracting the excess amount of TPMP$^+$ associated with cells at zero ψ from the total TPMP$^+$ associated with cells at different ψ values and then estimating $[\text{TPMP}^+]_i$ may not be entirely valid. (ii) An accurate estimate of $[\text{TPMP}^+]_i$ requires a reliable means of estimating the intracellular water space. In some cells, such as white adipocytes, it is not always feasible to do this with enough accuracy and precision (Perry & Hales, 1969; Minemura et al., 1970; Gliemann et al., 1972; Zierler, 1972; Horn, Rogus & Zierler, 1973; Horn & Zierler, 1975) and therefore Eq. (1) cannot be used directly to estimate ψ.

In this report, we present a method of estimating resting ψ's and ψ changes induced by perturbations by following the intracellular accumulation of TPMP$^+$ without measuring the intracellular water space. The methods of calculation employed in this study are as follows:

$$\Delta\psi = \psi_c - \psi_e = \frac{RT}{zF} \ln \frac{[\text{TPMP}^+]_o^c}{[\text{TPMP}^+]_i^c} - \frac{RT}{zF} \ln \frac{[\text{TPMP}^+]_o^e}{[\text{TPMP}^+]_i^e}, \tag{2}$$

$$\Delta\psi = 59 \log \frac{[\text{TPMP}^+]_o^c / [\text{TPMP}^+]_i^c}{[\text{TPMP}^+]_o^e / [\text{TPMP}^+]_i^e}, \tag{3}$$

where ψ_c and ψ_e are the resting ψ's of the control and experimental conditions, respectively. The experiments can be performed under such conditions that the amount of TPMP$^+$ taken up by the cells is negligible when compared to the total amount of TPMP$^+$ (i.e.,

$[TPMP^+]_o$ is approximately the same before and after a perturbation). Therefore, if $[TPMP^+]_o^e = [TPMP^+]_o^c$, then

$$\Delta\psi = 59 \log \frac{[TPMP^+]_i^e}{[TPMP^+]_i^c}. \quad (4)$$

From Eq. (4) $\Delta\psi$ is a function of log $[TPMP^+]_i^e/[TPMP^+]_i^c$ and is independent of the level of the resting ψ. Therefore, if $[TPMP^+]_i^e > [TPMP^+]_i^c$ hyperpolarization has occurred and if $[TPMP^+]_i^e < [TPMP^+]_i^c$ depolarization has occurred. If the intracellular water space of the cells is assumed to be changed negligibly by the perturbation (*see* Results and Discussion II), then Eq. (4) can be simplified as follows:

$$\Delta\psi = 59 \log \frac{[TPMP^+]^e_{\text{cell associated}}}{[TPMP^+]^c_{\text{cell associated}}}. \quad (5)$$

Equation (5) can also be used to calculate the resting ψ of cells under physiological conditions. If conditions are adjusted such that ψ_e = zero, then $\Delta\psi = \psi_c$ or the resting ψ.

II. *Methods*

A) Erythrocyte Preparation. Human and turkey blood were drawn daily by venipuncture from healthy young human males and 6-month-old male turkeys (Quaker Farms, Pa.), respectively, into heparinized tubes and centrifuged immediately at 4 °C ($1000 \times g$ for 10 min). After the plasma and buffy coat were removed by aspiration, the erythrocytes were washed ($1000 \times g$ for 10 min) and resuspended four times with a buffer containing (in mM): 148, NaCl; 5, KCl; and 17, Tris-Base; pH 7.4 (normal buffer). The final washed erythrocytes were resuspended in normal buffer and kept on ice until use. Erythrocytes used in Cl^- calibration studies were washed in a buffer containing (in mM): 60, NaCl; 105, Na-tartrate; and 17, Tris-Base; pH 7.4 (Hoffman & Laris, 1974). Cells used in K^+ calibration studies were washed in K^+-free buffer (153 mM choline Cl and 17 mM Tris-Base, pH 7.4). Cells used for $SO_4^=$ flux measurements were washed in $SO_4^=$ buffer containing (in mM) (93, Na_2SO_4; 2.5, K_2SO_4; and 17, Tris-Base, pH 7.4). Cells used for Rb^+ flux measurements were washed in Normal Buffer with RbCl replacing the KCl. Cells used for 3-0-methylglucose transport studies were washed in normal buffer supplemented with 5 mM 3-O-methylglucose. Erythrocytes were counted in a hemocytometer and hematocrits were determined after centrifugation.

B. Adipocyte Preparation. In all experiments, male Sprague-Dawley rats (150–200 g) which had free access to food and water were utilized. Tris Buffer of the following composition was used in preparation of adipocytes and all other studies employing adipocytes (except where stated otherwise) (in mM): 124.0, NaCl; 4.8, KCl; 1.0, $CaCl_2$; 1.0, $MgSO_4$; 1.0, KH_2PO_4; 24.6 Tris-HCl, 1.0, glucose and 3% bovine serum albumin (Fraction V) (BSA), pH 7.4. Isolated adipocytes were prepared essentially according to the method of Rodbell (1964). Specifically, minced epididymal fat pads from 3–5 rats were digested with collagenase at a concentration of ~1 mg/ml in 10–15 ml Tris buffer. The tissue was incubated for 40–60 min at 37 °C in a metabolic shaker at a reciprocating speed of 80 cycles per min. At the end of the incubation, the contents of the incubation flask were filtered through two layers of coarse cheese cloth and then through one layer of fine mesh silk or nylon screen to remove debris. Cells were washed (400 rpm; Model UV International Centrifuge) four times with Tris buffer and suspended in the same buffer solution with a final cell concentration of about 30–40% by volume. Cells used for Rb^+ flux measurements were washed in Tris buffer with RbCl replacing the KCl.

