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pK_a VALUES AND PARTITION COEFFICIENTS OF NITROXIDE SPIN PROBES FOR MEMBRANE BIOENERGETICS MEASUREMENTS

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Knowledge of pK_a's is necessary to calculate intracellular/intravesicular pH values from nitroxide accumulation in cells or vesicles as detected with electron spin resonance (ESR) spectroscopy. pK_a values were confirmed in lipid vesicles of known internal pH. To help select probes that do not accumulate in lipid membranes, octanol/buffer partition coefficients of uncharged nitroxides were determined. As an application of selected probes, pH gradients and internal aqueous volumes were analyzed in mitochondria (one internal compartment) and in the cyanobacterium Synechococcus 6311 (two internal compartments). The combination of 3-carboxy-, 3-amino- and 3-aminocarbonyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl was found to be most satisfactory for determinations of internal pH and volumes.

KEY WORDS: nitroxides, dissociation constants, partition coefficients, pH indicators, mitochondria, Synechococcus.

ABBREVIATIONS: bis-Tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]-propane; CAPS, 3-[cyclohexylamino]-1-propane-sulfonic acid; CCCP, meta-chlorocarbonylcyanophenylhydrazone; CHES, 2-[N-cyclohexylamino]-ethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; KΦ, 2,5-dihydro-2,2,5,5-tetramethyl-3-[(triphenylphosphonio)methyl]-1*H*-pyrrol-1-yloxyl bromide; MES, 2-[N-morpholino]-ethanesulfonic acid; NiTEPA, nickel tetraethylenepentamine sulfate; Tris, tris[hydroxymethyl]-aminomethane.

INTRODUCTION

The measurement of transmembrane pH gradients can provide information for understanding energy conversion mechanisms. Major techniques to measure intracellular pH values are: a) radioactive probes, analyzed in conjunction with flow dialysis,[1] b) non-fluorescent substances which are converted to fluorescent probes in cells by

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cytosolic hydrolases,[2] and c) spin labeled probes (nitroxides) analyzed by ESR spectroscopy.[3] These techniques avoid separation of cells from the suspension medium prior to the pH measurement. Each method has its drawbacks. With method a), membrane binding is difficult to assess. With method b), long loading times of cells with the non-fluorescent probe and fluorescence quenching might pose problems. Method c) may pose difficulties because of a loss of nitroxide signal due to reduction of nitroxides to ESR silent hydroxylamines[4] and line broadening of the internal signal. The latter problems can be corrected for (see Materials and Methods). The advantages of the ESR method include small samples, high sensitivity, and easy detection of membrane-bound probes (including binding due the membrane surface potential),[5] thus yielding concentrations in the osmotically active compartments. Control of dissolved gases can be achieved as well by use of gas permeable tubing.

For determination of the internal pH with ESR,[3,6–11] nitroxide-labeled weak amines and weak acids are used in combination with pH insensitive volume probes. The external signal is broadened by a membrane-impermeable quenching agent (e.g. by NiTEPA). By comparing the height of the ESR signal in the absence and presence of the quencher, the intracellular/intravesicular fraction of the probe is obtained.[3]

From a) the distribution ratios of acids and amines between the vesicle/cell interior and the surrounding medium, b) the volume ratio of both compartments and c) the *pK* values the intravesicular/intracellular pH can be calculated for one[12] and two compartment systems.[13] Weak amines accumulate in more acidic aqueous compartments whereas acids concentrate in more alkaline compartments. The method relies on the assumption that only the uncharged species is membrane permeable.

MATERIALS AND METHODS

Chemicals

Asolectin (soy bean phospholipids) was from Associated Concentrates, Woodside, NY. CCCP and the buffer substances were obtained from the Sigma Chemical Company, St. Louis, Missouri. Stock solutions of nitroxides at 10 mM were prepared in water (**1, 11** and **17–25, KΦ**), in 5 mM NaOH (**2–6**), or in 5mM HCl (**7–10,12–16**). (For correlating numbers with chemical structures see Tables I and II). Nickel tetraethylene-pentamine sulfate (NiTEPA) was prepared by mixing equimolar amounts of nickel sulfate and tetraethylenepentamine and subsequent titration to pH 7.0 with sulfuric acid. The nitroxides **1,7** and **19** were obtained from Molecular Probes, Junction City, Oregon. The nitroxides **5,6, 12–14, 21–24** were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin, nitroxide **8** was obtained from Sigma Chemical Company, St. Louis, Missouri. The nitroxides **2**[14], **9, 10** and **KΦ**[4], and **18**[15] were prepared as described. The nitroxides **3** and **4** were a gift of Dr. Zh. Wang and Dr. J.F.W. Keana, University of Oregon Eugene.[16] The synthesis of the nitroxides **17**, **20** and **25** will be published elsewhere (Hankovszky, in preparation).

