Biochimica et Biophysica Acta, 723 (1983) 59–70
Elsevier Biomedical Press

BBA 41278

STUDIES ON THE RELATIONSHIP BETWEEN ATP SYNTHESIS AND TRANSPORT AND THE PROTON ELECTROCHEMICAL GRADIENT IN RAT LIVER MITOCHONDRIA

MARIO ZORATTI, DANIELA PIETROBON and GIOVANNI FELICE AZZONE

C N R Unit for the Study of the Physiology of Mitochondria and Institute of General Pathology, University of Padua, Via Loredan 16, 35100 Padua (Italy)

(Received October 12th, 1982)
(Revised manuscript received January 17th, 1983)

Key words ATP synthesis, Nucleotide translocation, Chemiosmosis, Proton gradient, (Rat liver mitochondria)

The effect of ATP synthesis on $\Delta\tilde{\mu}_H$ in rat liver mitochondria has been analyzed by separating the steps of adenine nucleotide translocation and ATP synthesis in the matrix. Either exchange of ATP, synthesized by substrate level phosphorylation in the matrix of oligomycin-treated mitochondria, for external ADP, or activity of the membrane-bound ATP synthase complex results in $\Delta\tilde{\mu}_H$ depression with respect to resting state levels. This depression appears to be more pronounced, under strictly comparable conditions, when arsenate is used to stimulate ATP synthase activity than when the ornithine-citrulline conversion reaction is used for the same purpose.

Introduction

While it is generally accepted that operation of the redox and ATPase H^+ pumps gives rise to the formation of an H^+ electrochemical gradient, $\Delta\tilde{\mu}_H$, across the energy-transducing membrane of mitochondria, chloroplasts and bacteria, the mechanism by which these pumps are coupled is still a matter of debate. The answer as to whether $\Delta\tilde{\mu}_H$ acts as the sole competent intermediate coupling the redox and ATPase H^+ pumps may be sought by analyzing the flow-force relationship during ATP synthesis [1–5]. This analysis has recently been initiated by our group [6] and results have been obtained which cannot be accounted for by the chemiosmotic hypothesis in its simple, classical form The present work is an extension of the preceding study.

If $\Delta\tilde{\mu}_H$ is the only intermediate in energy transduction, it is predicted that when operation of one pump is driven backwards or, strictly speaking, thermodynamically reversed, by operation of the other, a depression of $\Delta\tilde{\mu}_H$ should occur This has in fact been observed, and it is now generally accepted that in the stationary state for phosphorylation the level of $\Delta\tilde{\mu}_H$ is lower than in static head (State 4) mitochondria. The extent of $\Delta\tilde{\mu}_H$ depression is controversial, ranging from 2–5 mV [1,7] to 50 mV [8]. (In our hands about 15–20 mV [6].). In the chemiosmotic view this $\Delta\tilde{\mu}_H$ depression is essentially a consequence of charge translocation across the membrane.

Two distinct charge-translocating processes which occur during phosphorylation of external ADP tend to lower $\Delta\tilde{\mu}_H$: (1) the ATP-ADP exchange which results in the extrusion of one negative charge per ATP exported via the adenine

Abbreviations TPMP, triphenylmethylphosphonium, DMO, 5,5-dimethyloxazolidine-2,4-dione, $\Delta\Delta\tilde{\mu}_H$ ($\Delta\Delta\psi$, $\Delta\Delta$pH), variation of $\Delta\tilde{\mu}_H$ ($\Delta\psi$, ΔpH) with respect to State 4 levels, J_o, rate of oxygen consumption, J_{ATP}, rate of ATP production (translocation), Mops, 4-morpholinepropanesulfonic acid

nucleotide translocase [9] and (ii) the influx of two to three H^+ for the synthesis of one ATP via the ATPase H^+ pump [10–12] The fraction of available energy used by each process should be proportional to the number of charges translocated.

Determination of $\Delta\tilde{\mu}_H$ depression during ATP synthesis in the absence of transport has been carried out in submitochondrial particles [13], chloroplasts [14], chromatophores [15–17] and bacterial vesicles [18]. In rat liver mitochondria, stimulation of ATP synthase activity coupled to carbamoyl phosphate production has been used as a tool to obtain the same type of information. However, contrasting results have been reported: Duszynski et al. [10] found a considerable $\Delta\tilde{\mu}_H$ depression, while Williamson et al. [19] did not A clarification of the effects of ATP synthesis and transport on $\Delta\tilde{\mu}_H$ is important as a test of the chemiosmotic model and for the interpretation of the results presented in Ref. 6. $\Delta\tilde{\mu}_H$ depression was therefore measured under conditions either of net ATP extrusion via the adenine nucleotide carrier (without ATP synthase-catalyzed ATP synthesis) or of ATP synthesis (without transport)

Materials and Methods

The experimental system

Isolation of the effect of ATP-ADP exchange of $\Delta\tilde{\mu}_H$ requires generation of ATP within the matrix by a process independent of the ATPase H^+ punp, which must be kept blocked This can be done by taking advantage of substrate level phosphorylation, which generates one molecule of ATP for each molecule of α-ketoglutarate converted to succinate [20]. The reaction is favored by maintaining the $NAD(P)^+/NAD(P)H$ pool oxidized through the reductive amination of α-ketoglutarate to glutamate (Krebs-Cohen dismutation) [21,22]. For this purpose NH_4^+ and malonate are added to the medium. In addition to inhibiting succinate oxidation, malonate favors α-ketoglutarate transport [23]. The ATPase H^+ pump is blocked by oligomycin while transport of endogenously synthesized ATP is facilitated by maintaining a low ATP/ADP ratio in the outer aqueous phase. The effect of adenine nucleotide exchange on $\Delta\tilde{\mu}_H$ can then be estimated by comparing the values of $\Delta\tilde{\mu}_H$ under conditions of maximal rate of transport and of transport inhibition by atractyloside.