C. Uptake of TPMP$^+$ by Erythrocytes. The uptake of [^{3}H]-TPMP$^+$ by human erythrocytes was studied at 37 °C. Uptake was carried out in plastic tubes and was initiated by adding 1 volume of erythrocytes at a 10% hematocrit to 9 volumes of prewarmed (37 °C) buffer containing [^{3}H]-TPMP$^+$ and TPB$^-$ [final concentrations were 10 μM (sp act: 0.10 Ci/mmol) and 2 μM, respectively]. Other compounds, if present, were also added to the prewarmed buffer prior to the addition of cells, except where stated otherwise. Valinomycin, ouabain, and A23187 were introduced from stock solutions in absolute ethanol (prepared daily, final concentration of ethanol ≦0.5% vol/vol in all cases). 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) dissolved in deionized water was introduced from stock solutions which were prepared daily and protected from light. Forty seconds after the addition of erythrocytes (unless otherwise indicated) three (triplicate sampling) 0.2 ml aliquots of cell suspension were layered over 0.2 ml silicone oil (d=1.05 g/cc) in 0.5 ml polyethylene microfuge tubes. The tubes were immediately centrifuged for 15 sec in a Beckman Model 152 microfuge. After centrifugation the bottoms of the microfuge tubes were cut just above the cell pellets with a sharp scalpel and placed into scintillation vials. The erythrocyte pellets were lysed with 0.2 ml of deionized H_2O, the hemolysates decolorized with 0.4 ml of 30% vol/vol H_2O_2, and radioactivities determined by liquid scintillation counting in the presence of 10 ml of Scintiverse (Fisher Scientific). Extracellular trapping within cell pellets was estimated in separate experiments with [^{3}H]-inulin. [^{3}H]-TPMP$^+$ uptake was then corrected for this volume of extracellular fluid trapped within the pellet.

D. Uptake of TPMP$^+$ by Rat White Adipocytes. The uptake of [^{3}H]-TPMP$^+$ by isolated adipocytes was measured in plastic tubes containing Tris buffer at 37 °C using the oil flotation separation technique described by Gliemann et al. (1972). Reactions were started by adding 1 volume of 30–40% cell suspension to 9 volumes of prewarmed (37°) Tris buffer containing [^{3}H]-TPMP$^+$, TPB$^-$, and other compounds, if so indicated. The final concentrations of [^{3}H]-TPMP$^+$ and TPB$^-$ in the reaction mixture were 10 μM (sp act: 0.10 Ci/mmol) and 2 μM, respectively. Where indicated, TPB$^-$ was not included as this did not alter TPMP$^+$ uptake by the adipocytes (*see* Results and Discussion, II). The uptake was terminated by transferring three (triplicate sampling) 0.2-ml aliquots of reaction mixture to 0.5 ml polyethylene microfuge tubes and immediately centrifuging the aliquots under 0.2 ml of dinonyl phthalate for 20 sec in a Beckman Model 152 microfuge. After centrifugation, the tops of the microfuge tubes were cut just under the cell cakes and placed into scintillation vials containing 1 ml of 10% wt/vol sodium dodecyl sulfate. The vials were shaken for 20 min at room temperature, and radioactivities were determined by liquid scintillation counting in the presence of 10 ml ScintiVerse.

E. Determination of Resting ψ's. From Eq. (2), when ψ_e = zero then $\psi_c = \Delta\psi$ or the resting ψ. ψ_e = zero can be achieved by suspending cells in a buffer having a $[K^+]$ equal to $[K^+]_i$ in the presence of the K^+ ionophore, valinomycin. $[K^+]_i$ of human erythrocytes and rat white adipocytes are ~153 mM (Hoffman & Laris, 1974) and ~140 mM (Perry & Hales, 1969), respectively. For all determinations TPMP$^+$ uptake was measured under two conditions: (i) zero ψ and (ii) resting ψ. In all cell systems, cells were washed and resuspended in buffers appropriate to the conditions required. For human and turkey erythrocytes the zero ψ buffer was 153 mM KCl, 2.2 μM valinomycin, 17 mM Tris base, pH 7.4, and the resting ψ buffer was normal buffer. Uptake was measured as usual (Experimental II*C*), except that for turkey erythrocytes 30 min were required for TPMP$^+$ uptake to reach equilibrium. For rat white adipocytes the zero ψ buffer was a modified Tris buffer containing no NaCl, 140 mM KCl and 2.2 μM valinomycin, and the resting ψ buffer was the usual Tris buffer. Uptake was measured as usual (Experimental II*D*).

III. *Materials*

[^{3}H]-TPMP$^+$Br$^-$ (3.60 Ci/mmol), $Na^{35}SO_4$ (640 mCi/mmol), ^{86}RbCl (3.3 mCi/mmol), ^3H_2O (1.0 mCi/g), [^{3}H]-inulin (350 mCi/g), [^{3}H]-2-deoxy-D-glucose (500 mCi/mmol), and [^{3}H]-3-O-methylglucose (420 mCi/mmol) were obtained from New England Nuclear. Unlabeled TPMP$^+$Br$^-$ and dinonyl phthalate (d= 0.98 g/cc) were obtained from K & K Laboratories. Valinomycin, ouabain, and BSA (Fraction V) were obtained from Sigma Chemical Co. A23187 was obtained from E. Lilly and Co. Na$^+$TPB$^-$ and silicone oil (d=1.05 g/cc) were obtained from Aldrich Chemical Co. DIDS was obtained from Pierce Chemical Co. Sephadex G-50 was obtained from Pharmacia Fine Chemicals. Male Sprague-Dawley rats (150–200 g) were purchased from Charles River Laboratories. Crude Bacterial Collagenase (Type I) was obtained from Worthington Biochemical Corporation. All other chemicals were reagent grade obtained from commercial sources.