3-[[N-[2-(Dimethylamino)-ethyl]-N-methylamino]methyl]-2,5-dihydro-2,2,5,5-tetramethyl-1 H -pyrrol-1-yloxyl (11)

2,5-dihydro-3-formyl-2,2,5,5-tetramethyl-1*H*-pyrrol-1-yloxyl[15] (337 mg, 2.0 mol) N,N,N′-trimethylethylenediamine (Aldrich) (307 mg, 3.0 mmol) and sodium cyanoborohydride (126 mg, 2.0 mmol) were stirred in dry methanol for one day, then

TABLE I

Hyperfine splitting constant a_N, pK_a values and partition coefficients of spin probes. For types of nitroxides see Results. pK_a values for **11** were determined by titration. For partition coefficients mean values of three measurements are presented, the standard deviation was less than 5%.

Nitroxide	Type	*R*	a_N (Gauss) protonated form	a_N (Gauss) unprotonated form	pK_a	Partition coefficient at pH 2.5	pH 7	pH 12
Acids								
1	I	*R*1: –COOH, *R*2 = *R*3: –H	16.31	16.49	3.90 ± 0.10	6.07	0.01	
2	I	*R*3: –COOH, *R*1 = *R*2: –H	16.13	16.34	4.65 ± 0.12	3.02	0.03	
3	I	*R*1 = *R*2: –COOH (cis), *R*3: –H	15.98(pH < 1.0)	16.39(pH > 6.5)	see Discussion	2.90	0	
4	I	*R*1 = *R*2: –COOH (trans), R3: –H	16.01(pH < 2.7)	16.20(pH > 6.0)	see Discussion	0.32	0	
5	II	–COOH	16.17	16.39	3.65 ± 0.10	13.1	0.02	
6	III	–COOH	17.25	17.33	4.00 ± 0.35	13.3	0.03	
Amines								
7	I	*R*1:–NH_2, *R*2 = *R*3: –H	15.89	16.33	7.45 ± 0.05		0.20	10.8
8	I	*R*1: –CH_2NH_2, *R*2 = *R*3: –H	16.25	16.53	8.80 ± 0.12		0.02	2.27
9	II	–$CH_2N(CH_3)_2$	16.35	16.51	8.20 ± 0.30		1.98	5.00
10	II	–CH_2N⬡	16.24	16.50	8.70 ± 0.07		6.34	76.4
11	II	–$CH_2N(CH_3)CH_2CH_2N(CH_3)_2$	n.d	n.d	pK_{a1}: 3.60 pK_{a2}: 8.20		0.15	8.15
12	III	–NH_2	17.18	17.38	9.10 ± 0.10		0.04	3.50
13	III	–$NHCH_3$	n.d	n.d	9.06 (Ref.[22])		0.05	4.78
14	III	–$N(CH_3)_2$	n.d	n.d	8.57 (Ref.[22])		0.05	5.29
15	IV	–CH_2N⬡	16.85	17.04	9.20 ± 0.15		2.65	37.8
16	IV	–CH_2N⬡(O)	16.87	17.05	6.60 ± 0.20		9.47	13.8

TABLE II
Partition coefficients of volume probes between 1-octanol and buffer and internal volumes found with liposomes. For types of nitroxides see Results.

Volume probe	Type	*R*	Partition coefficient	$V_{liposomes}/V_{total}$
17	I	*R*1 = *R*2 = *R*3: – H	10.1	n.d.
18	I	*R*1: – CH_2OH, *R*2 = *R*3: – H	2.20	0.036
19	I	*R*1: – $CONH_2$, *R*2 = *R*3: – H	0.61	0.034
20	II	–H	20.6	0.096
21	II	$-CONH_2$	1.79	0.035
22	III	–H	70.8	0.144
23	III	–OH	4.22	n.d.
24	III	= O	1.32	n.d.
25	IV	–H	60.8	n.d.

evaporated to dryness in vacuo. The residue was diluted with saturated sodium chloride solution, treated with aqueous (10%) sodium hydroxide, and extracted with chloroform (3 × 20 ml). The organic phase was dried ($MgSO_4$) and evaporated in vacuo. The residue was purified on silica gel (Merck Silica gel 60) preparative plate with chloroform-ether (1:1) to give **11** as thick yellow oil. Yield 346 mg (68%). Anal. calcd. for $C_{14}H_{28}N_3O$ (M^+ 254), C, 66.10; H, 11.09; N, 16.52. Found: C, 66.18; H, 10.98; N, 16.43%.

