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Membrane potential difference of isolated plant vacuoles: positive or negative? II. Comparison of measurements with microelectrodes and cationic probes

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Values of the transmembrane potential difference (E_m) of isolated vacuoles were shown to differ markedly when measured on the same vacuolar suspension with microelectrodes ($E_m = +13$ mV), or with the lipophilic cation tetraphenylphosphonium (TPP^+) ($E_m = -60$ mV). The extent of TPP^+ binding on vacuolar preparations was evaluated using the binding parameters determined previously on the tonoplast [15], and assuming a positive E_m value. The results show that TPP^+ binding represents 46–96% of the overall accumulation in vacuole suspensions. This binding, when confused with an accumulation of free ions, simulates the existence of a negative transmembrane potential, and accounts for the discrepancy between negative E_m values obtained with TPP^+ and positive E_m values measured with microelectrodes. Furthermore, modulation of the apparent negative potential induced by different effectors (KCl, FCCP, Mg-ATP), and taken as evidence for negative E_m, was demonstrated to result from variations of probe binding. It is concluded that lipophilic cationic probes are unsuitable for measurement of E_m on vacuolar suspensions.

Introduction

A survey of literature (e.g., see Ref. 1) shows that the transtonoplast electrical potential difference (E_m) measured on isolated vacuoles is reported to be positive when measured with microelectrodes and negative when calculated from equilibrium distribution of cationic probes. From measurements with microelectrodes on vacuoles isolated in simple ionic media, tonoplast E_m is in the range +5 mV to +20 mV [2–8], whereas the method based on the accumulation of cationic probes systematically indicates negative E_m values in the range −50 to −80 mV [9–14].

This paper compares, for the first time, the use of microelectrodes and of the cationic probe tetraphenylphosphonium (TPP^+) for determining tonoplast E_m on the same vacuolar preparation (non-energized vacuoles).

The first part of this study [15] demonstrated binding of cationic probes on liposomes and on vacuoles. The extent of binding may affect the E_m value calculated from the distribution of the probe between the vacuoles and their suspension medium, and may consequently be a source of discrepancy between microelectrode and probe measurements. Thus, the relative importance of the binding in the

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; TPP, tetraphenylphosphonium; DiS-C_2-(5), 3,3′-diethylthiadicarbocyanine iodide; ANS, 8-anilino-1-naphthalene sulphonate.

overall probe accumulation by vacuoles has to be quantified.

Materials and Methods

Vacuoles from *Acer pseudoplatanus* cells were isolated from a cationic treatment of protoplasts as described elsewhere [16,17]. Vacuoles from *Catharanthus roseus* cells were purified from protoplasts as described in the first paper of this series [15]. Vacuoles from beet root tissue were isolated mechanically [5].

Lipids were assayed colorimetrically by the sulphophosphovanillin method, using a dioleoylphosphatidylcholine standard [18]. The assay of fatty acids by gas chromatography gave similar results. Proteins were assayed by the method of Bradford [19], using a bovine serum albumin standard. A modified Lowry method [20] gave similar results.

To estimate the ratio of tonoplast-bound proteins to the total vacuolar proteins, vacuoles were completely lysed by shaking in final 0.21 M sorbitol, and membranes were sedimented by centrifugation for 1 h at $100000 \times g$. Proteins were then assayed in the pellet and the supernatant.

The measurement of E_m on isolated vacuoles with microelectrodes has been described elsewhere [5].

Equilibrium dialysis experiments on isolated vacuoles were performed with 1 ml Teflon cells (Dianorm) and cuprophane membrane. Vacuoles were incubated with tetra[U-^{14}C]phenylphosphonium bromide (spec. act. 31.4 mCi/mmol) purchased from Amersham (reference CFA 651) and tritiated water. Each dialysis was run for 30 min, then aliquots (five samples of 100 μl per half dialysis cell) were mixed with 5 ml scintillation liquid (Picofluor 30) and counted in the double-labelling configuration (Intertechnique SL 4000). The percentage of recovery of the radioactivity at the end of dialysis was higher than 99%. TPP^+ concentrations were proportional to the ratio $^{14}C/^3H$. Apparent vacuolar concentrations of TPP^+ were calculated assuming that TPP^+ molecules absorbed by the vacuoles were freely distributed in the internal vacuolar volume. This internal volume was estimated by counting the vacuoles and measuring 100 individual diameters. The mean vacuolar volume was the mean of individual volumes. The accumulation ratios of apparent internal concentrations to external concentrations were used to calculate the corresponding Nernst potentials.