Results and Discussion

I. *Kinetics of TPMP$^+$ Accumulation by Human Erythrocytes*

In the absence of TPB$^-$ the rate of TPMP$^+$ uptake by human erythrocytes was slow (Fig. 1). Equilibrium was not reached until 2 hr had elapsed. The rate of TPMP$^+$ uptake was accelerated >100-fold by the addition of 2 μM TPB$^-$ (i.e., equilibrium was attained in <1 min). The equilibrium level of TPMP$^+$ in human erythrocytes was slightly higher in the presence of TPB$^-$ than in its absence. However, after 4 hr incubation, both conditions gave the same final equilibrium level of TPMP$^+$ uptake. Furthermore, the addition of 2 μM TPB$^-$ to erythrocytes which had been equilibrated with TPMP$^+$ for 4 hr did not change the level of TPMP$^+$ accumulation. These results suggest that the higher equilibrium level of TPMP$^+$ accumulation observed in erythrocytes initially exposed to 2 μM TPB$^-$ did not result from an observable ionophoric effect of TPB$^-$. Rather, the decrease in TPMP$^+$ uptake with long incubation (4 hr) may reflect a changing ψ.

II. *Kinetics of TPMP$^+$ Accumulation by Rat White Adipocytes*

Maximal accumulation of TPMP$^+$ by adipocytes was observed at ~30 min and remained constant for at least another 30 min (Fig. 2). Therefore, all studies with rat white adipocytes have been performed with incubation times of 40–50 min unless otherwise specified. TPMP$^+$ uptake was not altered if TPB$^-$ was omitted from the incubates (data not shown).

III. *Effects of TPMP$^+$ and TPB$^-$ on Cell Integrity, Ion Fluxes, and Volume*

A) Human Erythrocytes. ψ is determined by the distribution and permeability of ions across the cell mem-

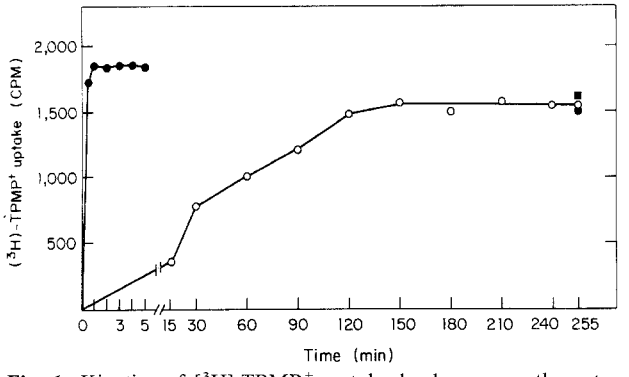

Fig. 1. Kinetics of [^{3}H]-TPMP$^+$ uptake by human erythrocytes in the presence and absence of TPB$^-$. Reactions were initiated by adding 1 volume of a 10% suspension of normal human erythrocytes in normal buffer to 9 volumes of prewarmed (37 °C) normal buffer containing [^{3}H]-TPMP$^+$ in the presence (●) and absence (○) of TPB$^-$ (final hematocrit=1%). The final concentrations of [^{3}H]-TPMP$^+$ and TPB$^-$ (if present) in reaction mixtures were 10 μM (sp act: 0.10 Ci/mmol) and 2 μM, respectively. The reactions were terminated at times indicated and the uptake of radioactivity determined as described in Experimental. An aliquot of the cell suspension incubated at 37 °C for 4 hr in the presence of [^{3}H]-TPMP$^+$ but no TPB$^-$ was treated with TPB$^-$ (2 μM) 40 sec prior to assay of [^{3}H]-TPMP$^+$ uptake at 255 min (■)

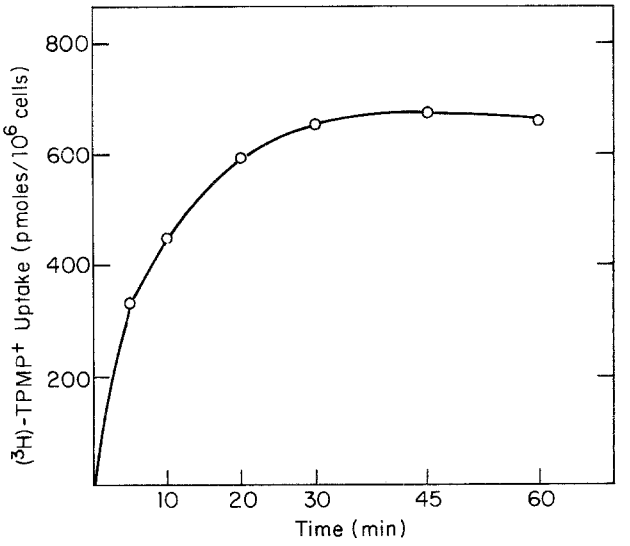

Fig. 2. Kinetics of [^{3}H]-TPMP$^+$ uptake by isolated rat white adipocytes. TPMP$^+$ uptake was measured in Tris-buffer plus 10 μM [^{3}H]-TPMP$^+$ (sp act: 0.10 Ci/mmol) and 2 μM TPB$^-$, at 37 °C. Conditions were described in Experimental. At the times shown, the reactions were terminated and radioactivity determined as described in Experimental

brane (Katz, 1966). Therefore, a ψ probe must not alter the integrity of the cell membrane. Incubation of human erythrocytes with 10 μM $TPMP^+$ and 2 μM TPB^- at 37 °C for up to one hour did not cause significant hemolysis. However, at concentrations $\geqq$10 μM TPB^- hemolysis as well as significant hyperpolarization (increased $TPMP^+$ uptake) were evident (data not shown). The uptake of [^{3}H]-3-O-methylglucose and $^{35}SO_4^=$ by human erythrocytes was not affected by 10 μM $TPMP^+$ and/or 2 μM TPB^-. $TPMP^+$ (10 μM) had no effect on the influx of $^{86}Rb^+$ either in the presence or absence of 0.5 mM ouabain. However, the influx of $^{86}Rb^+$ was increased by ~50% by 2 μM TPB^- after a 1-hr incubation (with or without 0.5 mM ouabain) (data not shown). Since Cl^- permeability is 100 times greater than K^+ permeability in the human erythrocyte (Hunter, 1971), and the Cl^- Donnan equilibrium determines the ψ of the erythrocyte, the increase of $^{86}Rb^+$ influx by 2 μM TPB^- should not affect the ψ.