*1,2,5,6-Tetrahydro-2,2,6,6-tetramethyl-4-(1-piperidinylmethyl)-pyridin-1-yloxyl (**15**)*

1,2,5,6-Tetrahydro-4-formyl-2,2,6,6-tetramethyl-pyridin-1-yloxyl[17], (365 mg, 2.0 mmol) and piperidine hydrochloride (1.46 g, 12.0 mmol) were stirred in dry methanol (60 ml) with sodium cyanoborohydride (126 mg, 2.0 mmol) at room temperature for one day then worked up as described above for **11** to give **15** as orange oil. Yield

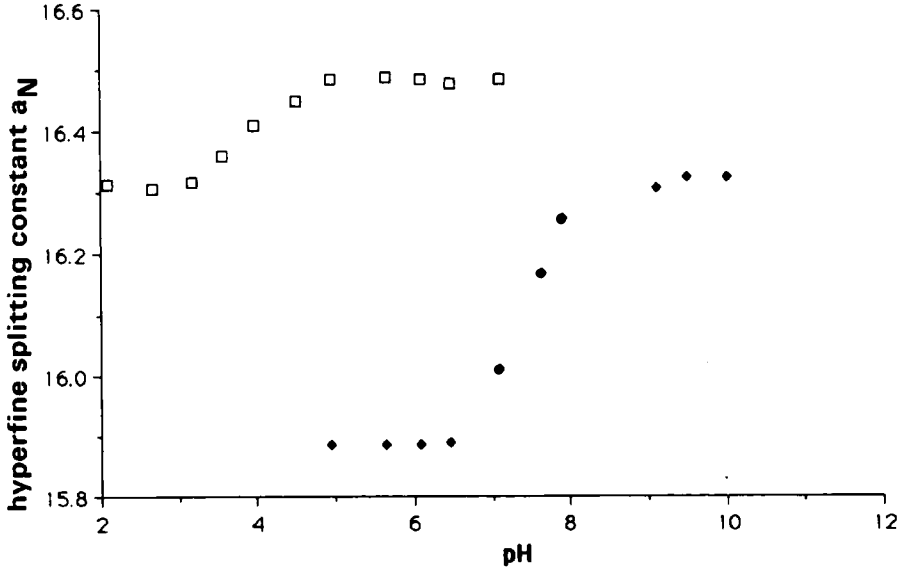


FIGURE 1 Hyperfine splitting constant a_N (in Gauss) as a function of pH for nitroxides **1** (□) and **7** (◆). The standard deviation for a_N was ±0.05 G, that for pH was ±0.05 pH units.

412 mg (82%). Anal. calcd. for $C_{15}H_{27}N_2O$ (M^+ 251), C, 71.67; H, 10.83; N, 11.14. Found: C, 71.72; H, 10.90; N, 11.07%.

*1,2,5,6-Tetrahydro-2,2,6,6-tetramethyl-4-(4-morpholinylmethyl)-pyridin-1-yloxyl (**16**)*

Compound **16** was prepared as described above with morpholine hydrochloride as the amine component to give **16** as deep yellow oil. Yield 355 mg (79%). Anal. calcd. for $C_{14}H_{25}N_2O_2$ (M^+ 253), C, 66.37; H, 9.95; N, 11.06. Found: C, 66.28; H, 10.02; N, 11.12%.

pK_a Values

pK_a values of nitroxides were determined at 25° C from the pH dependent shift of a_N as described.[18,19] An example is shown in Figure 1. An advantage of this method is the small sample volume (50 μl) and the low concentrations needed (10^{-5} to 10^{-4} M). The following buffers were used after titration with KOH and adjustment with KCl to yield an ionic strength of 0.2 M: oxalic acid (pH 1.0–2.5), citric acid (pH 2.5–6.0), MES (pH 6.0–6.7), HEPES (pH 6.7–8.0), Tris (8.0–9.0), CHES (pH 9.0–10.0), CAPS (pH 10.0–11.1), phosphoric acid (pH 11.1–12.0). The pK_a value for **11** was determined by titration of a 10 mM solution in water with 50 mM HCl. For pH measurements an Orion combination pH electrode 91-1 (Boston, Massachusetts) connected to a Corning pH meter 130 was used. Measurements were repeated three times.