The methods for measuring the electrophoretic mobility of vacuoles and calculating zeta potentials, surface charge densities and surface concentrations were described in the first paper [15].

Results and Analysis

Measurement of E_m on isolated vacuoles with microelectrodes

In media containing a neutral osmoticum (sorbitol or mannitol), organic buffers (Hepes, Mes, Tris), no inorganic anion and 0–20 mM K^+, the transtonoplast E_m measured with microelectrodes was positive irrespective of the precise composition of the buffer solution and of the plant species. The mean E_m values obtained on vacuoles isolated from cell suspensions, in a medium comprising 0.7 M mannitol/25 mM Tris-Mes (pH 7.2), were $+13.6 \pm 0.2$ mV ($n = 98$) for *A. pseudoplatanus* and $+10.6 \pm 0.4$ mV ($n = 29$) for *C. roseus*. The E_m value of vacuoles spontaneously released from protoplasts was not significantly different from that obtained with vacuoles purified after protoplast lysis induced by an osmotic shock (*C. roseus*) or by a polycation treatment (*A. pseudoplatanus*). In this latter material, E_m remained unchanged upon storage over ice up to 20 h. On vacuoles isolated from beet root tissue in 1 M sorbitol, 10–50 mM Hepes-KOH (pH 8.0), E_m values were $+17.3 \pm 0.2$ mV ($n = 58$) for sugar beet, $+9.6 \pm 0.1$ mV ($n = 50$) for fodder beet, and $+9.5 \pm 0.3$ mV ($n = 95$) for red beet [4,5,8].

After insertion of the microelectrode into the vacuole, the signal remained stable up to 20 min. For a given vacuole, the E_m value was maintained during a series of successive insertions and withdrawals of the microelectrode. It did not depend on the nature of the electrolyte used for filling the microelectrode (1 M KCl, 1 M NaCl or 0.5 M K_2SO_4).

Similar positive E_m values have been obtained on *A. pseudoplatanus* isolated vacuoles by Rona and Cornel [21,22], who demonstrated unambiguously by measuring the membrane resistance that the electrode tip was located inside the vacuole.

Measurement of E_m on isolated vacuoles with TPP^+

The accumulation of [^{14}C]TPP^+ by vacuoles isolated from *A. pseudoplatanus* or *C. roseus* cells was measured in equilibrium dialysis experiments, with external TPP^+ concentrations ranging from 2 to 50 μM (Table I). Apparent internal probe concentrations were calculated using the results of dialysis, the mean internal vacuolar volume obtained from optical determination, and assuming that the probe molecules were freely distributed inside this vacuolar volume. The corresponding Nernst potentials were in the range -40 to -90 mV, with mean values of -67 mV and -59 mV for *A. pseudoplatanus* and *C. roseus,* respectively. The important point is that transmembrane potentials measured on each preparation with microelectrodes were always positive ($+14$ mV).

Thus, for the first time, these contradictory E_m values have been obtained on the same vacuolar preparations with the two techniques, lipophilic cations or microelectrodes. This establishes clearly that the contradiction lies in the methods themselves, and is not linked to differences between plant species or procedures of vacuole isolation, as suggested by Leigh [1].

Degree of membrane binding in the overall accumulation of TPP^+: determination of the membrane surface area in vacuolar preparations

The validity of the E_m estimation obtained from TPP^+ is crucially dependent on the degree of membrane binding in the overall accumulation of TPP^+. The amount of membrane-bound TPP^+ in vacuolar preparations depends on two factors, namely the membrane surface area and the kinetic parameters of the binding (affinity constant and number of binding sites per unit membrane surface area). The latter were determined in the first paper of this series [15]. The former was studied on the one hand by optical counting, measuring the individual diameters of the visible vacuoles (i.e., diameters greater than 2 μm), and calculating a mean surface area from individual surface areas (Table II). On the other hand, small (0.3 μm) vesicles were observed in freeze-etching electronic micrographs (*C. roseus*). Since these vesicles were observed with laser scattering at 633 nm in the native vacuolar suspension, it was inferred that they were not produced by the freezing step. However, none of these methods enabled simultaneous estimation of the membrane surface areas of the