Isolation in intact mitochondria of the effect of ATP synthesis through the ATPase H^+ pump, in the absence of adenine nucleotide exchange, has been achieved in two ways. One involves the use of arsenate, a well known uncoupler of oxidative phosphorylation [24]. Addition of 1–2 mM arsenate to mitochondria results in a marked atractyloside-insensitive, oligomycin-sensitive stimulation of respiration [25] The commonly accepted explanation for this effect considers arsenate as an analog of P_i. Hence arsenate can be utilized by the ATP synthases to generate an analog of ATP, AsADP, which undergoes rapid hydrolysis [26,27] (denoted also as 'arsenolysis') in the mitochondrial matrix. The rate of respiration is therefore a function of the rate of AsADP synthesis, which in turn is equal to the rate of hydrolysis. Thus, addition of arsenate to atractyloside-supplemented mitochondria is equivalent to generating an endogenous ATPase activity.

The second approach used in the present study is based on the operation of part of the urea cycle. In mitochondria isolated from the liver of rats kept on a high-protein diet, the ATP-requiring conversion of ornithine to citrulline [28] proceeds at a fairly rapid rate [10,19]. The reaction leads to a lowering of the matrix ATP/ADP ratio [29] and to an atractyloside-insensitive, oligomycin-sensitive stimulation of respiration.

Materials

Liver mitochondria from male albino Wistar rats were prepared according to a standard procedure [30] in a medium containing 0 25 M sucrose, 10 mM Tris-HCl (pH 7 4), 0.1 mM EGTA and 0 1% bovine serum albumin. Bovine serum albumin and EGTA were omitted in the last washing. Rats utilized for the ornithine experiments were kept on a high-protein diet (approx. 40% total proteins) supplied by Piccioni (Brescia, Italy) for 2–8 weeks before killing. Protein concentration was determined by the biuret method vs. a bovine serum albumin standard All reagents and inhibitors used were of the maximum purity commercial grade. Triphenyl[^{14}C]methylphosphonium iodide was synthesized by Dr. M. Beltrame. Other radio-

actively labelled compounds were obtained from Amersham.

Medium composition is indicated in the legends to the tables. Care was taken not to vary the pH of the suspensions by any addition.

Monitoring the reactions

Rates of respiration in the arsenate and ornithine experiments were estimated from the decrease in oxygen concentration in the medium, which was followed polarographically with a Clark electrode (Yellow Springs) in a closed, thermostatically controlled vessel with magnitude stirring. The reactions were started by the addition of either arsenate or ornithine after 1–2 min of incubation. In the case of the ornithine reaction, the rate of respiration increased gradually over an approx 1 min period, and then stabilized Addition of arsenate induced a respiration increase essentially without any lag time. The new rate of oxygen consumption remained constant over the time span of the experiment (3–4 min).

To determine the rate of adenine nucleotide exchange in the translocation experiments, suspensions of mitochondria (2 or 4 mg/ml) were incubated in the substrate level phosphorylation medium (see legend to Table I) in a thermostatically controlled vessel with magnetic stirring The medium contained either 1 mM EDTA to sequester Mg^{2+} and thus inhibit adenylate kinase activity, or $MgCl_2$, glucose and an excess of hexokinase. The reaction was started by the addition of ADP after a 3 min incubation 1-ml samples of the suspension were withdrawn at time intervals and quenched with perchloric acid. After neutralization with KOH/triethanolamine, ATP or glucose 6-phosphate formation was measured by standard enzymatic methods [31]. The data were corrected for the contribution by endogenous nucleotides and the adenylate kinase reaction using the results of duplicate experiments conducted in the presence of an excess of both oligomycin and atractyloside. Controls were carried out to ascertain that the concentrations of oligomycin and atractyloside used were sufficient to block completely phosphorylation and transport, respectively. In the case of experiments involving substrate level phosphorylation, complete blockage of the ATP synthases was checked by incubating mitochondria in a succinate (+rotenone, +oligomycin) medium and verifying that no ATP was produced