Partition Coefficients

1-Octanol/buffer partition coefficients were determined as described.[20]

Liposomes

Liposomes (40 mg asolectin/ml were prepared as described[20] but 250 mM sodium phosphate pH 6.45 was used. After centrifugation 50 μl of the supernatant, 50 μl of 250 mM sodium phosphate (ph 7.45) and 1 μl of 10 mM nitroxide were mixed and the total ESR signal was measured in glass capillaries (VWR micropipettes, VWR Scientific Inc., San Francisco, CA). For determination of the internal signal 10 μl of 1.7 M of NiTEPA were included. The final external pH after addition of the quencher was 6.76.

Mitochondria and Cyanobacteria

Rat liver mitochondria were prepared as described earlier[21] and kept on ice till further use. Axenic cultures of Synechococcus PCC 6311 were grown photoautrophically and preconditioned for ESR spectroscopy as described.[8]

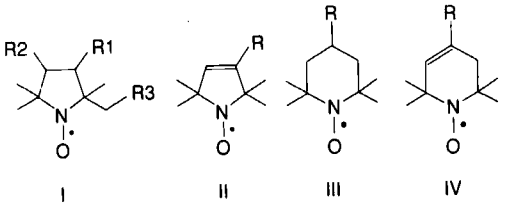
ESR Measurements of Internal pH and Volume

Measurements and calculations were performed as described previously.[8] Signal heights were extrapolated to the time point of mixing to the sample to correct for nitroxide reduction. Depending on the nitroxide a loss in internal signal height

between 5 and 30% within 5 min was observed in mitochondria and illuminated *Synechococcus* (not shown). Internal heights were corrected for line broadening using calibration curves signal height versus oxygen concentration. The distribution ratios between the interior of mitochondria/bacteria and the external medium were independent of the concentration of added nitroxides up to 250 μM.

RESULTS

pK_a values and partition coefficients of the following nitroxides were determined (for *R* see Tables I and II):



To test the consistency of the pK_avalues, a pH gradient of 0.31 pH units (inside acidic) was formed across the membrane of liposomes. Since acids and amines are distributed in an opposite manner, the accuracy of measurements for acids was too low if a greater pH gradient was adjusted. All of the probes yielded internal pH values between 6.33 and 6.53 which compares to an expected internal pH of 6.45 (not shown).

TABLE III

Concentration ratios of nitroxides ($C_{internal}/C_{external}$) and pH gradients in mitochondria and cyanobacteria.

Rat liver mitochondria (state 4): Mitochondria kept on ice were diluted with 275 mM sucrose + 5 mM sodium phosphate and 10 mM Tris-HCL pH 7.0 + sodium salts of α-ketoglutarate, pyruvate, malate, β-hydroxybutyrate (2.5 mM each)[21] to a density of 1.2 mg protein/ml and incubated for 1 min at 25° C. CCCP (dissolved in dimethylsulfoxide) was added to a final concentration of 1 μM. Δp = protonmotive force = $-(2.3RT/F)\Delta pH + \Delta\psi$ (R,T and F are the gas constant, absolute temperature and Faraday constant, respectively). The standard deviation was ± 0.07 pH units for ΔpH and ± 4 mV for $\Delta\psi$ (n = 4).

Cyanobacteria: Cells were suspended in 50 mM HEPES/Bis-tris-propane, pH 7.0 to a density of 0.25 mg chlorophyll/ml, equivalent to 6.3 mg protein/ml. Steady state pH gradients were measured 1 minute after preincubation (6.3 mg protein/ml) under different bioenergetic conditions. The standard deviation was ± 0.07 or ± 0.10 pH units for the pH gradient across the cytoplasmic and the thylakoid membrane, respectively (n = 4).

Subscripts: ext = external, int = internal, cyt = cytoplasmic, thyl = thylakoid.

