The proton-per-electron stoichiometry of 'site 1' of oxidative phosphorylation at high protonmotive force is close to 1.5

Piet C. DE JONGE and Hans V. WESTERHOFF

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, P.O. Box 20151,
1000 HD Amsterdam, The Netherlands

(Received 18 September 1981/Accepted 31 December 1981)

The maximum redox potential difference between the NAD^+/NADH couple and the succinate/fumarate couple generated during ATP-energized reduction of NAD^+ by succinate in submitochondrial particles was measured, together with the electrochemical potential difference for protons ($\Delta\tilde{\mu}_{\text{H}^+}$). The presence of cyanide, the time-independence of the redox potential difference and the irrelevance of the initial redox state of the NAD^+/NADH couple ensured that the experimental situation corresponded to a 'static-head condition' with $\Delta\tilde{\mu}_{\text{H}^+}$ as the input force and the redox potential difference as the output force, the flow of electrons having reached dynamic equilibrium. Consequently, the observed value of 1.6 for the ratio $\Delta G_e/\Delta\tilde{\mu}_{\text{H}^+}$ is interpreted as indicating that the $\tilde{\text{H}}^+/\text{e}^-$ stoichiometry at 'site 1' is 1.5 and that therefore the mechanism of the proton pump at 'site 1' is not of the group-translocation type (no direct $\tilde{\text{e}}^- - \tilde{\text{H}}^+$ coupling).

The chemiosmotic hypothesis (Mitchell, 1961) has now generally been accepted as the best working hypothesis for the mechanism of oxidative phosphorylation (Boyer *et al.*, 1977). Yet, two of its most important aspects are still being discussed (Wikström & Krab, 1980). One is the thermodynamic competence of the protonmotive force with respect to the phosphate potential that can be generated (see, e.g., Sorgato *et al.*, 1980; Westerhoff *et al.*, 1981b). The other is the mechanism by which the protons are pumped across the membrane (see, e.g., Mitchell, 1977; Wikström & Krab, 1979).

As to the latter aspect, the proposed mechanisms can be grouped into two classes. In one class pumping occurs according to a direct (Mitchell, 1977) chemiosmotic mechanism: the protons pumped are both substrate and product of the redox (or ATPase) reaction itself, and this reaction has an orientation in space so that the substrate protons are derived from one side of the membrane and the product protons from the other (Mitchell, 1961,

1977). For a redox proton pump, such as the respiratory chain, such a situation may arise from the alternate positioning of 'H' and electron carriers (Mitchell, 1961). We refer below to this type of mechanism as 'direct' (Mitchell, 1977) $\tilde{\text{e}}^- - \tilde{\text{H}}^+$ coupling.

The second class of mechanisms, i.e. the indirect chemiosmotic ones (Mitchell, 1977), implies that the redox reaction itself may essentially proceed isotropically, but that it results in the action of a proton-pumping protein, which, owing to its three-dimensional positioning, always pumps in the same direction (Wikström & Krab, 1979).

One of the points of experimental distinction between the two classes of proposed mechanisms is the ratio of $\tilde{\text{H}}^+/\text{e}^-$ predicted (Mitchell, 1977; Wikström & Krab, 1980). The direct $\tilde{\text{e}}^- - \tilde{\text{H}}^+$ coupling mechanisms have been introduced (Mitchell, 1961) as predicting 1 $\tilde{\text{H}}^+/\text{e}^-$ per complete redox loop or 'site' of oxidative phosphorylation. For cytochrome *c* oxidase, which would catalyse only part of such a loop, the stoichiometry would be predicted to be 0 H^+/e^- and 1 charge/ e^- (Moyle & Mitchell, 1978; Wikström & Krab, 1980). Failure to observe such stoichiometries has led to rejection of the direct $\tilde{\text{e}}^- - \tilde{\text{H}}^+$ coupling mechanisms for cytochrome *c* oxidase (cf. Wikström & Krab, 1980). The postulate of extra chemiosmotic loops at a certain 'site' (cf. Mitchell, 1976) may increase the $\tilde{\text{H}}^+/\text{e}^-$

Abbreviations used: $\tilde{\text{H}}^+/\text{e}^-$, translocated proton per redox electron; $\Delta\tilde{\mu}_{\text{H}^+}$, electrochemical potential difference for protons; $\Delta\psi$, the electrical potential across the membrane; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mops, 4-morpholinepropanesulphonic acid; 'site 1', proton-translocating segment of $\text{NADH}:\text{ubiquinone oxidoreductase}$.

stoichiometry, but, then again, the resulting number should be an integer. It follows that the determination of $\bar{H}^+/e^-$ for any 'site' of oxidative phosphorylation yields information whether or not there may be direct $\bar{e}^- - \bar{H}^+$ coupling at that site.