Determination of $\Delta\tilde{\mu}_H$

The transmembrane electrical potential gradient, $\Delta\psi$ was determined from the distribution between mitochondrial matrix and medium of the labeled lipophilic cation triphenyl[^{14}C]methylphosphonium ([^{14}C]TPMP) (0 02 μCi/ml) [32] The transmembrane proton affinity gradient, ΔpH, was evaluated from the distrubition of 5,5-[^{14}C]dimethyloxazolidine-2,4-dione ([^{14}C]DMO) (0.05 μCi/ml) [33] or of [^{3}H]acetate [34] Mitochondria were incubated, in the appropriate medium, in polyethylene centrifuge tubes (4 mg protein in 2 ml), kept in a thermostatically controlled bath. The reaction of interest was started by the addition of either ADP, arsenate or ornithine A sufficient time (2–3 min) was allowed to elapse to ensure attainment of steady state for respiration or adenine nucleotide exchange. The mitochondria were then separated from the suspension medium by centrifugation in the S-12 rotor of a Sorvall RC12 Supercentrifuge at $18–19 \cdot 10^3$ rpm. for 2 min 100-μl aliquots of the supernatant were placed in scintillation vials along with 2 ml of Insta-Gel scintillation fluid (Packard) The walls of the centrifuge tubes were carefully dried with filter paper and the pellets dissolved by incubation with 0.5 ml of 1 mM EDTA, 0.1% NaCl and 0.9% sodium deoxycholate for 2–3 h at room temperature. The centrifuge tubes were sealed with parafilm during this time. The dissolved pellets were quantitatively transferred to scintillation vials containing 100 μl H_2O together with 4 ml Insta-Gel, and counted along with the supernatant samples on a Packard 300C scintillation spectrometer.

The cpm output was automatically converted to dpm by means of an external standard and preset calibration curves to correct for fluorescence quenching effects. All measurements were done at least in duplicate (see tables). Each experiment reported was performed utilizing one mitochondrial preparation and measuring rates of respiration or adenine nucleotide translocation and $\Delta\tilde{\mu}_H$ in parallel. ΔpH measurements are strongly dependent on the correction applied to compensate for the contribution of the medium trapped in the pellet. To estimate better the in-

termitochondrial volume, all incubations containing ^{14}C-labelled probes were supplemented with $^{3}H_2O$ (0.2 μCi/ml). The intermitochondrial space for each pellet was then estimated from the difference between the total pellet volume, measured by tritiated water, and the matrix volume (see below). This procedure could not be followed when [^{3}H]acetate was used to measured ΔpH. For this reason, acetate-based ΔpH measurements showed considerable scatter among duplicate samples.

The distribution of labelled DMO was measured in State 4 mitochondria either at a constant concentration of [^{14}C]DMO and with increasing concentrations of unlabelled DMO (up to 0.5 mM), or at increasing concentrations of labelled probe and without added unlabelled compound. In the former case the amount of radioactivity found in the pellet did not change. For the latter case, it is straightforward to derive the relationship

$$dpm^{p}_{tot} = \frac{dpm^{p}_{distr}}{dpm^{s}} \times dpm^{s} + dpm^{p}_{bind}$$

where dpm^{p}_{tot} is the total amount of ^{14}C radioactivity found in the pellet, dpm^{p}_{distr} the part of this radioactivity due to ΔpH-driven DMO accumulation and any 'non-saturable' binding, dpm^{p}_{bind} the amount incorporated because of saturable binding phenomena, and dpm^{s} the radioactivity found in 1 μl of supernatant. In agreement with Ref. 35, a plot of dpm^{p}_{tot} vs. dpm^{s} gave a straight line going through the origin, as expected if ΔpH does not vary and DMO does not bind in a saturable manner. On the basis of the experimental approach described in Ref. 6, we tend to discount the possibility of unsaturable binding as well. Therefore, no binding corrections were applied to the DMO data. Similar controls were not performed for acetate.

The approach to the problem of TPMP binding is described elsewhere [6,36]. It compensates for what appears to be a saturable binding of this probe to deenergized mitochondria. The extent of the correction is small, corresponding at most to 1–2% of the radioactivity found in coupled mitochondria. Other authors [37,38] have concluded that a large and constant fraction of the TPMP taken up by energized mitochondria is bound. A correction of this type would lead to lower $\Delta\psi$ values, but has no effect on differences between them. For example, take entries 1 and 2 in Table I (Expt. 1). If two-thirds of the radioactivity found in the pellets were considered to be due to binding, the $\Delta\psi$ values would change from 169 to 141 and from 175 to 147 mV, respectively. The difference between the two measured values, 6 mV, would be unaffected. The differences are similarly not affected by the error arising from the explicit, and probably erroneous, assumption that the activity coefficients and the standard chemical potentials for the probe molecules in the inner and outer compartments are equal.

In a few experiments $\Delta\psi$ was continuously followed with a TPMP-sensitive electrode, contructed essentially according to Ref. 39. An Orion Ion Analyzer 92-20 was used as electrode body, and a Beckman combination H^+ electrode as reference. Initial TPMP concentration in these determinations was 10 μM. Results obtained utilizing this method were in excellent agreement with those obtained using [^{14}C]TPMP.

Mitochondrial matrix volume is most often determined from the difference between $^{3}H_2O$-permeable and [^{14}C]sucrose-permeable spaces [40]. Since the permeability of the inner membrane to sucrose is not negligible [6,36,41,42], [^{14}C]ATP was used instead. 200 μM atractyloside and 2 mM adenine nucleotides were included in the medium to prevent operation of the translocator and binding of the labelled compound. [^{14}C]ATP was added to the mitochondrial suspension immediately before centrifugation. The protein content of the pellets was determined in a parallel sample. With this method mitochondria were found to behave as osmometers: a plot of matrix volumes vs. the reciprocal of medium osmolarity gave a straight line with an intercept of about 0.2 μl/mg. The volumes measured for State 4 mitochondria and mitochondria supplemented with ornithine, ornithine + oligomycin, arsenate or arsenate + oligomycin in the appropriate reaction media were all the same within experimental error, namely, 0.5–0.55 μl/mg protein. In all our calculations we therefore used this value. The method cannot be applied to adenine nucleotide-translocating mitochondria, for which a volume of 0.5 μl/mg was also assumed.

