TRANSMEMBRANE ELECTRICAL AND PH GRADIENTS OF *PARACOCCUS DENITRIFICANS* AND THEIR RELATIONSHIP TO OXIDATIVE PHOSPHORYLATION

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1. Introduction

Two different general mechanisms have been entertained to account for oxidative phosphorylation: the chemical coupling hypothesis and the chemiosmotic hypothesis. In the chemical coupling hypothesis the free energy available in the transfer of reducing equivalents from a more negative oxidation–reduction potential (such as NADH) to a more positive oxidation–reduction potential (such as cytochrome *c*) is transduced into chemical 'high energy' bond(s) which then drives ATP synthesis. This hypothesis which began with the discovery of oxidative phosphorylation [1,2] has evolved continuously over the years and produced several 'different' versions (e.g. [3–5]). The chemiosmotic coupling hypothesis, in contrast to the chemical coupling hypothesis, proposes a vectoral organization of the oxidation–reduction reactions in the membrane (fuel cell) which results in electrogenic transport of hydrogen ions from one side of the membrane to the other [6,7]. This creates a combined transmembrane electrical potential/pH gradient which acts as a 'high energy' intermediate.

If the membrane potential and pH gradient are to be functional intermediates between the respiratory chain and ATP synthesis, the inequality:

$$-\Delta G_{o-R} \geqslant -\Delta G_{H^+} \geqslant -\Delta G_{ATP} \qquad (1)$$

must hold for conditions of net ATP synthesis. That is, the negative free energy change accompanying transfer of reducing equivalents from the more negative E_h value to the more positive E_h value (ΔG_{o-R}) must be equal to or greater than the negative free energy change of transferring the protons down their electrochemical gradient ($-\Delta G_{H^+}$) and both must then be equal to or greater than the negative free energy change for hydrolysis of ATP ($-\Delta G_{ATP}$).

In the present communication we report an evaluation of the free energy relationships for oxidative phosphorylation in *Paracoccus denitrificans*, a unicellular prokaryotic organism with a respiratory chain very similar to that of mammalian mitochondria.

2. Experimental

Paracoccus denitrificans was grown aerobically at 30°C in a medium described [8]. The cells were harvested in the exponential growth phase and washed twice in Krebs-Henseleit saline containing 10 mM Tris–HCl, pH 7.4, instead of bicarbonate.

2.1. *Preparation of the spheroplasts*

The cells washed twice in 50 mM phosphate buffer pH 7.4 were suspended in 50 mM phosphate buffer containing 20% sucrose at a concentration which after 1:10 dilution in 50 mM phosphate buffer gave A_{750} 0.4 (Bausch and Lomb Spec 20, 1 cm light path). Lysosyme (3X recrystallized and lyophilized from Sigma, St Louis MO) 25 mg were added for 100 ml such cell suspension and the cells were incubated until the absorbance measured in distilled water after 1:10 dilution has dropped to below 10% of the initial

reading. The cell suspension was diluted 2-fold with Krebs-Henseleit–Tris medium and centrifuged for 10 min at 8000 rev/min in Sorvall refrigerated centrifuge. The pellet was washed once by centrifugation in the Krebs-Henseleit–Tris saline and suspended in the same medium.

2.2. *Incubation*

Intact cells or spheroplasts were incubated at 22°C in Krebs-Henseleit–Tris saline, pH 7.4, containing 10 mM glucose as substrate. Approx. 15 ml cell or spheroplast suspensions at 5 mg dry wt/ml was added to a 125 ml Erlenmeyer flask, the atmosphere changed to 100% O_2 and the flask shaken for 5–8 min. Aliquots taken at the end of the incubation had measured oxygen concentrations from 700–900 μM. The ATP, ADP, P_i, 3-OH-butyrate, acetoacetate and cytochrome *c* reduction were measured as in [9].

2.3. *Isotope distribution measurements*

Cells were preincubated as described above then the isotopically labeled compound added and incubation continued for the indicated time interval. The oxygen atmosphere was not maintained after isotope addition but an oxygen cathode was used to measure the respiratory rate and assure aerobiosis. The cells separated from the supernatant by centrifugation through versilube ® R-50 silicone oil from General Electric, Silicone Products Dept., Waterford NY using an Eppendorf microfuge or Beckman Model 152 microfuge. Aliquots of the suspension were transferred to centrifuge tubes and the centrifuge started at 2 min and 4 min after addition of the radioactive weak acids or weak bases. Complete separation of the cells through silicon oil was attained within 30 s starting the centrifuge. Accurately measured volumes of the supernatant fluid and the entire cellular pellet were transferred into aqueous counting solution (ACS) from Amersham/Searle Corporation and counting in a two channel Searle delta 300 liquid scintillation counter.