In principle there are three ways of determining the stoichiometry at which two reactions are coupled. The first is to measure the rate of reaction for the different substrates. In view of the possibility of back-leakage of protons this procedure must be conducted at low $\Delta\tilde{\mu}_H$ (the 'pulse' experiments; for a review see Wikström & Krab, 1980). The second [the static-head (Kedem & Caplan, 1965) force ratio] method (Nicholls, 1974) involves measurement of the free energies of the two reactions when the total reaction has come to equilibrium. If the coupled reaction contains no inherent slip, the ratio of the free energies equals the reaction stoichiometry. This method has been applied to mitochondrial H^+ -translocating ATPase (Nicholls, 1974; van Dam *et al.*, 1977; Azzone *et al.* 1978). A third method (van Dam *et al.*, 1980) has recently emerged from (mosaic) non-equilibrium thermodynamics (Westerhoff *et al.*, 1981c; Westerhoff & van Dam, 1982) and circumvents the limitation of the second method to the no-flux situation.

Of these methods only the first has been extensively applied to the determination of the $\bar{H}^+/e^-$ stoichiometries of the respiratory chain. The results do not give a coherent picture (for a review see Wikström & Krab, 1980). The third method has only been applied to $\bar{H}^+/e^-$ of the combination of 'site 2' and 'site 3' (van Dam *et al.*, 1980).

The second method has not yet been applied to the determination of $\bar{H}^+/e^-$ stoichiometries, partly because the respiratory chain as a whole cannot reach equilibrium. Attempts to obtain a flow of electrons from water 'upwards' into the respiratory chain have failed (Biefait, 1975).

Since, in practice, 'site 1' of oxidative phosphorylation is reversible (Chance & Hollunger, 1961), we set out to determine the $\bar{H}^+/e^-$ stoichiometry of 'site 1' by the static-head force ratio method. We conclude that the $\bar{H}^+/e^-$ stoichiometry for 'site 1' is 1.6 and that therefore the 'site 1' proton pump does not function through direct $\bar{e}^- - \bar{H}^+$ coupling.

Experimental

Preparation and characterization of submitochondrial particles

Mg/Mn/succinate/ATP submitochondrial particles were prepared from heavy bovine heart mitochondria (Smith, 1967) that had been frozen at 0.12 g of protein/ml in 0.25 M-sucrose/10 mM-Hepes/1 mM- Na_2ATP /1 mM- $MgCl_2$ /1 mM-succinate/KOH, pH 7.6, at 200 K. Preparation of the submitochon-

drial particles was essentially by the method of Hansen & Smith (1964), with the exception that Hepes/KOH buffer was used instead of Tris/HCl buffer and that the succinate dehydrogenase of the particles was activated (Kimura *et al.*, 1967) by incubating the crude particle fraction (supernatant after sedimentation of intact mitochondria by centrifuging) in the presence of 5 mM-potassium malonate for 30 min at 300 K. The particles were suspended at a concentration of about 25 mg of protein/ml in 0.25 M-sucrose/10 mM-Hepes/KOH buffer, pH 7.5, supplemented with 3% (v/v) glycerol, quickly frozen and stored in 1 ml portions under liquid N_2 (for up to 3 months).

Before an experiment, an appropriate amount of submitochondrial particles was quickly thawed, suspended in 'flow mix' (0.18 M-sucrose/50 mM-KCl/10 mM-Hepes/10 mM- $MgSO_4$ /5 mM- KH_2PO_4 /1 mM-EGTA/KOH buffer, pH 7.3), sedimented by centrifuging at 150 000 g for 30 min, homogenized and resuspended in 'flow mix'.

In 0.25 M-sucrose/10 mM-Hepes/3 mM- $MgCl_2$ /5 mM- KH_2PO_4 /KOH buffer, pH 7.5, and either 3 mM-NADH or 20 mM-succinate, respiration by submitochondrial particles at 0.7 mg of protein/ml could be stimulated 2.2-fold (S.E.M. ± 0.1 , $n = 3$) or 1.65-fold (S.E.M. ± 0.1 , $n = 3$) respectively by optimal carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone concentrations. Respiration was less than 10% sensitive to carboxyatractyloside. Oligomycin and (though less so) polylysine were able to increase respiratory control and succinate-driven 9-aminoacridine fluorescence quenching.