TABLE I

ABSORPTION OF TPP^+ BY VACUOLES ISOLATED FROM *ACER PSEUDOPLATANUS* OR *CATHARANTHUS ROSEUS* CELL SUSPENSIONS (EQUILIBRIUM DIALYSIS EXPERIMENTS), AND CALCULATION OF THE CORRESPONDING NERNST POTENTIALS

Vacuoles were incubated with [^{14}C]TPP^+ in a medium comprising 0.55 M Sorbitol/1 mM Na_2-EDTA/10 mM Hepes-KOH (pH 7.4). The TPP^+ absorbed by the vacuoles (nmol/ml vacuolar suspension) was transformed into equivalent internal concentrations assuming that TPP^+ molecules were freely distributed inside the internal vacuolar volume (estimated by counting the vacuoles, measuring the diameter of the vacuoles from an aliquot of the population, and calculating a mean vacuolar volume). The Nernst potential values were calculated from internal and external concentration values. E_m measured with microelectrodes in the same medium was $+14$ mV, for *A. pseudoplatanus* and *C. roseus* vacuoles.

Vacuolar prepn.	External concn. of TPP^+ (μM)	Absorbed TPP^+ (nmol·ml^{-1})	Equivalent internal concn. (μM)	Calculated Nernst potential (mV)
A. pseudo-platanus	2	0.04	10	-41
	7	0.37	95	-63
	15	1.17	300	-75
	34	1.75	448	-65
	50	7.00	1795	-91
C. roseus	1	0.19	20	-63
	8	0.89	92	-62
	15	0.88	91	-45
	32	3.85	396	-64
	49	5.27	543	-61

TABLE II

DETERMINATION OF THE MEMBRANE SURFACE AREA IN VACUOLAR PREPARATIONS OF *ACER PSEUDOPLATANUS* AND *CATHARANTHUS ROSEUS*

Surface areas, lipids and membrane-bound proteins are expressed in $Å^2$ or ng per visible vacuole. The visible surface area was calculated from individual diameters measured by microscopy in an aliquot of the vacuolar suspension and the corresponding individual surface areas. Lipids or proteins were assayed as described in Materials and Methods. The amount of membrane-bound proteins was calculated from the total (bound + free) proteins and the ratio (bound/bound + free) determined on *C. roseus* vacuoles. The total surface area was calculated from these amounts of lipids or proteins and the specific membrane areas: $3.1 \cdot 10^{13}$ $Å^2$ per ng lipids and $4.4 \cdot 10^{13}$ $Å^2$ per ng proteins.

Vacuolar preparation	Visible surface area ($Å^2$) ($\times 10^{-11}$)	Membrane lipids [c] or proteins [d] (ng)	Total surface area ($Å^2$) ($\times 10^{-12}$)
A. pseudoplatanus [a]	1.60	0.120 [c]	3.72
C. roseus [a]	1.74	0.051 [c]	1.58
C. roseus [a]	1.35	0.051 [d]	2.24
C. roseus [b]	3.68	0.042 [d]	1.85

[a] Vacuoles purified according to the classical procedure (flotation at $160 \times g$).
[b] Vacuoles purified without centrifugation (flotation at $1 \times g$).

vacuoles and of vesicles present in the same preparations. An alternative approach which did not depend on the size of the particles but relied only on the amount of membrane material in the preparation was used, as follows.

The specific membrane surface areas corresponding to a unit weight of lipids or proteins were calculated from three parameters, i.e., the tonoplast density, the protein-to-lipid ratio (w/w) and the membrane thickness. A good agreement exists between the results obtained by different authors for a tonoplast density of $1 \cdot 10$ g $\cdot$ cm^{-3} [23–26]. A mean value of 0.7 was calculated for the protein-to-lipid ratio from results of the literature on tonoplast isolated from red beet [27,28] or from yeast [29]. The specific membrane surface areas, calculated from the above values and assuming a membrane thickness of 50 Å, were $3.1 \cdot 10^{13}$ $Å^2$/ng lipid and $4.4 \cdot 10^{13}$ $Å^2$/ng protein (values for one side of the membrane). For estimating the whole membrane content in the vacuolar suspension, lipids or proteins were assayed on the vacuoles used in dialysis, in parallel with counting and measurement of diameters. The amount of membrane-bound proteins was calculated from the total (bound plus free) amount of proteins in the preparation as indicated in Materials and Methods. The total membrane surface area per visible vacuole was estimated from the specific areas and the amounts of lipids or membrane proteins per vacuole. The results of these calculations (Table II) indicate that the surface area determined from the amount of membrane material is 5-fold to 23-fold higher than that of the visible vacuoles.