The [^{3}H]triphenylmethyl phosphonium bromide was a generous gift of R. Kaback, Roche Inst. Molec. Biol., Nutley, NJ 07110, and the [^{14}C]thiocyanate, ^3H_2O, [^{14}C]trimethylacetate, [^{14}C]methylamine, [^{14}C]dimethylamine, 5,5-di[^{14}C]methyl-2,4-oxazolidimedione and [^{14}C]polyethylene glycol (4000–6000) were obtained from New England Nuclear.

3. Methods

3.1. *Transmembrane pH gradient*

The transmembrane pH gradient was determined by measuring the distribution of a weak acid, trimethylacetate and weak bases, methylamine and dimethylamine. The method relies on high permeability of the uncharged species relative to the charged species so that most of the total flux across the membrane is due to the uncharged species [10,11]. Distribution of a weak acid will be according to eq. (2)

$$pH_i = pK_a + \log [T_i/T_e (10^{(pH_e - pK_a)} + 1) - 1] \quad (2)$$

and a weak base will be according to eq. 3

$$pH_i = pK_a - \log [T_i/T_e (10^{(pK_a - pH_e)} + 1) - 1] \quad (3)$$

where T_i and T_e are the total concentration of acid or base in the suspending medium and inside the cell respectively.

3.2. *Transmembrane electrical potential*

The transmembrane electrical potential was measured using both positively and negatively charged ions which are permeable to the membrane. An ion which is in electrochemical equilibrium across a membrane is distributed according to the Nernst equation

$$E = -\frac{RT}{nF} \ln \frac{[C]_{in}}{[C]_{out}}$$

where E is the transmembrane electrical potential, $[C]_{in}$ and $[C]_{out}$ the activities of the ion on the inside and outside of the membrane respectively.

This equilibrium expression is derived for the case of an ion which is freely permeable. Its valid application in any membrane potential determination must meet two conditions:

(i) The ion must be passively permeable in its charged form only.

(ii) A true equilibrium must be established.

Any direct pumping of the ion or coupling of its transport to any other ions which are not in equilibrium invalidates its application and any binding to

components of the system introduces an error in the calculated membrane potential.

The accuracy of the method relies on the fact that whatever the sign of the transmembrane potential the positively and negatively charged ions should behave as mirror images, one being excluded from the intracellular space and the other being concentrated within the intracellular space. Because binding increases the amount of ion associated with the cellular components, it has the effect of increasing $[C]_{in}$, increasing the absolute value of the membrane potential calculated for the internally concentrated ion and decreasing the absolute value of the membrane potential calculated for the excluded ion. As long as *both* positive and negative ions are used, one will give a maximum value for the transmembrane potential and the other the minimum value. Where binding can be determined the correct value can then be calculated.

Most cell systems actively transport some inorganic ions such as (Na^+, K^+, Rb^+, Ca^{2+}, Mg^{2+}, Cl^- etc) and for the present study the nonphysiological lipid soluble ions triphenylmethyl phosphonium$^+$ [12] and thiocyanate$^-$ were chosen in order to avoid, in so far as possible, active transport.

3.3. *Intracellular spaces*

Tritiated water and [^{14}C]polyethylene glycol were added to the incubation medium and following centrifugation of the cells through silicone oil the total water volume of the pellet was determined from the content of ^{3}H while the extracellular water was determined from the content of ^{14}C.

4. Results

4.1. *The transmembrane pH gradient of* P. denitrificans *and its dependence on the external pH*

Cell suspensions incubated as in section 2.2 were distributed into individual stirred sample chambers and the isotopically labeled compounds added. The radioactive trimethylacetate (TMA) methylamine (MA), dimethylamine (DMA) and 5,5-dimethyl-2,4-oxazolidinedione (DMO) all attained their final cellular concentrations by 2 min after addition and no further change was observed at 4 min. The intracellular water spaces were found to be the same at 2 min and 4 min.