The internal volume of the submitochondrial particles was determined in 'flow-mix' as the sucrose-impermeable water-permeable space, essentially as described by Sorgato *et al.* (1978): particles were thawed, washed in 'flow mix' (with centrifugation at 150 000 g for 30 min) and resuspended at 30 mg of protein/ml in 'flow mix'. Then to 170 μ l 0.1 μ Ci of [^{14}C]sucrose (50 μ Ci/ml; 350 Ci/mol) and 0.75 μ Ci of [3H]water (250 mCi/l), both from The Radiochemical Centre (Amersham, Bucks., U.K.), were added, and the suspension was centrifuged at 130 000 g (Beckmann Airfuge) at room temperature. Then 100 μ l of the resulting supernatant plus 4 ml of 'flow mix' was added to 10 ml of scintillation fluid (Packard scintillator 299TM). The remainder of the supernatant was removed as much as possible; 'flow mix' was added to the pellet to a final volume of 170 μ l. After 15 min of mild sonication in a sonication bath, the above-mentioned centrifugation and sampling steps were repeated. The internal volume was calculated as:

$$V_i = (1000/[SMP]) \cdot [(H_r - C_r)/(1 - C_r)]$$

where V_i is expressed in μ l/mg of protein and [SMP] is the concentration of submitochondrial particles in

mg of protein/ml (in this case 30). H_r and C_r are the ratios of the radioactivities (c.p.m.) in the second, relative to the first, supernatant for ^3H and ^{14}C respectively. The results of four determinations were 1.1, 1.4, 1.2 and $1.2\mu\text{l}/\text{mg}$ of protein. The division by $1 - C_r$ in the equation above reflects the assumption that during incubation in the sonication bath [^{14}C]sucrose does not enter the intraparticle space. If this assumption is reversed, the values become 0.85, 1.1, 1.0 and 1.0. Since we do not know which of the assumptions above is correct, we assumed in our calculations an inner volume of $1.1\mu\text{l}/\text{mg}$ of protein.

Determination of $\Delta\bar{\mu}_{\text{H}^+}$ under reversal conditions

$\Delta\psi$ and ΔpH were calculated from the disappearance of thio[^{14}C]cyanate and amino[^3H]methane or amino[^{14}C]methane from the medium, assuming electrochemical equilibrium for SCN^- and CH_3NH_2 across the membrane of the submitochondrial particles. The disappearance was measured by the 'flow-dialysis' method, essentially as described by Arents *et al.* (1981). To a volume of 1.2 ml containing 0.14 M-sucrose, 50 mM-phosphocreatine, 37.5 mM-KCl, 7.5 mM-Hepes, 7.5 mM-MgSO₄, 3.75 mM-KH₂PO₄, 0.75 mM-EGTA, 1.0 mM-NaCN, 10 mM-succinate and 1.0 mM-NAD⁺, initial pH 7.3 (during the experiment the pH increased up to at highest a value of 8.0), at 298 K, submitochondrial particles (10 mg of protein/ml), 20 μM -thio[^{14}C]cyanate (60 Ci/mol) and 1 μM -amino[^3H]methane (11.4 Ci/mmol) (both from The Radiochemical Centre) were added. After 5 min, 2 mM-KADP plus various amounts of creatine kinase (0–0.7 mg, 0–20 units; Boehringer, Mannheim, Germany) were added. At $t = 10$ min 20 μg (in 20 μl of ethanol) of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and at $t = 15$ min 15 μl of 10% (v/v) Triton X-100 (in water) were added. The efflux of label through the dialysis membrane was monitored by taking samples at 30 s intervals from the 'flow mix' flowing through the lower compartment of the flow-dialysis vessel at 8.2 ml/min. The samples plus 10 ml of scintillation liquid each were analysed in a two-channel scintillation counter. The results were corrected for the effect of sampling and additions (H. V. Westerhoff, P. C. de Jonge & W. J. van der Zande, unpublished work) so that the correct reference line could be obtained through interpolation between the de-energized parts of the plot. By using this reference line, the membrane potential or the pH gradient was calculated.

During the experiments, once or twice per minute 25 μl samples were taken from the upper compartment of the flow-dialysis vessel and injected into 50 μl of ice-cold 0.5 M-KOH (alkaline quenching method). After at most 4 h storage on ice, the

samples were incubated for 5 min in a 335 K water bath. Then 75 μl of 0.6 M-NaHCO₃ was added, the incubation was continued for 30 min at 335 K and the mixture was stored for at most a fortnight at 277 K. This treatment resulted in complete degradation of NAD⁺, NADH remaining intact (Lowry *et al.*, 1961). Alternatively (acid quenching method), 25 μl samples were injected into 125 μl of ice-cold 0.6 M-HClO₄ and stored on ice for at most 4 h. Then the pH was adjusted to 7.0 ± 0.5 with 5 M-KOH + 1 M-Mops. In this way, all NADH is degraded, NAD⁺ remaining intact (Lowry *et al.*, 1961). For samples in which ADP was to be determined, 100 μl portions were injected into 50 μl of ice-cold 1.5 M-HClO₄, stored on ice and neutralized within 4 h with 5 M-KOH + 1 M-Tris to a final pH 8.0 ± 0.5 .