Results

The effect of adenine nucleotide translocation on $\Delta\tilde{\mu}_H$

A proper evaluation of the effect of adenine nucleotide translocation of $\Delta\tilde{\mu}_H$ is provided by comparison of the values obtained in the presence and absence of the specific transport inhibitor, atractyloside. Table I shows the results of two typical experiments; $\Delta\tilde{\mu}_H$ values for State 4 mitochondria are included for comparison. Due to the smallness of the change and its nature as a composite quantity, the translocation-induced $\Delta\tilde{\mu}_H$ depression was nearly always smaller than its standard deviation. The $\Delta\tilde{\mu}_H$ variation was not significant according to the Behrens-Student test [43] applied to any given experiment.

A more rewarding approach involves application of statistical significance tests to the complete set of our data. $\Delta\psi$ and ΔpH variations were measured in eight independent experiments, and the $\Delta\psi$ variation only in a ninth experiment. In all nine cases $\Delta\psi$ was lower in the absence than in the presence of atractyloside (with $\Delta\Delta\psi$ values ranging from -2.2 to -12.1 mV) ΔpH was lower in the absence of atractyloside in four cases, and higher in the other four (with $\Delta\Delta$pH values ranging from -2.8 to $+4.1$ mV). $\Delta\tilde{\mu}_H$ was lower in the absence of atractyloside in seven cases, and higher in one case (with $\Delta\Delta\tilde{\mu}_H$ values ranging from $+1.8$ to -8 mV). Application of the Wilcoxon signed-rank test [44,45] to these data indicates that $\Delta\psi$ and $\Delta\tilde{\mu}_H$ variations were significant ($P < 0.01$ and $P = 0.02$, respectively) while ΔpH variations were not ($P > 0.10$). It may therefore be concluded that adenine nucleotide translocation per se causes a depression of $\Delta\tilde{\mu}_H$ essentially by lowering the electrical component of $\Delta\tilde{\mu}_H$ If $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_H$ variations are computed with reference to State 4 values (i.e., incubations without ADP, hexokinase, etc) the same result is obtained.

However, the present data are not adequate to provide a reliable estimate of $\partial\Delta\tilde{\mu}_H/\partial J_{ATP}$, i.e, of the extent of $\Delta\tilde{\mu}_H$ decrease associated with a given rate of ATP translocation.

The effect of arsenolysis on $\Delta\tilde{\mu}_H$

Table II shows the results of two typical experiments, one with succinate and one with glutamate as substrate, utilizing arsenate to stimulate ATP synthase activity in the absence of adenine nucleotide transport. Phosphate was omitted from the medium in view of its competitive effect with respect to arsenate [24,46,47]. The effect of arsenate may be evaluated by comparing the data obtained in the presence and absence of oligomycin. Again,

TABLE I

THE DEPRESSION OF $\Delta\tilde{\mu}_H$ DUE TO ADENINE NUCLEOTIDE TRANSLOCATION

(Expt 1) Medium composition 50 mM KCl, 10 mM Tris-Mops, 40 mM sucrose, 10 mM NH_4Cl, 2 mM P_i-Tris, 10 mM α-ketoglutarate, 2 mM malonate, 1 mM EDTA, 2 μg/mg protein oligomycin, pH 7 4 When present ADP, 0 5 mM, atractyloside, 50 μM *T* 30°C (Expt 2) Medium as in Expt 1 except that 1 mM EDTA was substituted by 10 mM glucose, 4 mM $MgCl_2$, 0 5 mM EGTA When present ADP, 400 μM, hexokinase exceeding the amount needed to stimulate maximally respiration in a succinate-containing medium *T* 30°C Values are means ± S D for number of determinations in parentheses

	Additions	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_H$ (mV)	$\Delta\Delta\tilde{\mu}_H$ [a] (mV)	J_{ATP} (nmol/mg per min)
Expt 1	ADP	169 3±4 0 (6)	23 6±0 8 (6)	192 9±4 1	−3 6±	21
	ADP, atractyloside	175 3±1 4 (6)	21 2±1 6 (6)	196 5±2 1		–
	–	173 4/174 0	19 9/20 6	194 0		–
Expt 2	Hexokinase, ADP	166 0±3 6 (6)	16 6±2 5 (6)	182 6±4 4	−5 7±6 2	20
	Hexokinase, ADP, atractyloside	168 6±3 4 (6)	19 7±2 7 (6)	188 3±4 3		–
	–	172 9±1 3 (3)	18 2±2 2 (3)	191 1±2 6		–

[a] ($\Delta\tilde{\mu}_H$ in row 1)−($\Delta\tilde{\mu}_H$ in row 2)