The results are presented in fig.1 as the intracellular pH calculated from the distributions and the extracellular pH plotted against the extracellular (medium) pH. The calculated intracellular pH values derived from the weak acid and weak base distributions approach each other at more alkaline pH values. At an external pH of 7.7 the calculated internal pH values are 7.90 and 7.68 for TMA and DMO while that calculated for methylamine and dimethylamine is 7.35. As the external pH becomes more acidic the internal pH values calculated from the distribution

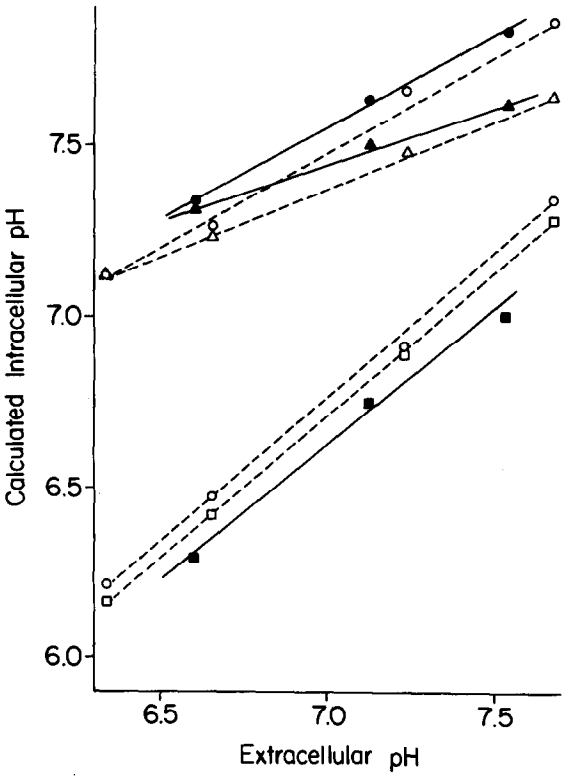

Fig.1. The intracellular pH calculated from the distribution of weak acids and weak bases and its dependence on extracellular pH. The *P. denitrificans* cells were suspended in Krebs-Henseleit medium plus 6 mM glucose and incubated as given in section 2.2. Small quantities of ^{14}C-labeled TMA (○,●), DMO (△,▲), DMA (■,□) or MA (○) were added and after 2 min incubation the cells were separated from the medium by centrifugation through silicon oil (see section 2.3). The transmembrane pH gradient and consequently the intracellular pH was calculated as described in section 3.1. In each experiment the external pH is that measured in the incubation mixture at the time the samples were centrifuged.

of weak acids and weak bases diverge. The weak acids show only a small change (TMA and DMO give equal calculated internal pH values of 7.2 at an external pH of 6.3) while the weak bases (MA and DMA) give calculated internal pH values which change in parallel to the external pH, attaining a value of 6.15 when the external pH is 6.3. Measurements at pH 7.1 using suspensions of spheroplasts gave results indistinguishable from those of suspensions of whole cells. The spheroplast suspension shows similar $[ATP]/[ADP][P_i]$ values and distributions of weak bases and weak acids to those of whole cells. Since spheroplasts do not have intact outer cell walls, they represent a test of the possible role of this structural element in the measured reagent distributions.

4.2. *The transmembrane electrical potential of* P. denitrificans *and its dependence on the pH of the suspending medium*

Cell suspensions taken from the same preincubation mixtures as for pH gradient measurements were treated with 5 μM $TPMP^+$ and 20 μM thiocyanate. Aliquots were removed at 2 min and 4 min and the ^3H and ^{14}C content determined for the cells and the suspending medium. Both compounds had achieved their equilibrium internal concentration after 2 min incubation and no changes were observed thereafter. The measured distribution data are given in table 1.

The $TPMP^+$ was concentrated inside the cells to give an internal concentration approx. 4 times greater than that of the suspending medium. The internal concentration of SCN^- was approx. 1/3 of the concentration in the suspending medium giving a calculated membrane potential of -18.8 ± 6.5 mV. Both positive and negative ions indicate that transmembrane potential exists which is negative inside. Varying the $TPMP^+$ concentration from 2–40 μM or the SCN^- from 10–100 μM did not change the measured concentration gradients of the calculated membrane potential. Moreover, addition of either probe at concentrations of 100 μM did not measurably change the intracellular [ATP/[ADP] or cellular respiration rate under the experimental conditions used. The measured distributions of $TPMP^+$ and SCN^- were pH-independent from external pH values of 6.2–7.7. Experiments using suspensions of spheroplasts at pH 7.1 gave distributions of $TPMP^+$ and SCN^- experimentally indistinguishable from those reported above for suspensions of intact cells.