ADP was determined through the pyruvate kinase and lactate dehydrogenase spectrofluorimetric assay (Jaworek *et al.*, 1970). NAD⁺ or NADH was determined in a catalytic assay (Bernofsky & Swan, 1973; modified by A. K. Groen & R. C. Vervoorn, unpublished work). At most 1 nmol of NAD(H) (i.e. NAD⁺ or NADH) was added to 1 ml of 0.15 M-Tris containing 0.15% Triton X-100, 3 mM-MgCl₂, 2.5 mM-Na₂EDTA, 0.30 M-ethanol, 0.10 mM-oxidized Nitro Blue Tetrazolium (Sigma), 0.38 mM-phenazine ethosulphate (Sigma), 20 μg of dialysed ethanol dehydrogenase (Boehringer) [dialysed against 50% (v/v) glycerol in water; 200 units/ml, 12.5 mg/ml] per ml and HCl, pH 8.0. After incubation at 303 K in the dark for a certain time, the absorbance at 570 nm was compared with that of an identically treated reference sample. Succinate, fumarate, NAD(H) and ATP concentrations were calculated from the measured NAD(H) and ADP concentrations. ΔG_e was calculated by using a ΔG_e^0 value of -30.7 kJ/mol of electrons (Clark, 1960), and ΔG_p^0 was taken to be -29.6 kJ/mol of ATP (Rosing & Slater, 1972).

Results

Although the definition of 'site' has become obsolete, we still use the term 'site 1' for the segment of NADH:ubiquinone oxidoreductase that catalyses redox-energized transmembrane proton movement. In view of the closeness of the midpoint potentials of the succinate/fumarate couple and the reduced/oxidized ubiquinone couple, it is generally assumed that no such site is present in between succinate and ubiquinone. Consequently, the $\bar{H}^+/e^-$ stoichiometry of site 1 (n_{H}^e) is identical with the $\bar{H}^+/e^-$ stoichiometry of the reduction of NAD⁺ by succinate, so that:

Here the subscripts m and c stand for matrix and cytosol respectively. We assume that the reaction as written here is fully coupled, i.e. that there is no slip in this proton pump (*pace* Pietrobon *et al.*, 1981). This does not mean, however, that we assume the entire system to be fully coupled; there may be passive proton leakage through the membrane, or even slips in the H^+ -translocating ATPase, as neither of these affects the equation above (the H^+ ions mentioned in this equation are exclusively those moving through the 'site 1' proton pump).

If we define ΔG_e as the difference in redox potential between the $NAD^+/NADH$ couple and the succinate/fumarate couple, the total free energy of the reaction, ΔG , is (in kJ/mol of electrons):

$$\Delta G = \Delta G_e + n_H^e \Delta \tilde{\mu}_{H^+} \quad (2)$$

Our approach in this study has been to wait until the reaction reached equilibrium and then to compare ΔG_e with $\Delta \tilde{\mu}_{H^+}$. When $\Delta G = 0$:

$$(\Delta G_e)/(\Delta \tilde{\mu}_{H^+}) = -n_H^e \quad (3)$$

Fig. 1 shows the concentration of NADH as well as $\Delta \tilde{\mu}_{H^+}$ as a function of time, when submitochondrial

Fig. 1. Time course of the ATP-energized reduction of NAD^+ by succinate: the NADH concentration and $\Delta \tilde{\mu}_{H^+}$ as a function of time

Experimental details are given in the text. NADH (O) was determined by using the alkaline quenching method. Owing to limitations in the kinetic resolution of the reading of $\Delta \tilde{\mu}_{H^+}$ (□) by the flow-dialysis procedure, the kinetics of the $\Delta \tilde{\mu}_{H^+}$ development should be regarded as an approximation. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

particles (Mg/Mn/succinate/ATP) are energized with ATP in the presence of cyanide, phosphocreatine, succinate and NAD^+ . The energization with ATP was brought about by the addition of creatine kinase and a small amount of ADP. Addition of large amounts of ATP to the flow-dialysis vessel causes artifacts in the reading of $\Delta \psi$ and ΔpH (H. V. Westerhoff, P. C. de Jonge & W. J. van der Zande, unpublished work; M. C. Sorgato, personal communication). We conclude from Fig. 1 that within 5 min the NADH concentration became constant and that measurement of $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ after 5 min reflects the equilibrium condition with respect to reaction (1).

In a separate flow-dialysis run with $[^{14}C]SCN^-$ ($\Delta \psi$ is the major contributor to $\Delta \tilde{\mu}_{H^+}$) it was checked that, in a similar incubation supplemented with oligomycin (25 $\mu g/mg$ of protein), neither creatine kinase nor carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone nor Triton X-100 had any effect on the flow-dialysis trace. The effect of 2 mM-KADP was estimated to change the efflux rate of SCN^- by less than 0.8%. Moreover, the flow-dialysis plots before and after energization could be extrapolated into each other (Fig. 2) when replotted in the appropriate way (H. V. Westerhoff, P. C. de Jonge & W. J. van der Zande, unpublished work; Hellingwerf *et al.*, 1979).