Degree of membrane binding in the overall accumulation of TPP^+: estimation of the extent of TPP^+-binding in a vacuolar preparation

The amounts of TPP^+ bound per unit of membrane surface area of *C. roseus* and *A. pseudoplatanus* vacuoles were calculated as follows: (i) the internal concentration of free probe was calculated from the external one (1 μM) and the E_m value measured with microelectrodes (+13 mV), using the Nernst law; (ii) the surface concentrations of probe were calculated from external and internal bulk concentrations and measured external surface potentials [15], assuming a symmetrical membrane and using the Boltzman law; (iii) the amounts of probe bound to external and internal surfaces were evaluated using these surface concentrations and the intrinsic binding parameters determined in microelectrophoresis experiments [16]. The total amount of probe bound on both faces of the membrane was obtained by adding these two amounts ($1.55 \cdot 10^{-20}$ nmol $\cdot$ $Å^{-2}$ for *A. pseudoplatanus* and $0.55 \cdot 10^{-20}$ nmol $\cdot$ $Å^{-2}$ for *C. roseus*).

TABLE III

COMPARISON BETWEEN APPARENT NERNST POTENTIALS, COMPUTED FROM APPARENT INTERNAL TPP^+ CONCENTRATIONS TAKING ACCOUNT OF TPP^+ BINDING TO THE TONOPLAST, AND NERNST POTENTIALS CALCULATED FROM EQUILIBRIUM DIALYSIS EXPERIMENTS ON *ACER PSEUDOPLATANUS* AND *CATHARANTHUS ROSEUS* VACUOLES

Apparent internal concentrations were calculated as the sum of internal concentration of free TPP^+ (calculated using the Nernst law, from the external concentration of 1 μM, and assuming that $E_m = +13$ mV) and internal concentration of bound TPP^+ (calculated from the amounts of probe bound per unit surface area, the total membrane surface area per vacuole and the mean volume of visible vacuoles). The amounts of membrane-bound TPP^+ (nmol·Å^{-2}) were calculated as described in the text, from the external concentration of TPP^+ (1 μM), a transtonoplast E_m of +13 mV, external and internal surface potentials of −66 and −14 mV, respectively, and the binding laws determined in microelectrophoresis experiments [15] (intrinsic binding parameters for *A. pseudoplatanus* vacuoles: $K_i = 0.68$ mM, $N = 0.446 \cdot 10^{-3}$ elementary charge·Å^{-2}, and for *C. roseus* vacuoles: $K_i = 1.2$ mM, $N = 0.281 \cdot 10^{-3}$ elementary charge·Å^{-2}). The value of the inner surface potential (−14 mV) was estimated using the measured internal pH and ionic strength, and assuming a symmetrical membrane. The inner surface potential was assumed to be the same as the one determined for the external surface in the same ionic conditions. Nernst potentials were calculated either from the apparent TPP^+ concentrations or from equilibrium dialysis data. The ratio of total surface to visible surface was calculated from the data of Table II.

Vacuolar preparation	Apparent internal TPP^+ concn. (μM) (% due to binding)	Nernst potentials (mV) calculated from:		Total surf./ visible surf.
		binding	dialysis	
A. pseudoplatanus [a]	14.0 (96)	−67	−67	23
C. roseus [a]	2.2 (73)	−20	−59	9
C. roseus [a]	4.2 (86)	−37	−21	17
C. roseus [b]	1.2 (50)	−4	−11	5

[a] Vacuoles purified according to the classical procedure (flotation at 160 × *g*).
[b] Vacuoles purified without centrifugation (floation at 1 × *g*).

TABLE IV

EFFECTS OF FCCP AND Mg-ATP ON TPP^+ BINDING ON ISOLATED VACUOLES (*ACER PSEUDOPLATANUS*) DETERMINED FROM MICROELECTROPHORESIS MEASUREMENTS

The effect of TPP^+ (2 mM) on surface charge densities (σ) was determined in control medium (10 mM KCl/0.7 M mannitol/1 mM Na_2-EDTA/10 mM Hepes (pH 7.4)), and in control medium supplemented with either FCCP (10 μM) or Mg-ATP (1 mM). The corresponding changes of surface charge density ($\Delta\sigma$) induced by TPP^+ were calculated. The effect of each factor (FCCP or Mg-ATP) on TPP^+ binding was expressed as variations (%) of $\Delta\sigma$ TPP^+.