TABLE II

THE DEPRESSION OF $\Delta\tilde{\mu}_H$ DUE TO ARSENOLYSIS

(Expt 1) Medium composition 75 mM KCl, 20 mM Tris-Mops, 2 mM $MgCl_2$, 10 mM succinate-Tris, 40 mM sucrose, 0 5 mM EGTA, 50 μM atractyloside, pH 7 4 When present arsenate, P_i, 2 mM, oligomycin, 1 μg/mg protein T 30°C (Expt 2) Medium composition 20 mM KCl, 120 mM sucrose, 5 mM $MgCl_2$, 5 mM glutamate, 0 5 mM EGTA, 50 μM atractyloside, pH 7 4 When present arsenate, 2 mM, oligomycin, 2 μg/mg protein T 25°C Values are means ±S D for number of determinations in parentheses

	Additions	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_H$ (mV)	$\Delta\Delta\tilde{\mu}_H$ (mV)	J_o (ngatom/mg per min)
Expt 1	Arsenate	179 4±0 5 (3)	20 3±1 4 (3)	199 7±1 5		56
					−6 2±2 3 [a]	
	Arsenate, oligomycin	185 2±1 5 (3)	20 7±0 7 (3)	205 9±1 7		20
					−10 6 [b]	
	P_i	185 4/191 2	23 1/20 9	210 3		19
Expt 2	Arsenate	169 3±1 5 (3)	28 0±1 1 (3)	197 3±1 9		27
					−19 5±2 4 [a]	
	Arsenate, oligomycin	187 1±1 0 (3)	29 7±1 1 (3)	216 8±1 5		6
					−20 9 [b]	
	–	175 5/177 1	43 1/40 6	218 2		6

[a] $\Delta\Delta\tilde{\mu}_H$ is given by $\Delta\tilde{\mu}_H$ in first row minus $\Delta\tilde{\mu}_H$ in second row
[b] $\Delta\Delta\tilde{\mu}_H$ is defined as ($\Delta\tilde{\mu}_H$ in first row)−(average $\Delta\tilde{\mu}_H$ in third row)

in most cases $\Delta\psi$, ΔpH or $\Delta\tilde{\mu}_H$ differences were not large enough, with respect to the associated standard deviations, to be considered significant on the basis of the Behrens-Student test. However, application of the Wilcoxon signed-rank test to the whole set of 19 experimental results shows that arsenolysis produced significant decreases in both $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_H$ ($P < 0\,01$ in all cases). The answer provided by the incubations in the presence of arsenate ±oligomycin is therefore unambiguous.

An unexpected observation made in these experiments is that addition of oligomycin to State 4 mitochondria resulted in a statistically significant decrease in $\Delta\psi$ ($P < 0.01$) (20 determinations) even though respiration was not affected or somewhat decreased (no such effect is caused by atractyloside with any statistical significance) Controls showed that this effect is not due to the ethanol added together with oligomycin. In Table II the $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_H$ values of State 4 mitochondria are also reported, which may be more appropriate to use as reference to evaluate the effect of arsenate. It should be remembered that $\Delta\psi$ and ΔpH values in State 4 depend on whether a permeable weak acid is present. It is well known (and cf. Table II) that P_i and arsenate, by acting as proton carriers, cause a decrease in ΔpH and a corresponding increase in $\Delta\psi$. $\Delta\Delta\psi$ and $\Delta\Delta$pH values obtained with reference to State 4 therefore depend on whether or not phosphate was present in the State 4 incubation. Only $\Delta\tilde{\mu}_H$ values may therefore be appropriately compared.

In a total of 25 determinations, $\Delta\tilde{\mu}_H$ was always lower in the presence of arsenate than in State 4, with values of $\Delta\Delta\tilde{\mu}_H$ ranging from −2 to −26 mV, and respiratory control ratios (referred to State 4 respiration) varying between 1 1 and 5.1, depending on substrate and concentration of arsenate. With succinate as substrate, and 2 mM arsenate, $\Delta\tilde{\mu}_H$ depression (referred to State 4) averaged 15 mV (six measurements) with respiratory control ratios between 2.1 and 3 0. When referred to arsenate and oligomycin-supplemented mitochondria $\Delta\tilde{\mu}_H$ depression averaged about 10 mV. Thus, the presence of arsenate induces a decrease in $\Delta\tilde{\mu}_H$ levels, whether State 4 mitochondria or mitochondria supplemented with both arsenate and oligomycin are taken as the reference state. A rough correlation between the extent of

$\Delta\tilde{\mu}_H$ depression and stimulation of respiration was observed.

It may be mentioned that with the same mitochondrial preparations the ADP-induced State 4–State 3 transition, with succinate as substrate, was accompanied by a $\Delta\tilde{\mu}_H$ depression of about 15 mV and a respiratory control ratio between 3 and 4 (Cf. also Ref. 6). The question may arise as to whether the depression of $\Delta\tilde{\mu}_H$ is due to operation of the ATP synthases or to some unspecific membrane damage due to arsenate The latter hypothesis is unlikely in view of the low concentrations of arsenate used (maximum 2 mM) of the relatively short time of incubation and of the sensitivity of both $\Delta\tilde{\mu}_H$ depression and respiratory stimulation to oligomycin. In control experiments, addition of up to 10 mM arsenate to oligomycin-treated mitochondria resulted in no detectable increase in the rate of respiration for up to 4 min