Table 1 shows the values of the different thermodynamic forces in five sets of experiments of the type described above. In the first two sets, the ratio of ΔG_e to $\Delta \tilde{\mu}_{H^+}$ turned out to be approx. 1.7. The third and fourth sets of experiments were performed to see whether the total concentration of (nicotinamide) adenine nucleotides had a significant effect. The fourth and the fifth sets of experiments were performed to determine the variation of the ratio $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ with $\Delta \tilde{\mu}_{H^+}$, when the latter was varied by varying the ATP concentration; the experiments were performed with different concentrations of creatine kinase.

The force ratio $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ was 1.6 (S.E.M. ± 0.05) (cf. Table 1). We conclude that this ratio does not strongly depend on the total concentrations of $NAD^+ + NADH$ or $ATP + ADP + AMP$. Also, at least when $\Delta \tilde{\mu}_{H^+}$ is varied by varying the creatine kinase concentration, this force ratio is independent of $\Delta \tilde{\mu}_{H^+}$ for values of the latter ranging between 13 and 19 kJ/mol.

Although Fig. 1 shows that the rate of reversal slowed down gradually before the point at which we determined $\Delta G_e/\Delta \tilde{\mu}_{H^+}$, it might still be put forward that the decrease in reversal rate with time is not due to the approach to dynamic equilibrium between the protonmotive force and the redox potential difference. A kinetic limitation might have the same effect. As a result the observed $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ ratio would be an underestimation of the actual $\bar{H}^+/e^-$

Fig. 2. ATP-energized SCN^- ($\square$) and CH_3NH_2 ($\circ$) uptake by submitochondrial particles as monitored by flow dialysis. A plot of c.p.m. (t) versus $\Sigma_{\tau=0}^t$ c.p.m. (t) is shown for Expt. 1 in Table 1. The straight lines are linear least-squares lines, calculated for the points before energization and after Triton X-100 de-energization. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Table 1. Thermodynamic forces under static-head reversal conditions

Thermodynamic forces under static-head ('State -4') reversal conditions at different creatine kinase concentrations are given. The average of the $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ values is 1.59 (S.E.M. ± 0.05 , $n = 12$). The experiments were performed as described in the Experimental section; NADH concentrations were determined 5 min after the addition of ADP and creatine kinase by using the alkaline quenching method, except for Expt. 3, where NAD^+ was determined by using the acid quenching method. Remarks: 'More NAD' means 5 mM- $\text{NAD}^+ + \text{NADH}$, rather than 1 mM; 'Less ADP' means 0.7 mM, rather than 2 mM. Abbreviation: n.d., not determined. In Expt. 3 $30 \mu\text{M}$ - $[^{14}\text{C}]\text{SCN}^-$ and $60 \mu\text{M}$ - $[^{14}\text{C}]\text{CH}_3\text{NH}_2$ (22 Ci/mol) were used in parallel runs, rather than $[^3\text{H}]\text{CH}_3\text{NH}_2$ in the same run. ΔG_e is in kJ/mol of electrons.

Expt. no.	Remark	Forces (kJ/mol)				Force ratios	
		ΔG_p	$\Delta \psi$	$\Delta \tilde{\mu}_{H^+}$	ΔG_e	$\Delta G_p/\Delta \tilde{\mu}_{H^+}$	$\Delta G_e/\Delta \tilde{\mu}_{H^+}$
1		n.d.	12.7	18.7	32.3	n.d.	1.7
2		n.d.	9.1	12.5	21.8	n.d.	1.7
3	No NAD	n.d.	12.5	19.8	n.d.	n.d.	n.d.
		n.d.	12.1	19.3	32.2	n.d.	1.7
4	More NAD	n.d.	11.9	18.6	28.8	n.d.	1.5
		46	13.5	18.7	28.3	2.5	1.5
		43	13.0	16.8	26.4	2.6	1.6
		n.d.	11.4	14.9	21.1	n.d.	1.4
	Less ADP	n.d.	10.6	13.4	18.7	n.d.	1.4
5		n.d.	10.6	16.8	25.9	n.d.	1.5
		n.d.	10.1	14.9	25.2	n.d.	1.7
		44	9.6	13.9	23.2	3.1	1.7
		n.d.	8.2	11.8	19.6	n.d.	1.7

Table 2. $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ at 'State -4', this state being approached from either side. Experimental conditions were as given in Table 1, except that in the last two experiments 1 mM-fumarate + 9 mM succinate, rather than 10 mM-succinate, were present.