Medium	Surface charge density (σ) (e·Å^{-2}) (×10^4)	$\Delta\sigma$ induced by TPP^+ (e·Å^{-2}) (×10^4)	Variation of $\Delta\sigma$ TPP^+ (%)
Control	−7.04	–	
Control + TPP^+	−4.45	+2.59	
Control + FCCP	−6.93	–	
Control + FCCP + TPP^+	−5.07	+1.86	−28
Control	−8.32	–	
Control + TPP^+	−5.49	+2.83	
Control + Mg-ATP	−9.05	–	
Control + Mg-ATP + TPP^+	−5.16	+3.89	+38

Calculation of apparent Nernst potentials

The amounts of probe bound per vacuole were estimated from the amount of bound probe per unit surface area and the total membrane surface (Table II). Total amounts of bound plus free TPP^+ were transformed into apparent concentration using the volume of the visible vacuoles (Table III). The corresponding Nernst potentials deduced from the ratio of the apparent internal TPP^+ concentration to the external concentration were negative, ranging from -4 to -67 mV (Table III). The values of the apparent E_m calculated in this way are in good agreement with those directly obtained from dialysis data (Table III). These calculations demonstrate that apparent negative potentials due to TPP^+ binding on membranes could be obtained on isolated vacuoles with the TPP^+ method, even when the true E_m is taken as positive.

Effect of FCCP and Mg-ATP on TPP^+ binding

Microelectrophoresis measurements on *A. pseudoplatanus* vacuoles demonstrated that 10 μM FCCP did not significantly modify the negative surface charge of vacuoles (Table IV). It apparently inhibited the TPP^+ binding, as seen by the reduction of the TPP^+-induced surface charge shift in vacuoles. In contrast, Mg-ATP increased both the net surface charge of vacuoles and the efficiency of the lipophilic cation to depolarize the surface of vacuoles, probably by favouring its binding (Table IV).

Discussion

Binding of lipophilic cations to the membrane surface is responsible for the apparent negative E_m

The binding of TPP^+ to vacuolar membrane demonstrated in the precedent paper results in an overestimation of the ratio of the intravacuolar concentration of free TPP^+ to the extravacuolar one, and thus in a shift of the calculated E_m towards negative values. Marin et al. [10,11] and Komor et al. [13] proposed that binding of probe on vacuoles was less than 10% of probe accumulation. In these reports the extent of binding was estimated from the amount of probe associated with broken lutoids [10] or boiled vacuoles [13], assuming that these treatments did not modify membrane properties. We used a different approach to evaluate the extent of binding of TPP^+ in a vacuolar preparation by calculating its value from the binding parameters measured on intact vacuoles and reported in the preceding paper [15].

The total membrane surface computed from lipid or protein levels in vacuolar preparations was markedly higher than the surface of visible vacuoles calculated from diameters measured under the microscope. The laser diffractometry data suggest that this discrepancy is due to a large size heterogeneity in vacuolar preparations. The purity of vacuolar suspensions, assessed by enzymatic measurements, was higher than 95% (to be detailed elsewhere). Thus, it was inferred that small vesicles result from vacuole bursting and are excluded from the so-called 'visible' vacuoles. Assuming that the suspensions contain 0.3 μm vesicles and 20 μm vacuoles, simple calculations with the results of Table III show that the contribution of the vesicles to the total membrane surface is 75-fold to 100-fold larger than their contribution to the total internal volume.

Vacuoles of *C. roseus* were isolated very gently to minimize bursting (isolation by floatation at 1 g). They were large (mean diameter 33 μm, from protoplasts of mean diameter 35 μm), with a small difference between the surface area calculated from the lipid and protein contents and that of visible vacuoles. Table III shows that such vacuoles presented a low TPP^+ potential (-11 mV). Conversely, visible vacuoles isolated by the classical procedure (flotation at 160 g) were smaller (mean diameter 20 μm), with a large difference between the surface calculated from lipid or protein contents and the surface of visible vacuoles. Table III shows that such a difference between surfaces is associated to a high potential value (-60 mV). As indicated by the theoretical Nernst potentials calculated from binding data, the relation between this excess of surface and the Nernst potential calculated from dialysis data is probably due to the TPP^+ binding component.