4.3. *The effect of anaerobiosis on the transmembrane electrical and pH gradients*

Under conditions used *P. denitrificans* is an obligate aerobe and under anerobiosis the intracellular energy supply ($[ATP]/[ADP][P_i]$) rapidly falls from its normal aerobic values near 3×10^3 M^{-1} [8] to less than 100 M^{-1}. The transmembrane electrical potential was measured taking aliquots of cell suspensions which were aerobic, anaerobic for 6 min and then reoxygenated for 3 min. Table 2 summarizes measurements made at external pH values of 6.3–7.6 as calculated electrical potentials. The $TPMP^+$ distribu-

Table 1
The measured transmembrane electrical potential and pH gradient of *P. denitrificans* cells suspended at pH 7.1

Membrane potential (mV)		pH Gradient			
TPMP	SCN^-	DMO	TMA	MA	DMA
-39.9 ± 4.4 (10)	-18.8 ± 6.5 (8)	$+0.30 \pm 0.17$ (7)	$+0.48 \pm 0.21$ (7)	-0.24 ± 0.13 (2)	-0.26 ± 0.13 (5)

The *P. denitrificans* cells were suspended as given in section 2.1 with an external pH of 7.1. The indicated isotopically labeled compounds were added and 2–2.5 min aliquots taken and the distribution of the radioactivity measured (see section 2.3). The data are presented as the mean ± the standard deviation of the data when expressed as either an electrical potential ($TPMP^+$ and SCN^-) or transmembrane pH gradient. The number of experiments is indicated in parenthesis.

Table 2
The effect of anaerobiosis on the measured transmembrane electrical potential and pH gradient of *P. denitrificans* cells

	Metabolic	Membrane potential (mV)		pH Gradient			
pH	condition	TPMP[c]	SCN[c]	DMO[b]	TMA[b]	DMA[a]	MA[b]
	aerobic	−40.1 ± 5.9	−22.7 ± 7.5	0.00 ± .17			−0.31 ± .11
7.6	anaerobic	−57.3 ± 7.2	−28.9 ± 8.0	0.19 ± .13			−0.53 ± .16
	reoxygenated	−40.6 ± 2.8	−23.5 ± 1.6	0.11 ± .19			−0.49 ± .11
	aerobic	−41.2 ± 3.5	−15.1 ± 2.8	0.49 ± 0.12	0.69 ± .24	−0.36	
7.1	anaerobic	−57.6 ± 2.7	−12.9 ± 3.1	0.22 ± .06	0.44 ± .10	−0.48	
	reoxygenated	−42.5 ± 4.3	−13.9 ± 2.5	0.55 ± .13	0.68 ± .25	−0.34	
	aerobic	−31	−23.6	0.75			
6.3	anaerobic	−54.4	−24	0.25			
	reoxygenated	−35.1	−19.4	0.74			

[a] 1 exp.
[b] Av. 2 exp.
[c] Av. 3 exp.

At pH 6.3 only one experiment (duplicate determinations) was run

The *P. denitrificans* cells were suspended as given in section 2.1 with the indicated pH of the external medium. The cells were incubated with glucose as given in section 2.2 and then the radioactive probes added. After 2.5 min of aerobic incubation the samples (Aerobic) were taken and the cell suspension allowed to become anaerobic. After 6 min anaerobiosis samples were taken (anaerobic) and oxygen gas used to reoxygenate the suspension and maintain it oxygenated for 3 min. (reoxygenated)

tion gave calculated transmembrane electrical potential of near −40 mV for cells in aerobic suspension with the value increasing to near −60 mV after anaerobiosis and returning to near −40 mV after reoxygenation (the intracellular [ATP]/[ADP] also return to the aerobic control value). The negative ion (SCN^-) was excluded under all conditions with the observed changes being experimentally insignificant.

The transmembrane pH gradients change markedly upon anaerobiosis at all of the measured external pH values (table 2). The weak acids DMO and TMA both move out of the cells after anaerobiosis and back into the cells upon reoxygenation. The weak bases MA and DMA both move into the cells after anaerobiosis and back out of the cells upon reoxygenation. At pH 7.1, the acidification of the internal cellular space is 0.1–0.3 pH units as measured by both the weak acids and weak bases. Thus although giving different values for the absolute pH gradient, both the weak acids and weak base show internal acidification upon anaerobiosis. Although the average standard deviations are ±0.15 pH units for the pH gradient, reflecting variation from experiment to experiment, within each experiment the changes in pH gradient from aerobic to anaerobic states are very reproducible, that is, at an external pH of 7.1 the standard deviations of the change in intracellular pH which occurs on anaerobiosis as measured with DMO is 0.30 ± 0.06, and that measured with TMA is 0.24 ± 0.11.