Start with	ΔG_e (kJ/mol of electrons)	$\Delta \tilde{\mu}_{H^+}$ (kJ/mol)	$\Delta G_e/\Delta \tilde{\mu}_{H^+}$
NAD ⁺	33	21	1.6
NAD ⁺	30	18	1.7
NADH	30	18	1.7
NADH	31	18	1.7

stoichiometry. Table 2 discards this possibility; the ratio $\Delta G_e/\Delta \tilde{\mu}_{H^+}$ was not different when the final ΔG_e was reached from a higher, rather than a lower (cf. Table 1), initial ΔG_e .

Discussion

Our experiments indicate that at the 'static-head' condition of reversed electron transport between NAD(H) and succinate/fumarate ('State -4'), the ratio of the difference in redox potential between

these two couples and the protonmotive force is approx. 1.6. Since this 'static head' comprises an equilibrium situation for the protonmotive-force-driven reduction of NAD^+ by succinate (reaction 1), it follows (cf. eqn. 3) that the stoichiometry of this reaction is approximately $3 \bar{\text{H}}^+$ per 2 electrons. This number is not in agreement with any simple scheme of a directly $\bar{\text{e}}^- - \bar{\text{H}}^+$ -coupled proton pump. Thus an indirectly $\bar{\text{e}}^- - \bar{\text{H}}^+$ -coupled proton pump mechanism (Mitchell, 1976, 1977), such as the vectorial Bohr mechanism (Papa, 1976; Wikström & Krab, 1979), would appear to be a more appropriate working hypothesis.

A number of uncertainties in the present experiments should be discussed in terms of the influence that they might have with regard to the final conclusion. One is the determination of the protonmotive force in submitochondrial particles by using the flow-dialysis technique, with thiocyanate and aminomethane as $\Delta\psi$ and ΔpH probes respectively. This technique has been amply applied to submitochondrial particles (Sorgato & Ferguson, 1979; Sorgato *et al.*, 1978, 1980, 1981; van Dam *et al.*, 1978; Westerhoff *et al.*, 1981a).

It should be noted that there is also some variance in the internal volume of submitochondrial particles. For a similar medium, Sorgato *et al.* (1978, 1980) first took $1 \mu\text{l}/\text{mg}$ as the internal volume, but later redetermined it as $0.75 \mu\text{l}/\text{mg}$ of protein (Sorgato *et al.*, 1981). In our calculations, we used an inner volume of $1.1 \mu\text{l}/\text{mg}$ of protein, based on determined values of 1.0, 1.0, 1.1 and 1.4. If the true value were as low as $0.75 \mu\text{l}/\text{mg}$ of protein, we would have underestimated the $\Delta\tilde{\mu}_{\text{H}^+}$ values by some 1.9 kJ/mol . The $\Delta G_e/\Delta\tilde{\mu}_{\text{H}^+}$ ratio would have been overestimated by less than 15%: the true value would be $1.4 \text{ H}^+/\text{e}^-$. We conclude that errors in internal volume will not interfere with our conclusion that there is no direct $\bar{\text{e}}^- - \bar{\text{H}}^+$ coupling.

A related, but more serious, point is the possibility that the activity coefficients for SCN^- or CH_3NH_3^+ are different inside the particles from those outside. The concentration of the probe inside the particles, however, never exceeded 3 mM, and the compositions of sonication and flow-dialysis media were similar. Therefore large deviations of the activity coefficients for this reason may be considered unlikely.

An as yet unsolved problem is the degree to which the tritiated water space inside submitochondrial particles can be considered as a true solvent (Garlid, 1979). If it is not, our measured 'internal volume' would be a gross overestimation. Also, it should be borne in mind that submitochondrial particles are probably osmotically inactive and that, as a consequence, a hydrostatic pressure gradient across their membrane may develop. Multiplication of the

magnitude of this pressure gradient by the partial specific volume of a substance will yield an additional term in the electrochemical gradient. Consequently, the assumption that the distribution of SCN^- across the membrane reflects the electric potential difference may fall short at high pressure gradients. The partial specific volumes of the present substances are, however, so low (Weast, 1974) that only at, as yet, ununderstandably high pressure gradients could this error become relevant.

After a review of these more technical problems associated with the determination of $\Delta\tilde{\mu}_{\text{H}^+}$ in submitochondrial particles, a more fundamental problem concerns the proposal (cf. Wiechmann *et al.*, 1975; Azzone *et al.*, 1978; van Dam *et al.*, 1978; Kell, 1979; see, however, Westerhoff *et al.*, 1981b) that the 'relevant proton electrochemical gradient' ($\Delta\tilde{\lambda}_{\text{H}^+}$) may be localized close to the membrane. The measured bulk-to-bulk $\Delta\tilde{\mu}_{\text{H}^+}$ would then be an underestimate of this relevant $\Delta\tilde{\lambda}_{\text{H}^+}$, the degree of underestimation varying with the proton permeability of the membrane (van Dam *et al.*, 1978).