Further evidence for the lack of a specific interaction of $TPMP^+$ with the human erythrocytes was obtained by demonstrating that the cation does not significantly associate with hemoglobin or red cell membranes. Human erythrocytes (20% hematocrit) were incubated with 10 μM [^{3}H]-$TPMP^+$ for 5 min, the cells were hemolyzed by freeze-thawing, and the lysate passed over Sephadex G-50 with normal buffer as the eluant, Lack of a high affinity interaction between hemoglobin and $TPMP^+$ was demonstrated by the lack of coelution of radioactivity with hemoglobin ($Absorbance_{417nM}$).

In similar experiments erythrocytes were incubated with [^{3}H]-$TPMP^+$, aliquots were taken for determination of cell-associated $TPMP^+$, the remaining cells hemolysed, and aliquots of the lysate centrifuged over silicone oil for 3 min in a Beckman Model 152 microfuge. Less than 0.5% of the cell-associated $TPMP^+$ was membrane associated when the erythrocytes were lysed.

To measure ψ reliably by the methods described, it had to be demonstrated that the intracellular water space changed negligibly under the experimental con ditions employed. The intracellular water space of the human erythrocyte, as determined from the distribution of ^3H_2O, remained constant ($\pm$5%) for $\leqq$15 min after the addition of 10 μM $TPMP^+$ and/or 2 μM TPB^-. Furthermore, valinomycin (2.2 μM) or A23187 (2 μM) plus Ca^{++} (0.5 mM) did not cause significant changes ($\pm$7%) in the volume of the human erythrocyte for $\leqq$2 min after addition.

B) Turkey Erythrocytes. Incubation of turkey erythrocytes with 10 μM $TPMP^+$ and 2 μM TPB^- at 37 °C for up to 1 hr did not cause significant hemolysis. The uptake of $^{35}SO_4^=$ and $^{86}Rb^+$, as well as the intracellular water space, were unaffected ($\pm$5%, $\pm$4%, and $\pm$7%, respectively) by 10 μM $TPMP^+$ and/or 2 μM TPB^-. Furthermore, valinomycin (2.2 μM) did not alter the intracellular water space of turkey erythrocytes ($\pm$2%) when incubated in zero ψ buffer (high-K^+).

C) Rat White Adipocytes. Incubation of rat white adipocytes with 10 μM $TPMP^+$ and 2 μM TPB^- at 37 °C for up to 2 hr did not cause significant lysis (determined by cell count). The uptake of $^{86}Rb^+$ was unaffected ($\pm$10%) by 10 μM $TPMP^+$ and/or 2 μM TPB^-. Insulin and epinephrine stimulation of 2-deoxy-glucose uptake by rat white adipocytes was not prevented by incubating rat white adipocytes with 10 μM $TPMP^+$. [^{3}H]-2-deoxyglucose uptake was measured by the method of Livingston and Lockwood (1974).

Furthermore, $TPMP^+$ was shown not to associate with the fat globule or plasma membrane of adipocytes. Adipocytes (4% vol/vol) were incubated with [^{3}H]-$TPMP^+$, aliquots were taken for a determination of cell associated $TPMP^+$, the remaining cells were lysed by freeze thawing, and aliquots were centrifuged under dinonyl phthalate (20 sec in Beckman Model 152 microfuge). Less than 1.0% of the cell associated $TPMP^+$ was membrane associated or fat globule associated.

In separating free floating adipocytes, the small amount of cell water is usually within experimental error of the measured total water and extracellular water (Zierler, 1972). Thus, this parameter cannot be directly controlled for in experiments with rat white adipocytes. It was therefore assumed, by comparison with the erythrocyte data, that the intracellular water space of the adipocytes remained constant.

IV. *Calibration of ψ Responses in Human Erythrocytes*

A) Changing $[Cl^-]_o$ and External pH (pH_o). The distribution of small anions (e.g., Cl^-, HCO_3^-, OH^-, etc.) between human erythrocytes and their surrounding media follows Donnan's Law (Katz, 1966). Accordingly,

$$\frac{[Cl^-]_i}{[Cl^-]_o}=\frac{[HCO_3^-]_i}{[HCO_3^-]_o}=\frac{[OH^-]_i}{[OH^-]_o}=\frac{[H^+]_o}{[H^+]_i}. \quad (6)$$

Therefore, by changing $[Cl^-]_o$ and pH_o simultaneously, the ratio $[Cl^-]_i/[Cl^-]_o$ is altered while $[Cl^-]_i$ and $[H^+]_i$ remain constant. The ψ of human erythrocytes is dominated by the ratio, $[Cl^-]_i/[Cl^-]_o$ (Katz, 1966; Hoffman & Laris, 1974). It should, therefore, be a