The effect of ornithine metabolism on $\Delta\tilde{\mu}_H$

Table III shows the results of three representative experiments aimed at determining the effect of the ornithine-citrulline reaction on the values of $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_H$ and on the rate of respiration. Again, the values of these parameters in mitochondria metabolizing ornithine may be compared to those in mitochondria supplemented with both ornithine and oligomycin, or in State 4 mitochondria (without ornithine or oligomycin). The comparison $\pm$oligomycin was obtained for $\Delta\psi$ in 24, for ΔpH in 26 and $\Delta\tilde{\mu}_H$ in 22 cases. The signed-rank test indicates that ornithine metabolism induced a statistically nonsignificant decrease in $\Delta\psi$ ($P > 0.05$), a statistically significant increase in ΔpH ($P < 0.02$) and a statistically nonsignificant increase in $\Delta\tilde{\mu}_H$ ($P > 0.10$). Since the slight oligomycin-induced $\Delta\tilde{\mu}_H$ depression, observed in this system also, might have masked the ornithine effect, the latter was further analyzed by taking the values of $\Delta\psi$, ΔpH and $\Delta\tilde{\mu}_H$ of State 4 mitochondria as a reference in 16 independent experiments The statistical significance test shows in this case significant decreases in $\Delta\psi$ and $\Delta\tilde{\mu}_H$ ($P < 0.01$) and increase in ΔpH ($P < 0.05$) induced by ornithine metabolism. The extent of $\Delta\tilde{\mu}_H$ depression and of respiratory stimulation varied from one mitochondrial preparation to the other,

TABLE III

THE EFFECT OF ORNITHINE METABOLISM ON $\Delta\tilde{\mu}_H$

Medium composition 50 mM KCl, 10 mM (Expts 1 and 2) or 20 mM (Expt 3) Tris-Mops, 40 mM sucrose, 16 mM $KCHO_3$, 2 mM $MgCl_2$, 2 mM (Expts 1 and 2) or 1 mM (Expt 3) P_i-Tris, 10 mM succinate-Tris, 10 mM NH_4Cl, 0 5 mM EGTA, 50 μM (Expts 1 and 2) or 10 μM (Expt 3) atractyloside, pH 7 4 *T* 30°C When present ornithine, 10 mM, oligomycin, 1 μg/mg protein Values are means $\pm$S D for number of determinations in parentheses

	Additions	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_H$ (mV)	$\Delta\Delta\tilde{\mu}_H$ (mV)	J_o (ngatom/mg per min)
Expt 1	–	189 2/187 9	17 1/16 4	205 3		36
					−5 3 [a]	
	Ornithine	178 4±1 2 (4)	21 6±1 7 (4)	200 0±2 1		60
					−0 5±4 1 [b]	
	Ornithine, oligomycin	183 2±2 7 (4)	17 3±2 3 (4)	200 5±3 5		21
Expt 2	–	189 5±1 4 (3)	19 3±0 5 (3)	208 8±1.5		46
					−11 4±1 9 [a]	
	Ornithine	178 4±0 2 (3)	19 0±1 2 (3)	197 4±1 2		99
					−1 2 [b]	
	Ornithine, oligomycin	183 0/182.5	15 8±0 3 (3)	198 6		36
Expt 3	–	190 7±1 5 (4)	22 5±2 2 (4)	213 2±2 7		36
					+0 8±4 2 [a]	
	Ornithine	184 1±2 7 (4)	29 9±1 7 (4)	214 0±3 2		56

[a] Row 2 minus row 1 values

[b] Row 2 minus row 3 values

TABLE IV

COMPARISON OF THE EFFECTS OF ARSENOLYSIS AND ORNITHINE METABOLISM ON $\Delta\tilde{\mu}_H$

Medium composition as in Table III, Expt 3, except that phosphate was omitted When present ornithine, 10 mM, P_i, 1 mM, arsenate 150 μM (Expts 1 and 2), 1 mM (Expt 3) or 0 7 mM (Expt 4) $\Delta\psi$ and ΔpH values are means $\pm$S D for three (Expt 1, 2 and 4) or four (Expt 3) determinations

	Additions	$\Delta\psi$ (mV)	ΔpH (mV)	$\Delta\tilde{\mu}_H$ (mV)	$\Delta\Delta\psi$ (mV)	$\Delta\Delta$pH (mV)	$\Delta\Delta\tilde{\mu}_H$ (mV)	$\Delta\Delta\Delta\tilde{\mu}_H$ [a] (mV)	J_o (ngatom/mg per min)
Expt 1	–	182 4±0 1	37 2±1 5	219 6±1 5					35
					−8 4±0 3	+3 7±1 6	−4 7±1 6		
	Ornithine	174 0±0 3	40 9±0 4	214 9±0 3				−2 6±3 1	48
					−6 8±2 0	−0 5±1 7	−7 3±2 6		
	Arsenate	175 6±2 0	36 7±0 7	212 3±2 1					45
Expt 2	–	185 3±1 1	32 3±1 7	217 6±2 0					26
					−5 7±1 7	+4 3±1 7	−1 4±2 3		
	Ornithine	179 6±1 2	36 6±0 3	216 2±1 2				−7 9±3 3	36
					−3 2±1 8	−3 2±1 8	−9 3±2 3		
	Arsenate	179 2±1 0	29 1±0 6	208 3±1 2					37
Expt 3	–	175 8±1 1	39 0±0 8	214 8±1 4					31
					−1 7±2 9	−9 1±1 9	−10 8±3 5		
	Ornithine, P_i	174 1±2 7	29 9±1 7	204 0±3.2				−4 1±5 2	56
					−4 2±3 1	−10 7±3 3	−14 9±3 8		
	Arsenate	171 6±1 5	28 3±3 2	199 9±3 5					52
Expt 4	–	179 4±1 4	35 8±0 8	215 2±1 6					27
					−1 4±1 5	−10 9±1 5	−12 3±2 1		
	Ornithine, P_i	178 0±0 4	24 9±1 3	202 9±1 4				−1 0±3 1	51
					−4 5±1 6	−8 8±1 7	−13 3±2 3		
	Arsenate	174 9±0 7	27 0±1 5	201 9±1 7					49