In experiments very similar to those reported here, in which we measured the ratio of ΔG_e to $\Delta\tilde{\mu}_{\text{H}^+}$ as a function of protonophore concentration (Westerhoff *et al.*, 1981a), we did indeed observe that this ratio increased with increasing amounts of protonophore. We concluded that for 'site 1' of oxidative phosphorylation in submitochondrial particles the notion of 'localized chemiosmosis' may be realistic (contrast Westerhoff *et al.*, 1981b). It might be suggested that the endogenous proton permeability of the membranes of our submitochondrial particles was so high that already in the absence of added protonophore $\Delta\tilde{\mu}_{\text{H}^+}$ underestimated significantly $\Delta\tilde{\lambda}_{\text{H}^+}$, so that a ratio of $\Delta G_e/\Delta\tilde{\lambda}_{\text{H}^+} = n_{\text{H}}^e = 1$ would be consistent with the observed ratio of $\Delta G_e/\Delta\tilde{\mu}_{\text{H}^+} = 1.6$. Branca *et al.* (1981) examined the dependence of the measured protonmotive force in succinate-energized submitochondrial particles on the medium composition. The medium composition used in the experiments reported in the present paper yielded (Branca *et al.*, 1981) the highest values of the protonmotive force, which would suggest that this medium minimizes the difference between $\Delta\tilde{\mu}_{\text{H}^+}$ and $\Delta\tilde{\lambda}_{\text{H}^+}$. Furthermore, a plot of $\Delta G_e/\Delta\tilde{\mu}_{\text{H}^+}$ versus $\Delta\tilde{\mu}_{\text{H}^+}$ for an experiment in which $\Delta\tilde{\mu}_{\text{H}^+}$ was varied with protonophore (Westerhoff *et al.*, 1981a) shows that, at $\Delta\tilde{\mu}_{\text{H}^+}$ values of 15 kJ/mol and higher, the ratio $\Delta G_e/\Delta\tilde{\mu}_{\text{H}^+}$ became almost independent of $\Delta\tilde{\mu}_{\text{H}^+}$. Consequently, we suppose that the phenomenon of 'localized chemiosmosis' was not influential in the present experiments and that the ratio $\Delta G_e/\Delta\tilde{\mu}_{\text{H}^+}$ did not grossly overestimate the $\bar{\text{H}}^+/\text{e}^-$ stoichiometry of 'site 1'.

It may be of interest to compare the present conclusion concerning the stoichiometry of $\bar{\text{H}}^+/\text{e}^-$

at 'site 1' to conclusions obtained by using other methods. The oxygen-pulse technique for measuring $\bar{H}^+/e^-$ ratios has been applied to submitochondrial particles: Hinkle & Horstmann (1971) observed a difference between NADH and succinate of 1.0 $\bar{H}^+/e^-$, but only at pH values below 7.0. Their explanation of lower $\bar{H}^+/e^-$ ratios observed at higher pH values being due to penetrating amines may well be extended to indicate that the $\bar{H}^+/e^-$ values at 'site 1' around pH 6.5 may exceed 1.0. Mitchell & Moyle (1966) presented mitochondria with pulses of oxygen or ferricyanide with either 3-hydroxybutyrate or succinate as the substrate. The difference between the two $\bar{H}^+/e^-$ values was 1. Lawford & Garland (1972) pulsed with quinones in the presence of 3-hydroxybutyrate and observed $\bar{H}^+/e^-$ values of 0.38, which, after correction for rotenone-insensitive oxidation, approached the value of 1.0. Brand *et al.* (1976a) showed, however, that inhibition of phosphate transport increased the observed $\bar{H}^+/e^-$ value of 'site 1' (actually 'sites 1 + 2 + 3' minus 'sites 2 + 3') in oxygen pulses to 1.5. As in our experiments phosphate movement would not interfere with the stoichiometry determination, this value seems to be confirmed by the present paper. Yet Pozzan *et al.* (1979), who also inhibited phosphate transport, measured a difference of 2.0 in H^+/e^- between endogenous substrate and succinate as substrate. Also, in view of the higher $\bar{H}^+/e^-$ values obtained by Brand *et al.* (1976b) when pulsing with reductant, it must be concluded that the results of pulse experiments are not conclusive with regard to the $\bar{H}^+/e^-$ stoichiometry of 'site 1'. Reasons for this (Wikström & Krab, 1980) include the possibilities that the transhydrogenase reaction, scalar Bohr protons or ion permeation interfere. Also, the extrapolations to correct for proton back-leakage during the proton-extrusion phase may be incorrect owing to rapid changes in actual membrane potential in the course of pulse experiments (Mitchell, 1968; Heinz *et al.*, 1981). Finally, pulse experiments generally suffer from the disadvantage that only a few turnovers of the respiratory chain take place: proton association or dissociation, connected to the charging of the membrane's capacitance (cf. Kell, 1979), will complicate the interpretation of the experimental results. Also, pulse experiments yield the stoichiometries for different substrates at low $\Delta\bar{\mu}_{H^+}$ values whereas those at high $\Delta\bar{\mu}_{H^+}$ values are physiologically more relevant. Brand *et al.* (1978) devised a method to compare stoichiometries for different substrates at arbitrary values of the protonmotive force. For the $\bar{H}^+/e^-$ values at 'site 1', 'site 2' and 'site 3' they found the proportions 0.5:0.5:1.0. The stoichiometries at physiological values of the protonmotive force at 'sites 2 and 3' are still under discussion. Consequently, also, this experiment did