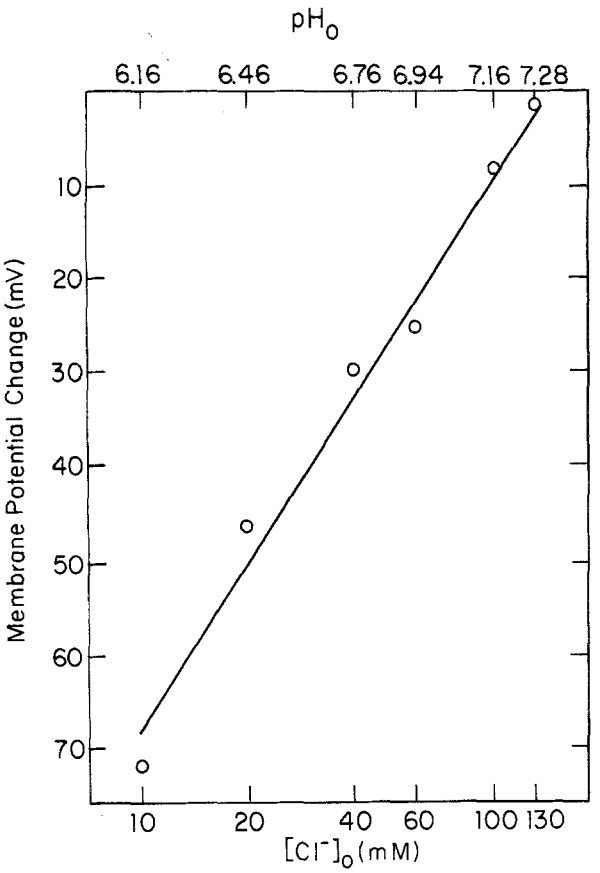

Fig. 3. Change of membrane potential of human erythrocytes by varying $[Cl^-]_o$ and pH_o. Fresh human blood was washed 4 times with buffer containing 60 mM NaCl, 105 mM Na-tartrate and 17 mM Tris-base, pH 7.4. The packed erythrocytes were suspended in buffer containing Tris base (17 mM) and various concentrations of Cl^-, and tartrate$^-$ ($[Cl^-]_o + [\text{tartrate}^-]_o = 165$ mM, in all buffers), pH 7.4 (final hematocrit = 1%). The pH's were adjusted, if necessary, with 0.5 N H_2SO_4 or 1.0 N NaOH. The final $[Cl^-]_o$ and pH_o were 10 mM at pH 6.16, 20 mM at pH 6.46, 40 mM at pH 6.76, 60 mM at pH 6.94, 100 mM at pH 7.16, 130 mM at pH 7.28, and 165 mM at pH 7.38. Under these conditions $[Cl^-]_i$ and pH_i are assumed to remain constant (120 mM at pH 7.20). The uptake of TPMP$^+$ was measured immediately after pH adjustments as described in Experimental. The membrane potential changes were calculated as described in Experimental using the uptake at 165 mM $[Cl^-]_o$ as a reference point ($[TPMP^+]^{\epsilon}_{\text{cell associated}}$). The slope of the line is 58.5 mV/decade ("Nernst" slope)

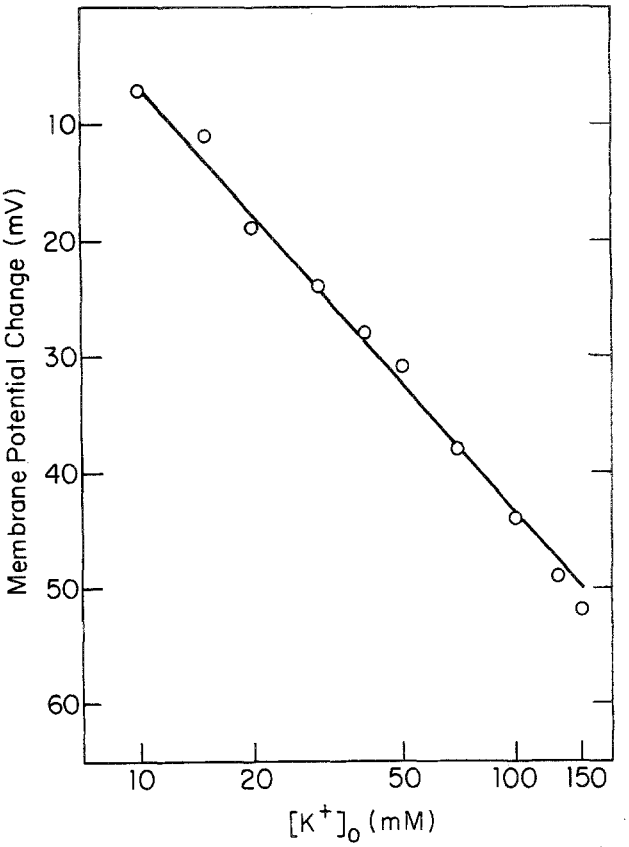

Fig. 4. Change of membrane potential of human erythrocytes by changing $[K^+]_o$ in the presence of valinomycin. Fresh human blood was washed 4 times with buffer containing 153 mM NaCl and 17 mM Tris-base, pH 7.4. Cells were suspended in 17 mM Tris-base, pH 7.4, containing various concentrations of cholineCl and KCl ($[\text{choline}^+]_o + [K^+]_o = 153$ mM in all buffers) (final hematocrit = 1%). The uptake of TPMP$^+$ was measured as described in Experimental with a final concentration of 2.2 μM valinomycin. The membrane potential changes were calculated as described in Experimental using the uptake at 5 mM $[K^+]_o$ as a reference point ($[TPMP^+]^{\epsilon}_{\text{cell associated}}$). The slope of the line is 37 mV/decade

linear function of log $[Cl^-]_o$. As shown in Fig. 3, the ψ changes calculated from TPMP$^+$ accumulation using Eq. (5) are linear with log $[Cl^-]_o$ (within the range tested). Furthermore, a slope of 58.5 mV was obtained for a 10-fold change in $[Cl^-]_o$. These results demonstrate that the TPMP$^+$ uptake by human erythrocytes reflects ψ changes in a "Nernst" fashion.