[a] $\Delta\Delta\tilde{\mu}_H$(arsenate)− $\Delta\Delta\tilde{\mu}_H$(ornithine)

and depended on whether phosphate was present in the medium. Phosphate (1–2 mM) induced higher stimulated and State 4 respirations with correspondingly lower $\Delta\tilde{\mu}_H$ values. The effect on the State 4 parameters is presumably due to some ATP synthase activity occurring even in the absence of ornithine, since it is blocked by oligomycin. State 4 respiration was lowered by oligomycin whether or not P_i was present, so that respiratory control ratios obtained by addition of oligomycin were higher than those calculated on the basis of the resting state respiration. In the presence of 1 or 2 mM P_i the former varied between 2 and 3 (in one case 3.2) and the latter between 1.3 and 2.2 (in one case 2.5) while $\Delta\tilde{\mu}_H$ variations (referred to State 4) ranged between -2 and -14 mV (average -7 mV)

ΔpH was measured by acetate distribution in a few experiments. Acetate systematically gave ΔpH values about 20 mV higher than those obtained by DMO distribution. This discrepancy has already been observed by others (Ref 42; cf., however, Refs. 37 and 38). Its origin is open to speculation. The ΔpH variation pattern, however, remained the same.

Comparison of the effects of ornithine metabolism and arsenolysis on $\Delta\tilde{\mu}_H$

The $\Delta\Delta\tilde{\mu}_H$ data obtained during arsenate and ornithine metabolism suggest that these compounds might depress $\Delta\tilde{\mu}_H$ to different extends even while causing similar increases in the rate of respiration. To be valid, the comparison must be performed on the same mitochondrial preparations Furthermore, both resting state and stimulated respiration must be the same, and the same medium ought to be used throughout the experiment. A series of experiments satisfying these requirements was performed, four of which are presented in Table IV The effect of ornithine may be determined both in the absence and presence of exogenous P_i. In the former case the comparison with the effect of arsenate is straightforward when using a concentration of arsenate which gives the same rate of respiration as 10 mM ornithine (see Expts 1 and 2 in Table IV). In the latter case a correct comparison requires a concentration of arsenate which gives the same respiration as ornithine plus 1 mM P_i. As mentioned in the preceding section, phosphate causes a certain amount of ATP synthase activity in State 4 mitochondria isolated from liver of rats kept on a protein-rich diet. $\Delta\Delta\tilde{\mu}_H$ values are then properly calculated by subtracting the $\Delta\tilde{\mu}_H$ measured in State 4 without phosphate from the values measured in the presence of either arsenate or ornithine plus P_i.

The effects of arsenate and ornithine without exogenous P_i were compared in five experiments. $\Delta\tilde{\mu}_H$ was lowered more by arsenate then by ornithine in four cases, and less in one case In eight further experiments we compared the effects of arsenate and ornithine $+P_i$. $\Delta\tilde{\mu}_H$ was lowered more by arsenate in seven cases and more by ornithine $+ P_i$ in one case Neither of these two sets of experiments, taken alone, proves that a statistically significant difference exists between the effects of arsenate and ornithine ($\pm P_i$) on $\Delta\tilde{\mu}_H$. If the two sets are combined, however, application of the Wilcoxon test indicates that a significant difference exists ($P = 0.02$). Values of $\Delta\Delta\Delta\tilde{\mu}_H$ ($= \Delta\Delta\tilde{\mu}_H$(arsenate) $- \Delta\Delta\tilde{\mu}_H$(ornithine)) range from $+3.5$ to -12.6 mV

If $\Delta\Delta\tilde{\mu}_H$ for the ornithine experiments is calculated with reference to P_i-supplemented State 4 mitochondria in those cases in which phosphate was present, the difference obviously remains significant ($P < 0.01$)

Discussion

Our results indicate that both adenine nucleotide translocation and ATP synthase-catalyzed ATP synthesis are accompanied by $\Delta\tilde{\mu}_H$ depression, with respect to State 4 levels, in rat liver mitochondria. This conclusion is in qualitative agreement with the chemiosmotic hypothesis. The variability of the $\Delta\Delta\tilde{\mu}_H$ measurements, a consequence of the small extent of the variations, does not allow the establishment with certainty whether the $\Delta\tilde{\mu}_H$ depression during adenine nucleotide translocation and ATP synthesis is proportional to the net number of charges believed to be transported across the mitochondrial membrane during each event (translocation or synthesis).