Mitochondria	pH_{ext}	C_{int}/C_{ext} nitroxide		pH_{int}	$\Delta pH_{int-ext}$	$\Delta\psi_{int-ext}$ (mV)	Δp (mV)
		1	**KΦ**				
control	6.9	0.71	70.4	6.8	−0.1	−109	−103
CCCP	6.9	0.48	1.4	6.6	−0.3	− 9	+ 9
Cyanobacteria	pH_{ext}	C_{int}/C_{ext} nitroxide		pH_{cyt}	pH_{thyl}	$\Delta pH_{cyt-ext}$	$\Delta pH_{cyt-thyl}$
		1	**7**				
light	7.0	3.13	1.02	7.5	6.1	0.5	1.4
dark aerobic	7.0	0.97	2.26	7.0	5.7	0.0	1.3
dark anaerobic	7.0	1.05	1.39	7.1	6.1	0.1	1.0

Mitochondria and Cyanobacteria

Intramitochondrial and intracyanobacterial pH values were measured using the nitroxides **1, 7** and **19** (Table III). No probe binding was found with any of these nitroxides. In mitochondria the electrical potential $\Delta\psi$ was determined similarly to pH measurements using **KΦ**. Data obtained with **KΦ** had to be corrected graphically for membrane binding.

DISCUSSION

pK_a Values of Nitroxides

pK_a values determined cover a range from 3.65 to 4.65 for the acids and from 6.60 to 9.20 for the amines. The magnitude of a_N and the accuracy of pK_a determinations decrease with increasing distance between the dissociable group and the nitroxide group (see standard deviations in Table I). The accuracy of pK_a determinations is ultimately limited by the standard deviation of 0.05 G for a_N. pK_a values of **5, 6** and **12** found from shifts of a_N are in good agreement with pK_a values determined from partition coefficients (3.72, 4.12 and 8.99, respectively[22]). pH dependent a_N shifts could not be used to separate the two pK_a's of **3** and **4**. Assuming equal contributions of both carboxylate groups to the a_N shift, a pK_{a1} of 3.3 and a pK_{a2} of 4.3 are suggested for both compounds.

Nitroxides for pH measurements should be selected with reference to Table I, choosing pK_a's that are not too far removed from the pH on either side of the membrane because, first, equilibration across the membrane might be too slow if the concentration of uncharged nitroxide is very low[23] and, second, an excessive accummulation of probe could change the internal buffer capacity, which in turn might affect the internal pH. With the broad range of available pK_a values, pH gradients can be assessed for organisms as diverse as from acidophilic to alkalophilic bacteria. Because of their extreme pK_a values dicarboxylic and diamino-nitroxides synthesized so far do not seem to be applicable to any organisms we are aware of. Future synthetic efforts should be directed towards probes with less extreme pK_a values.

Partition Coefficients

Ideally the probe does not accumulate in the lipid phase. By this criterion, nitroxides **18, 19, 21** (and **24**) are the preferred volume probes. All of these probes gave the same volumes with vesicles (Table II), whereas nitroxides **20** and **22** gave larger volumes, which could be attributed to unquenched membrane-bound signals (see Table II).

pH Measurement in Mitochondria and Cyanobacteria

In mitochondria there was no detectable ΔpH and $\Delta\psi$ was -108 mV at an external pH of 6.9. Using radiolabeled probes a ΔpH of 0.35 (inside alkaline) and a $\Delta\psi$ of -84 to -105 mV at an external pH of 7.00 have been reported for state 4 in the presence of 4.5 mM phosphate.[24] The discrepancy in ΔpH might have been caused either by differences in the medium composition, which has been demonstrated to have a large influence,[24] or by the use of a completely different set of indicators. A protonmotive force of nearly zero was observed in the presence of CCCP (Table III). As expected

the matrix pH became more acidic after addition of the uncoupler. For a recent report on ΔpH and $\Delta\psi$ in de-energized mitochondria using radiolabeled and fluorescent probes see Ref.[25] A study comparing radiolabelling, fluorescent and spinlabelling techniques under identical conditions would be desirable.

The cytoplasmic pH values of cyanobacteria (Table III) are in good agreement with previously published results where both compartments were analyzed.[4,26] The pH of the thylakoids is but less acidic than found with radiolabeled probes in *Synechococcus* PCC 6301.[26] Active transport of methylammonium is responsible for this discrepancy.[27]

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