B) Changing $[K^+]_o$ in the Presence of Valinomycin. The Cl^- permeability is about 100 times greater than the K^+ permeability in human erythrocytes (Hunter, 1971). In the presence of valinomycin, however, the K^+ permeability of the membrane is increased (Hunter, 1974; Knauf et al., 1977) and the ψ of the erythrocyte is therefore shifted from a Cl^- distribution dependence towards a K^+ distribution dependence (Hoffman & Laris, 1974; Knauf et al., 1977). In the presence of micromolar amounts of valinomycin, the accumulation of TPMP$^+$ by human erythrocytes should vary with $[K^+]_o$. The dependence of TPMP$^+$ uptake by human erythrocytes on $[K^+]_o$ (5–150 mM) in the presence of 2.2 μM valinomycin is shown in Fig. 4. The plot of log $[K^+]_o$ *vs.* $\Delta\psi$ is linear and has a slope of 37 mV for a 10-fold change in $[K^+]_o$. The slope is smaller than that obtained for the Cl^- calibration. However, this value is comparable to literature values obtained with ψ sensitive fluorescent probes (Hoffman & Laris, 1974) (*see* Results and Discussion, VII).

C) Changing $[K^+]_o$ in the Presence of A23187 plus Ca^{++}; Effect of DIDS. The K^+ permeability of human erythrocytes can be increased not only by the addition of the K^+ ionophore, valinomycin, but also by increasing $[Ca^{++}]_i$ (Sarkadi, Szasz & Gardos, 1976). The *Gardos Effect* can be achieved by the addition of extracellular Ca^{++} in the presence of the Ca^{++} ionophore, A23187. Therefore, by varying $[K^+]_o$ in the presence of 2 μM A23187 plus 0.5 mM Ca^{++} the ψ change calculated from TPMP$^+$ uptake [Eq. (5)] should be a function of log $[K^+]_o$. The data presented in Fig. 5 confirm this prediction. A slope of 48 mV per 10-fold change in $[K^+]_o$ was obtained.

Since the rate of K^+ flux (i.e. collapse of K^+ gradient) is limited by the permeabilities of anions (Knauf et al., 1977), the effect of an inhibitor of anion transport on the slope of the line was examined. The addition of 8 μM DIDS, a chloride transport inhibitor (Cabantchik & Rothstein, 1972, 1974), had no effect on the slope of the line. This result is not totally unexpected since DIDS, at $\leqq 10$ μM, primarily affects the anion exchange system (Cabantchik & Rothstein, 1972, 1974; Knauf et al., 1977) which is believed to be electrogenically silent (Knauf et al., 1977). If, however, the same experiment is carried out with human erythrocytes which have been pretreated with DIDS (1 mg/3 cm^3 packed cells) for 10 min at 37 °C, a slope of 57 mV/decade ("Nernst" slope) is obtained. This result suggests that higher concentrations of DIDS are required to inhibit passive Cl^- permeability (which may still be influencing the ψ during the Gardos Effect). Similar observations have been reported (Macey et al., 1978), and the suggestion by Knauf et al. (1977) that a common element may be involved in both net and exchange anion transport may explain these results.

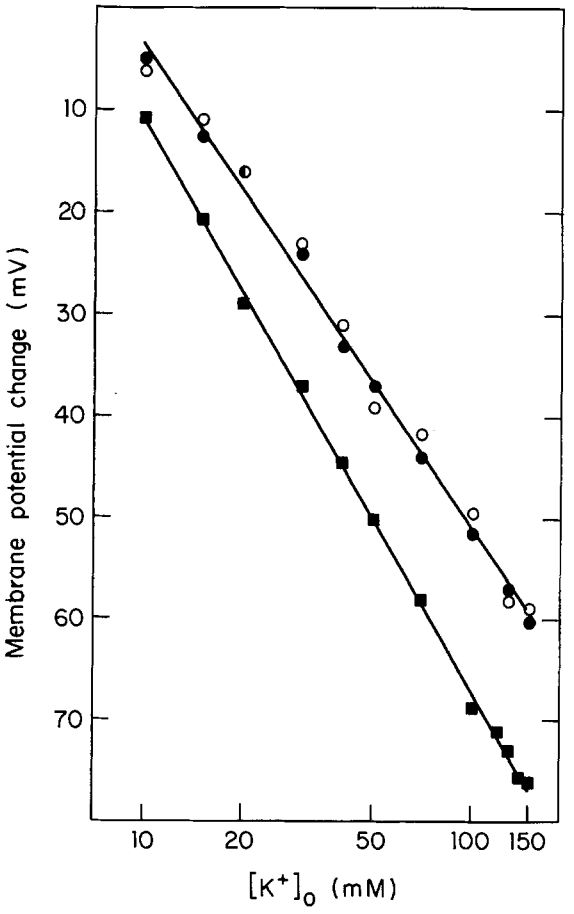

Fig. 5. Effect of DIDS on membrane potential changes of human erythrocytes induced by A23187 plus Ca^{++} while varying $[K^+]_o$. Experimental conditions and measurements of TPMP$^+$ uptake were as described for Fig. 3 except valinomycin was replaced by 2 μM A23187 plus 0.5 mM $CaCl_2$. Control (●); pretreated for 10 min at 37 °C with 8 μM DIDS (●); pretreated for 10 min at 37 °C with 1 mg DIDS/3 cm^3 packed cells (■). Slopes of the lines are 48 (⊖), 48 (●), and 57 (■) mV/decade

V. *Calibration of ψ Responses in Rat White Adipocytes*

The ions with dominant permeability (ions which determine ψ) across the plasma membrane of rat white adipocytes have not yet been unequivocally deter-