The acidification upon anaerobiosis is dependent on the external pH and is greatest at an extracellular pH of 6.3, least at pH 7.6 and intermediate at pH 7.1.

5. Discussion

Paracoccus denitrificans is a useful organism for studying the relationship between the oxidation–reductions of the respiratory chain and ATP synthesis and to examine the possible intermediates in the reaction to see if they fulfill the thermodynamic requirements of this role (e.g. [13]). Unicellular and

prokaryotic, these cells have minimal partitioning of cellular contents. The respiratory chain is essentially the same as that for mammalian mitochondria and is part of the cell membrane rather than of a specialized subcellular organelle. This means that the use of weak acids and weak bases to measure the transmembrane pH gradient and permeable positively and negatively charged ions to measure the transmembrane electrical gradient need be concerned primarily with possible binding artifacts and not with possible spatial compartmentalization. Limits on binding, since it is always a positive term giving increased amounts of compound in the cellular fraction (see section 3), can be readily determined by the use of oppositely responding compounds, i.e., weak acids and weak bases, positively charged ions and negatively charged ions.

5.1. *Measurements of the transmembrane pH gradient*

Superficially both the weak acids and weak bases appear to behave according to theoretical predictions when their distributions are measured separately and only direct comparison of their behavior patterns suggests that binding is significant. In the absence of independent methods for measuring the value of the internal pH the distribution of the weak acids and weak bases can be considered to establish only the possible acid and alkaline limits for the value. At external pH values more alkaline than pH 7 the difference is less and by pH 7.6 is small enough to establish quite well the intracellular pH (± 0.15 pH unit) and changes which occur (such as that upon anaerobiosis). The intracellular pH of *P. denitrificans* was estimated [14] to be 7.2–7.4 and essentially independent of the extracellular pH from 6–8. This would be most consistent with the behavior of DMO but even the latter suggests an internal pH change of approx. 0.7 units over the same range of external pH.

5.2. *Measurements of the transmembrane electrical potential*

The $TPMP^+$ and SCN^- give good agreement of the calculated transmembrane electrical potential at external pH values from 6.2–7.7. The excluded ion (SCN^-) becomes less precisely measured as the membrane potential becomes more negative because its internal concentration is low and the measurements include a trapped (extracellular) volume. Although the distribution of the included ion ($TPMP^+$) increases by 2-fold in the cellular fraction upon a change of membrane potential from –40 mV to –60 mV, the excluded ion (SCN^-) decreases by less than 10% in the cellular fraction. Thus the change in membrane potential calculated from $TPMP^+$ distribution which occurs on anaerobiosis lies within the error in the value calculated from SCN^- distribution. The reversible uptake of $TPMP^+$ upon anaerobiosis is good evidence that the $TPMP^+$ is permeable to the cell wall. Binding to the external surface of the membrane is unlikely to be a problem since there is no dependence of the $TPMP^+$ on the surface charge, i.e., the external pH (table 2). The $TPMP^+$ and SCN^- are almost certainly being accumulated or excluded according to the electrical gradient as evidenced by:

1. The close agreement of the electrical gradients calculated from the distributions of these oppositely charged ions.

2. The distributions of the $TPMP^+$ and SCN^- give calculated electrical gradients which are similarly independent of external pH.

3. The weak acids and weak bases which also have negatively and positively charged ionic species (in equilibrium with the uncharged species) show distributions which differ in magnitude and pH dependence from those of SCN^- and $TPMP^+$.

5.3. *The thermodynamic relationships of the oxidation–reduction reactions, the H^+ gradient and ATP synthesis*

The relevant parameters can be easily summarized. For convenience of comparison all three parameters may be expressed as the free energy change per equivalent transferred. The oxidation–reduction reactions of the respiratory chain from NADH to cytochrome *c* occur across an oxidation–reduction potential difference (ΔE) of 0.52 V, or 12 kcal/equiv. transferred, while synthesis of intracellular ATP requires 12 kcal/mol [9]. Experimentally of course the oxidation–reduction reactions encompass two phosphorylation sites and the overall stoichiometry is two equivalents transferred for 2 mol ATP synthesized. Thus the overall free energy change for the coupled reactions is zero within experimental error as shown [9].