not yield conclusive evidence for a certain $\bar{H}^+/e^-$ number for 'site 1' of oxidative phosphorylation. We conclude that our value of 1.6 $\bar{H}^+/e^-$ for 'site 1' is at present the only reliable value determined at high protonmotive force.

The present experiments also give some information about the $\bar{H}^+/\text{ATP}$ stoichiometry in submitochondrial particles. Table 1 shows the results of three phosphate potential determinations in the present experiments. As in these experiments the H^+ -translocating ATPase is not at equilibrium, but continuously pumping protons to compensate for proton back-leakage, the ratio of phosphate potential to $\Delta\bar{\mu}_{H^+}$ is an overestimate of the actual stoichiometry. Thus Table 1 suggests that the mechanistic $\bar{H}^+/\text{ATP}$ stoichiometry is lower than 2.5. This is in line with earlier conclusions by van Dam *et al.* (1978), but in conflict with a suggestion made by Sorgato *et al.* (1981). The pH gradient in their experiments being undetectably low, the ratio of phosphate potential to membrane potential had values of about 3.3. As indicated by these authors, however, the $\Delta G_p/\Delta\bar{\mu}_{H^+}$ ratios measured under conditions of ATP hydrolysis are expected to overestimate the actual stoichiometry. Therefore the ratio found by Sorgato *et al.* (1981) may still be consistent with an $\bar{H}^+/\text{ATP}$ stoichiometry of 2, as suggested by the experiments of Berry & Hinkle (1978), van Dam *et al.* (1978) and Branca *et al.* (1981) (the value of 1.9 in the medium of physiological osmolarity is cited; it should be noted that in other media the latter authors find ratios exceeding the value of 2, or even 3).

The consideration that the adenine nucleotide translocator is electrogenic (Klingenberg, 1980; see, however, Reynafarje & Lehninger, 1978), and that the $\bar{H}^+/\text{ATP}$ in rat liver mitochondria has been found to be even lower than 3 (Nicholls, 1974; Wiechmann *et al.*, 1975; Azzone *et al.*, 1978), suggests that a value of 2 for $\bar{H}^+/\text{ATP}$ in submitochondrial particles may at present be the best working hypothesis. An $\bar{H}^+/\text{ATP}$ of 2 and an $\bar{H}^+/2e^-$ of 3 for 'site 1' would yield a $\sim P/O$ ratio in submitochondrial particles of 1.5 for 'site 1' that does not differ much from the value of 4/3 suggested by Rottenberg & Gutman (1977). The $\sim P/O$ ratio in mitochondria for 'site 1' would be 1, which slightly differs from the 2/3 proposed by Hinkle & Yu (1979). Neither Rottenberg & Gutman (1977) nor Hinkle & Yu (1979) used methods that corrected for proton back-leakage through the mitochondrial membrane, and consequently both groups would have underestimated the true $\sim P/O$ theoretical stoichiometry.

To summarize: we interpret the experiments described in the present paper as indicative of $\bar{H}^+/e^-$ ('site 1') ≈ 1.5 , H^+/ATP (submitochondrial particles) = 2 and $\sim P/O$ ('site 1', submitochondrial

particles) = 1.5 at physiological magnitudes of the protonmotive force. These values are largely consistent with the available earlier measurements at physiological values of the protonmotive force.

We thank Mr. A. K. Groen and Mr. R. C. Vervoorn for their assistance in setting up the NAD(H) assays, Mr. A. L. M. Simonetti for expert technical assistance, Dr. M. C. Sorgato, Dr. D. Pietrobon and Dr. G. F. Azzone for allowing perusal of their manuscripts before publication, Dr. K. J. Hellingwerf for giving some of his amino- $[^3\text{H}]$ methane and for critical reading of the manuscript, and Ms. G. E. E. van Noppen and Ms. Y. Chong for their help in preparing the manuscript. Dr. K. van Dam has given invaluable theoretical support, for which we are very grateful. He, as well as Mr. G. van den Berg, Mr. A. K. Groen and Dr. M. C. Sorgato, is thanked for critical evaluation of the manuscript. This study was supported by a grant from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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