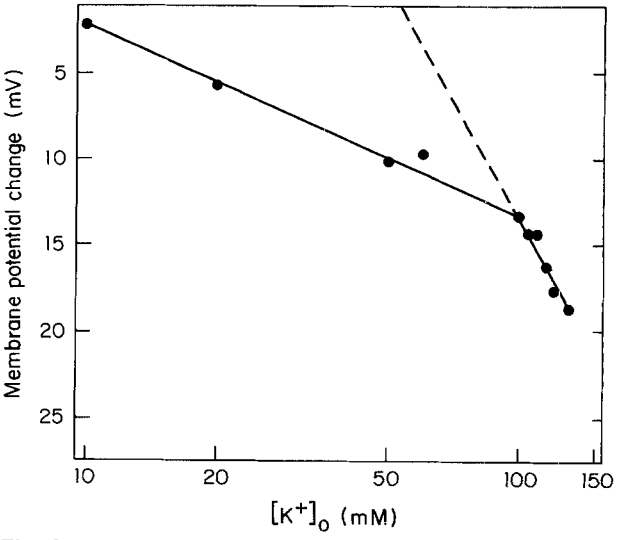

Fig. 6. Change in membrane potential of rat white adipocytes by changing $[K^+]_o/[Na^+]_o$. Isolated adipocytes were washed two times with Tris buffer and two times with a modified Tris buffer in which $[K^+]=60$ mM and $[Na^+]=69$ mM. Cells were resuspended in modified Tris buffer of the appropriate $[K^+]_o/[Na^+]_o$ compositions ($[K^+]_o+[Na^+]_o=129$ mM in all cases) and TPMP$^+$ uptake was measured as described in Experimental. TPB$^-$ was not included. The membrane potential changes were calculated as described in Experimental using TPMP$^+$ uptake at 5 mM $[K^+]_o$ as a reference point ($[TPMP^+]_{cell\ associated}$). The slope of the lines are 57 mV/decade above 100 mM K^+ and 11 mV/decade below 100 mM K^+

mined. Preliminary experiments (data not shown) in which the $[K^+]_o$ (substituting sucrose), $[Na^+]_o$ (substituting sucrose), $[Cl^-]_o$ (substituting tartrate$^-$), $[Mg^{++}]_o$ (substituting Na^+), and $[Ca^{++}]_o$ (substituting Na^+) were varied individually indicated that the ψ of rat white adipocytes is determined by the transmembrane K^+ and Na^+ gradients. The effects of other ions are negligible. The dependence of TPMP$^+$ uptake by rat white adipocytes on $[K^+]_o$ (substituting Na^+) is shown in Fig. 6. The plot of log $[K^+]_o$ *vs.* $\Delta\psi$ is curvilinear. If two linear approximations are made then the slopes obtained are 11 mV/decade for <100 mM K^+ and 57 mV/decade for >100 mM K^+. The Goldman-Katz equation (Katz, 1966) predicts that both the ion gradient and permeabilities of several ions can determine ψ. This suggests that the slope of 11 mV below 100 mM K^+ is a function of both the K^+ and Na^+ gradients. While the "Nernst" slope of 57 mV above 100 mM K^+ is a result of the K^+ gradient becoming the dominant ψ determining parameter; the data also imply that the permeability ratio for K^+ over Na^+ (P_{K^+}/P_{Na^+}) for rat white adipocytes is ~ 10. Therefore, TPMP$^+$ uptake by rat white adipocytes reflects $\Delta\psi$ in a fashion consistent with that predicted by the Goldman-Katz Equation.

VI. *Estimation of Resting ψ's of Human Erythrocytes, Turkey Erythrocytes, and Rat Adipocytes*

The resting ψ's of human erythrocytes, turkey erythrocytes, and isolated rat white adipocytes were estimated (*see* Table 1 and Experimental II*D*). The value obtained for human erythrocytes is in good agreement with values obtained by other methods (Fünder & Wieth, 1966; Hoffman & Laris, 1974). The resting ψ estimated for turkey erythrocytes agreed reasonably well with the value, -18 mV, obtained by Kimmich, Philo and Eddy (1977) for the pigeon erythrocyte. Miller, Schlosser and Beigleman (1966) have determined the ψ of isolated rat white adipocytes (embedded in 1% agar) using microelectrodes. The wide range of values that they report (15–40 mV) makes comparisons of their results with that reported here difficult. Furthermore, there are problems inherent in the microelectrode technique (*see* Results and Discussion, IX and the introduction). Perry and Hales (1969) determined the ψ of rat white adipocytes using the ion distribution method. The technique relied on an accurate determination of the intracellular water space which the authors state is rather difficult (*see* Results and Discussion, III). Assuming passive Cl^- equilibration, they estimated the resting ψ of the adipocytes to be -28.7 mV (from the Cl^- distribution ratio). This value is in poor agreement with that reported here but it relies on both the accuracy of their determination of the intracellular water space as well as the validity of their assumptions.

Table 1. Determination of resting membrane potential (ψ) using TPMP$^+$ accumulation

Cell type	Resting membrane potential, ψ (mV)[a]
Human erythrocyte	-8.4 ± 1.3 (5)
Turkey erythrocyte	-16.8 ± 1.1 (2)
Rat white adipocyte	-58.3 ± 5.0 (5)

[a] Based on percent change of cell-associated [^{3}H]-TPMP$^+$ under zero *vs.* resting membrane potential conditions (*see* Experimental and Eq. (5) for details).

Results expressed as mean ± SD (*n*).