This study demonstrates that the apparent values of TPP^+ potentials measured globally on a vacuolar preparation could be negative in spite of the true positive potential measured with microelectrodes on the visible vacuoles in the same preparation. However, it is necessary to evaluate

how far this conclusion is dependent on the values of the parameters used to calculate the extent of binding, i.e., (i) those concerning the intensity of binding per unit surface and (ii) those related to the estimation of the membrane surface which binds the probe.

As to (i), the first part of this study [15] discussed the concept that the values of these parameters probably correspond to an underestimation of the binding per unit surface, because intramembrane binding cannot be detected by microelectrophoresis. Correcting for this phenomenon would lead to more negative calculated E_m values in Table III and thus would reinforce the above conclusion.

As to (ii), the estimation of the total membrane surface can be affected by uncertainties on the parameters used in calculations. The protein and lipid contents of vacuolar preparations were assessed by different methods (colorimetric methods of Bradford or of Lowry for proteins, colorimetric method or gas chromatography for lipids) which gave similar results. The tonoplast density and the protein-to-lipid ratio have not been determined directly on the actual vacuolar suspensions used in dialysis experiments, but values obtained from the literature are in good agreement for various materials [23–29]. The ratio of tonoplast proteins to total vacuolar proteins (0.50) determined on *C. roseus* vacuoles agrees with published values, which vary between 0.5 and 0.7 [27,30,31]. As concerns the membrane thickness, no value is available specifically for tonoplast, so we took 50 Å. If a higher value (75 Å) is used in calculations, in order to increase the security of the conclusion, the computed membrane surface is decreased only by 27%, and the computed Nernst potentials are rendered less negative by 5 to 7 mV.

Thus, critical analysis of the parameters used in the calculations substantiates the conclusion that negative E_m values are artifactual.

Is probe binding to the vacuolar membrane responsible for the negative E_m values already published in the literature?

The above discussion emphasizes the necessity, when estimating the extent of probe binding to membranes, to take into account the total membrane surface of a vacuolar preparation and not simply that corresponding to the visible vacuoles. This problem was neglected in previous studies by authors using cationic probes for estimating tonoplast E_m where the internal cation concentrations were based on the measurement of the total vacuolar volume (the ^3H_2O-accessible, ^{14}C-impermeant volume). It is difficult to evaluate the importance of the probe binding in these studies as the necessary data are incomplete in the various papers. However, the following calculations concerning the experiments reporting negative E_m values sustain the hypothesis that these negative values could be artifactual due to probe binding.

Lipophilic cations have been used as membrane potential probes on vacuoles from different origins. Transtonoplast potential values obtained by Doll and Hauer [9] on vacuoles isolated from red beet roots, or by Komor et al. [13] on vacuoles isolated from sugarcane cell suspensions were −55 mV and −80 mV, respectively (Table V). These values were obtained at low external ionic strength (0–5 mM K^+), and thus attributed to a K^+-diffusion potential.

The extent of probe binding to the vacuolar membrane in these studies may be assessed as follows: the amount of probe bound per vacuole was calculated from the amount of bound TPP^+ per unit surface area and the membrane surface per vacuole. This area was estimated using the vacuolar protein content [27,31], ratios of membrane proteins to total proteins specific to beet or sugar-cane vacuoles (0.62 and 0.67, respectively [27,31], and the specific surface area per nanogramme protein. Unfortunately, no data are available on these red beet or sugar-cane preparations concerning the vacuolar size (volume or diameter), so calculations of apparent internal concentrations (bound + free probe) were made using three different diameters corresponding to a likely range of values (Table V). In each material, the range of apparent potential values overlaps the experimental values. It must be noted that these calculations were made assuming a real positive E_m (+13 mV), and demonstrate that the negative potential difference obtained on beet or sugar-cane vacuoles with lipophilic cations could be accounted for by the binding of these cations to membrane. Indeed, Thom and Komor [32,33] have recently published positive E_m values (0 to +3 mV) for

TABLE V

COMPARISON BETWEEN EXPERIMENTAL E_m VALUES, OBTAINED FROM CATIONIC PROBE ACCUMULATION IN *BETA VULGARIS* AND *SACCHARUM* SPECIES VACUOLES, AND APPARENT NERNST POTENTIALS COMPUTED FROM THE EVALUATION OF THE AMOUNT OF MEMBRANE-BOUND PROBE AND OF THE CORRESPONDING INTERNAL PROBE CONCENTRATIONS