The movement of protons across the cellular mem-

brane as H^+ is affected by both the transmembrane electrical potential and the transmembrane pH gradient. When the electrical potential is negative inside and the pH is alkaline inside these two terms are additive. The $TPMP^+$ gives the most negative calculated inside potential and DMO may be considered to give the most alkaline possible calculated internal pH (binding could cause overestimation of the internal pH by TMA). Under aerobic conditions at an external pH 7.0 these maximal values are –40 mV and 0.3 pH units, respectively, while when the external pH is 7.6 the maximal values are –40 mV and 0.0 pH units, respectively. The total 'protonmotive force' is thus –58 mV at pH 7.0 and –40 mV at pH 7.6, corresponding to –1.3 kcal/mol and –0.9 kcal/mol, respectively, for H^+ transfer from the external to the internal medium. Thus if H^+ transfer were to provide the energy for ATP synthesis, 9–12 mol H^+ must be transferred/mol ATP synthesized. In the same sense each two reducing equivalents transferred from NADH to cytochrome *c* must be coupled to the transfer of 18–24 mol H^+ from inside the cell to the outside medium.

Proton extrusion occurs in *P. denitrificans* coupled to oxygen utilization [14]. The reported stoichiometry was 6 H^+/O_2 or 2 H^+/phosphorylation site, approx. 1/5 the value required by the thermodynamic considerations in order for the proton gradient to function as an intermediate in the phosphorylation mechanism. A higher value of $3H^+$/site, still far below the values required by the thermodynamic considerations has been reported [15].

The overall behavior of the *P. denitrificans* transmembrane electrical and pH gradients are similar to those of eucaryotic mammalian cells [16]. It would appear that systems exist for maintaining the intracellular pH near 7.5 and the membrane potential near –40 mV. The cells used in this study were grown under conditions for which active transport of the substrates (amino acids) was not required. It will be of considerable interest to see if induction of active transport systems by growth under substrate limiting conditions will be accompanied by appearance of a large membrane potential and/or pH gradient and if these are functional in the active transport system.

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References

[1] Kalean, H. (1938) in: Fosforyleringer i Dyrisk Vaev (Forlag, N. N. and Busch, A. eds) pp. 126–128. Presented (1969) in: Biological Phosphorylations pp. 208–210, Prentice Hall.
[2] Belitser, V. A. and Tsybakova, E. T. (1939) Biokhimiya 4, 516–534. English: Kalkar, H. (1969) in: Biological Phosphorylations, pp. 211–227, Prentice Hall.
[3] Boyer, P. D. (1977) Ann. Rev. Biochem. 46, 957–966.
[4] Griffiths, D. E. (1976) Biochem. J. 160, 809–812.
[5] Wilson, D. F., Dutton, P. L. and Wagner, M. (1973) Current Top. Bioeng. 5, 233–269.
[6] Mitchell, P. (1966) Chemiosmotic coupling in Oxidative and Photosynthetic Phosphorylation, Glynn Research Ltd. Bodmin.
[7] Mitchell, P. (1977) Ann. Rev. Biochem. 46, 996–1005.
[8] Erecińska, M., Kula, T. and Wilson, D. F. (1978) FEBS Lett. 87, 139–144.
[9] Wilson, D. F., Stubbs, M., Oshino, N. and Erecińska, M. (1974) Biochem. J. 140, 57–64.
[10] Waddell, W. J. and Butler, T. C. (1959) J. Clinical Inv. 38, 720–729.
[11] Boron, W. F. and Roos, A. (1976) Am. J. Physiol. 231, 799–809.
[12] Bakeeva, L. E., Grinius, L. L., Jasaitis, A. A., Kuleine, V. V., Levitsky, D. O., Liberman, E. A., Severina, I. I. and Skulachev, V. P. (1970) Biochim. Biophys. Acta 216, 13–21.
[13] John, P. and Whatley, F. R. (1977) Biochim. Biophys. Acta 463, 129–153.
[14] Scholes, P. and Mitchell, P. (1970) Bioenergetics 1, 61–72.
[15] Meijer, E. M., Van Verseveld, H. W., Van der Beek, E. G. and Stonthamer, A. H. (1977) Arch. Microbiol. 112, 25–34.
[16] Altman, P. L. and Dittmer Katz, D. eds (1976) Cell Biology, Fed. Am. Soc. Exp. Biol., Bethesda, MD.