VII. *Rapid Responses to Perturbations of ψ*

The method is not limited to the estimation of resting ψ. As shown in Fig. 7, the level of TPMP$^+$ uptake by human erythrocytes responded rapidly to perturbations in ψ induced by ionophores. Figure 7*A* shows that upon addition of 2.2 μM valinomycin, human erythrocytes suspended in normal buffer rapidly (< 1 min) take up increased amounts of TPMP$^+$ (indicating the expected hyperpolarization) and that following the valinomycin-induced collapse of the K^+ gradient the level of cell-associated TPMP$^+$ decreases (indicating depolarization). Figure 7*B* demonstrates a similar result for the *Gardos Effect*, but differs in that the hyperpolarized state persists for a longer time (~ 4 min). Furthermore, the lack of stability of

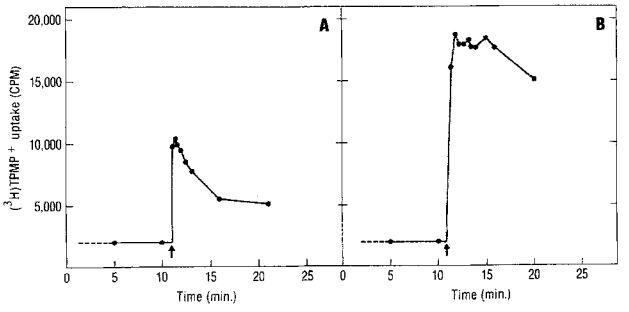

Fig. 7. Kinetics of TPMP$^+$ uptake by human erythrocytes in the presence of ionophores. (*A*): Valinomycin: Conditions are the same as in Fig. 1 when TPB^- (2 μM) was present except that where indicated (arrow) valinomycin was added to give a final concentration of 2.2 μM. (*B*): *Gardos Effect*: Conditions are the same as in Fig. 1 when TPB^- (2 μM) was present except that where indicated (arrow) A23187 and $CaCl_2$ were added to give a final concentration of 2 μM and 0.5 mM, respectively. Reactions in *A* and *B* were terminated at times indicated and the uptake of radioactivity determined as described in Experimental

Table 2. Changes in the membrane potential (ψ) of rat white adipocytes caused by agents which affect ion gradients and ion permeabilities

Agent	Membrane potential change, $\Delta\psi$ (mV)[a]
Ouabain (500 μM)	3.1 ± 0.3 (5) ↑
A23187 (2 μM)	1.6 ± 0.6 (2) ↓
Valinomycin (2.2 μM)	44.1 ± 4.5 (4) ↑

[a] Based on percent change of cell-associated [^{3}H] TPMP$^+$ using Eq. (5) to calculate $\Delta\psi$ ($\Delta\psi = \psi_c - \psi_e$).

Under perturbed *vs.* Tris buffer conditions. TPB$^-$ (2 μM) was not included in all cases. (*See* Experimental for details.) Results are expressed as mean ± SD. (*n*). ↑: Decrease towards zero potential (less negative inside, depolarization). ↓: Increase from zero potential (more negative inside, hyperpolarization).

the hyperpolarized state induced by valinomycin (Fig. 7*A*) may explain the apparent deviation from a "Nernst" slope observed in Fig. 4.

VIII. *Response to Perturbation of ψ by Rat White Adipocytes*

The effects of ouabain (500 μM), valinomycin (2.2 μM) and A23187 (2 μM) on the steady state ψ of rat white adipocytes (suspended in Tris buffer) as determined by TPMP$^+$ uptake are shown in Table 2. Ouabain, a Na^+-K^+ ATPase inhibitor (Dahl & Hokin, 1974), slightly depolarized adipocytes, while the Ca^{++} ionophore, A23187, did not significantly change the ψ. This suggests that the Na^+-K^+ ATPase, which is fully functional in isolated rat white adipocytes (Clausen, Rodbell & Durand, 1969), plays a very small, if any, apparent role in determining the resting ψ. Similar interpretations have been made in other systems e.g., *see* Lichtshtein, Kaback & Blume, 1979*b*). Valinomycin depolarized the adipocyte. This can be attributed to the collapse of the K^+ gradient induced by increased K^+ permeability. These results demonstrate that TPMP$^+$ uptake by rat white adipocytes responds to perturbations of ψ.

IX. *Usefulness and Advantages of the Method*

The methods described demonstrate that TPMP$^+$ accumulation can reliably be used to estimate both steady state and changing ψ's. The main advantages of this method are: (i) It is simple and accurate provided that TPMP$^+$ uptake is calibrated with ψ changes; (ii) the intracellular water space need not be measured; and (iii) it responds relatively rapidly to changes in ψ in a theoretically predictable manner.

With regard to advantages i and ii, it should be emphasized that only $(TPMP^+)_{\text{cell associated}}$ need be determined to calculate $\Delta\psi$ when comparing two conditions [*see* Eq. (5)]. As distinct from $(TPMP^+)_{\text{cell associated}}$, $[TPMP^+]_i$ can be calculated from estimates of the intracellular water space and by subtracting the excess amount of TPMP$^+$ which is associated with the cell at ψ = zero. When this is done for the human erythrocyte and absolute ψ's are calculated using Eq. (4), instead of Eq. (5), the slopes of the calibration curves as well as the estimated resting ψ's (data not shown) deviate from their expected values (Figs. 2–4 and Table 1, respectively). This emphasizes that the membrane-bound component of $(TPMP^+)_{\text{cell associated}}$ is changing with ψ. Since the resting ψ's obtained are correct and the technique calibrates in a "Nernst" fashion it may be concluded that all the significant components of $(TPMP^+)_{\text{cell associated}}$ are proportional to ψ.

The practical use of this method is best demonstrated in the white adipocyte. Due to its small water space and large fat globules, it is not possible to use the microelectrode method to measure ψ's in this system (*unpublished observations*). The ψ-sensitive fluorescent probes are difficult to use in studies of white adipocyte ψ. This is due to flotation of the cells in the fluorescence cuvette (*unpublished observations*). But one can study hormonally induced changes in the ψ of rat white adipocytes[2] using a modified version of the present TPMP$^+$ distribution method.

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