Experimental E_m values are those obtained from lipophilic cation accumulation in *B. vulgaris* [9] and *Saccharum* sp. [13] vacuoles. The amount of probe bound per vacuole was calculated from the amount of bound TPP^+ per unit surface area ($1.55 \cdot 10^{-20}$ nmol·Å^{-2}) and the membrane surface area per vacuole. This area was estimated using the protein content per vacuole [27,31], the ratio of membrane-bound proteins to the total vacuolar proteins (0.62 for beet vacuoles [27], and 0.67 for sugar-cane vacuoles [31]) and the specific surface area $4.4 \cdot 10^{13}$ Å^2/ng protein. The apparent internal probe concentrations were calculated using vacuolar volumes deduced from three diameters: a, 20 μm; b, 15 μm; and c, 10 μm. The corresponding Nernst potentials were then calculated.

Vacuolar preparation	Experimental E_m (mV)	Computed Nernst potential					
		Apparent int. probe conc. (μM)			Nernst potential (mV)		
		a	b	c	a	b	c
Beta vulgaris	−55	2.6	5.4	16.8	−25	−43	−71
Saccharum sp.	−80	4.0	8.6	27.8	−35	−55	−85

sugar-cane vacuoles (measured with the lipophilic anion thiocyanate). Although the resolution of lipophilic probes is questionable around 0 mV, this result suggests that those they had obtained with TPP^+ (−80 mV) were artifactual [13].

The quenching of the fluorescent cation diS-C_2-(5) [9,14] and the insensitivity of the fluorescence of the anion ANS upon addition of a vacuole suspension to the medium [9] have been considered as indicative of negative E_m in vacuoles. Concerning the former probe, it is classically admitted that the quenching may result from interaction with the membrane as well as from accumulation in the internal volume [34]. The interaction with membranes is known to exist in absence of any transmembrane potential on sarcoplasmic reticulum, with 50% quenching [35,36]. This phenomenon occurs in vacuoles [15]. Due to this drawback, the experimental procedures to estimate E_m must necessarily correct for the E_m-independent quenching [36]. The methods used on vacuoles [9,14] did not allow this correction, so the data on the quenching of diS-C_2-(5) upon addition of vacuole suspensions are not evidence for negative E_m. Concerning ANS, the lack of quenching of fluorescence [9] cannot be taken as indicating negative E_m. Experimental conditions were not suitable for detecting a change in fluorescence upon the effect of positive E_m, since the free probe is highly fluorescent in viscous medium (1 M sorbitol) [37].

Effect of classical E_m effectors on negative transtonoplast potentials measured with cationic probes

The effect of high K^+ concentrations on vacuolar E_m measured with cationic probes is illustrated by Fig. 1, taken from the work of Komor et al. [13] on sugar-cane vacuoles. The depolarization of E_m induced by increasing external K^+ was taken by these authors as evidence that negative E_m corresponded to a K^+-diffusion potential [9,13]. This argument does not hold [15], as KCl primarily

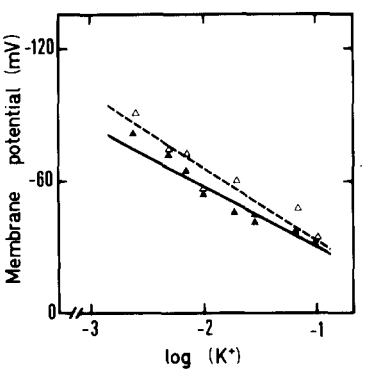

Fig. 1. Effect of external KCl concentrations on membrane potentials measured on *Saccharum sp.* vacuoles with the lipophilic cation TPP^+ (△) (taken from Komor et al. [13]), and on apparent Nernst potentials computed for *A. pseudoplatanus* vacuoles taking account of TPP^+ binding on the tonoplast (▲). The amounts of TPP^+ bound to *A. pseudoplatanus* vacuoles were calculated using the binding law determined in microelectrophoresis experiments and the effect of KCl on the binding of TPP^+ [15]. The amount of internal free probe was calculated assuming a real transtonoplast positive E_m (+13 mV). Apparent Nernst potentials (mV) were calculated from the total amounts of bound and free probe transformed to apparent internal concentrations using the internal volume of visible vacuoles. Slopes of the curves: *A. pseudoplatanus* 30 mV per unit log (K^+); *Saccharum* sp. 35 mV per unit log (K^+).

suppresses TPP^+ binding to liposomes and vacuoles: increasing KCl concentration decreased the TPP^+ binding in a way that could be misinterpreted as an action on diffusion potentials. Apparent Nernst potentials were computed for *A. pseudoplatanus* vacuoles, varying the external concentration of K^+ and taking into account the effect of K^+ on TPP^+ binding [15]. The amounts of TPP^+ bound per vacuole were calculated as described above, assuming constant values for E_m (+13 mV) and internal surface potential, and using values of external surface potentials described in Ref. 15 as a function of external K^+. The corresponding Nernst potentials were then calculated and compared to the experimental results of Komor et al. plotted in Fig. 1. The results show (Fig. 1) that the apparent negative potential is linearly decreased by KCl, with a slope of 30 mV per unit log[K^+], in good agreement with the experimental results of Komor et al. (slope of 35 mV per unit log[K^+]) [13]. Values around 30 mV were also obtained on beet vacuoles by Doll and Hauer [9] and Miller et al. [14]. Here again, our calculations demonstrate that the changes induced by the external K^+ on potentials measured with lipophilic cations can be accounted for by the binding phenomenon.

FCCP and ATP, considered as classical transmembrane potential effectors, were shown to modify the tonoplast E_m measured with cationic probes [9]. The variations induced by these effectors on TPP^+ binding (−28% for FCCP and +37% for Mg-ATP) (Table IV) are in the same range as the variations of potential induced by the same effectors on red beet vacuoles (−42% for FCCP, and +36% for Mg-ATP) [9]. From these results, it is clear that the apparent decrease or increase in accumulation of the cationic probe could not be construed as proof of membrane depolarization due to the uncoupler, or of membrane hyperpolarization due to tonoplast energization [9].

Conclusion

In summary, the simultaneous measurements of E_m with both microelectrodes and TPP^+ have demonstrated that the results of the two methods are truly contradictory.

On the one hand, the above analysis shows that membranes bind lipophilic cations in such a way that they simulate free ion accumulation driven by transmembrane potential [15]. Extensive binding of lipophilic cations to membrane or other cell components has been recognized by many authors and put forward to explain discrepancies between potential values measured with these probes and those obtained by other techniques [38–40]. Although several correction procedures have been applied (reviewed in Ref. 41), few authors have quantified the extent of probe binding taking account of the effect of surface potential on this binding [39,41,42]. The relative importance of TPP^+ binding to vacuole suspensions is high enough (50–96% of the overall accumulation) to simulate the existence of negative transmembrane potentials, and to account for the discrepancy between negative values measured with this probe and positive potentials obtained with microelectrodes. Furthermore, modulation of the apparent negative potential induced by different effectors and taken as consistent with negative E_m have herein been demonstrated to result from variations of probe binding. The characteristics of lipophilic cation binding have elsewhere been observed to be analogous to those expected for an accumulation of free ions, and such reports have concluded that these probes were unsuitable to measure transmembrane potentials on *Chlorella* [43] or *Phaseolus* [44] cells. Similarly, the results presented in this paper demonstrate that methods using lipophilic cationic probes are inadequate to measure transmembrane potentials on vacuolar suspensions.

On the other hand, the E_m values obtained with microelectrodes are not significantly affected either by membrane puncture with the electrode or by putative electrolyte leakage from the electrode tip inside the vacuole. They are of the same order irrespective of the procedure used to isolate the vacuoles (protoplast lysis for cell suspensions or mechanical rupture for beet root tissue). The developmental stage of the beet roots did not affect the results [4,5]. Nevertheless, the values obtained were correlated with the sugar-accumulating capacity of beet genotypes [4,5], which suggests that they are related to physiological properties. The influence of the medium composition on transtonoplast E_m has been studied on *A. pseudoplatanus* vacuoles [45]. The observed PD

variations were in accordance with a transmembrane origin, and may be accounted for by the hypothesis of a preferential transport system for endogenous carboxylates. This hypothesis was supported by the influence of external anions (especially citrate^{3-}) on E_m values and by the ionic composition of vacuolar sap. Further investigations are needed to characterize the transport systems involved.

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