ATP-ADP exchange lowers $\Delta\tilde{\mu}_H$ by decreasing its electrical component, $\Delta\psi$, as expected of an electrophoretic process and in agreement with pre-

vious findings [9,48]. The data obtained in experiments involving ornithine metabolism may be compared to those recently reported in the literature. Duszynski et al [10] observed, before addition of ornithine, a $\Delta\psi$ of 166 mV and a ΔpH of 52 mV During ornithine metabolism $\Delta\psi$ was depressed by 14 mV to 152 mV, while ΔpH remained constant. On the other hand, Williamson et al. [19] found a much smaller $\Delta\psi$ depression, say 3–4 mV, essentially balanced by the increase in ΔpH. Hence, in these latter experiments, citrulline synthesis and the accompanying respiratory stimulation were not paralleled by $\Delta\tilde{\mu}_H$ depression.

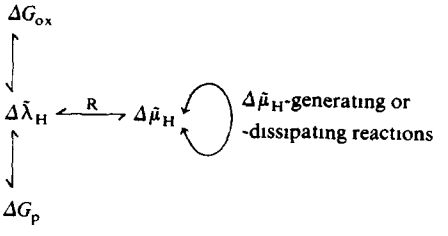
Among our experiments some may be found which agree rather closely with the results of Duszynski et al. (e.g., Expt 2 in Table III) and others which resemble instead those of Williamson et al (e.g, Expt. 3 in Table III) Experimental variability may thus account for such discrepancies. Statistical analysis of a large body of experimental results leads to the conclusion that ornithine metabolism causes both a ΔpH increase and a $\Delta\tilde{\mu}_H$ decrease with respect to State 4, as illustrated by Expts. 1 in Table III and 1 and 2 in Table IV

The increase in ΔpH following ornithine addition is presumably linked to its transport, the mechanism of which has not been completely clarified. Gamble and Lehninger [49] proposed an electrophoretic uptake in response to the negative-inside $\Delta\psi$, but more recently evidence has been put forward in favor of an ornithine/H^+ antiport (or uptake of the uncharged form in ornithine) [50] and an ornithine/citrulline exchange [51]. The rise of ΔpH supports the view that ornithine uptake occurs at least partly in exchange for protons. The simultaneous flows of ammonia and bicarbonate may also affect ΔpH. Metabolite transport would thus help to sustain $\Delta\tilde{\mu}_H$ Analogous processes have already been observed (e.g., see Ref. 52)

Our data also indicate that somewhat different changes of $\Delta\tilde{\mu}_H$ are associated with the same stimulation of respiration depending on whether they are brought about by supplying arsenate or ornithine to mitochondria from the liver of rats kept on a high-protein diet. The two separate sets of comparisons mentioned in Results (with ornithine $\pm P_i$), taken separately, indicate that this conclusion in uncertain. However, this may well be due to the small size of the samples. Their combination into one set is permissible, given the way the $\Delta\Delta\tilde{\mu}_H$ values were calculated, and when this is done the result becomes significant.

This observation is in constrast with the classical chemiosmotic model, and it is reminescent of the different correlations between rate of respiration and $\Delta\tilde{\mu}_H$ reported by some authors [1,3,38,53] depending on whether respiration was stimulated by uncouplers or by the induction of ATP synthesis. In the present work respiration is stimulated via the ATP synthases in both cases, so that allosteric effects by the adenine nucleotides [1,54] cannot provide an explanation

The discrepancy between the arsenate and ornithine data may become intelligible if other mechanistic schemes for energy transduction, e.g , Scheme I, are considered. Scheme I [54] represents



Scheme I

in essence a 'micro' or 'localized chemiosmosis' model. The assumption made is that the pertinent intermediate for energy transfer between ΔG_{ox} and ΔG_p is a localized transmembrane electrochemical proton gradient, $\Delta\tilde{\lambda}_H$ [54,55], which 'communicates' with bulk-phase $\Delta\tilde{\mu}_H$ but never reaches thermodynamic equilibrium with is due to the presence of a resistance to equilibration and of $\Delta\tilde{\mu}_H$-dissipating and -generating reactions, represented by the curved arrow. The model predicts phosphorylation to cause a depression of bulk-phase $\Delta\tilde{\mu}_H$. This depression is furthermore predicted to be the same, at equivalent rates of respiration and respiratory stimulation, in the two experiments (arsenolysis and ornithine metabolism). The discrepancy with this prediction can be explained on the basis of the following reasoning. Arsenolysis and ornithine metabolism at equivalent rates lead to the same depression of $\Delta\tilde{\lambda}_H$, the relevant intermediate. The disequilibrium between $\Delta\tilde{\lambda}_H$ and

$\Delta\tilde{\mu}_H$ is, however, smaller during ornithine transport, due to the ornithine-H^+ exchange reaction, which 'generates' ΔpH (i.e., $\Delta\tilde{\mu}_H$), partially offsetting $\Delta\tilde{\mu}_H$-dissipating reactions. Thus, equal extents of respiratory stimulation are accompanied by the same decrease in $\Delta\tilde{\lambda}_H$, but by different changes of $\Delta\tilde{\mu}_H$.

In conclusion, the present data (a) confirm that the adenine nucleotide exchange is an electrophoretic process and (b) provide further information as to the flow-force relationship during oxidative phosphorylation. Due to the smallness of the $\Delta\tilde{\mu}_H$ changes during activation of ATP synthesis, caution is needed before drawing firm conclusions as to the role of $\Delta\tilde{\mu}_H$ as the only relevant intermediate in oxidative phosphorylation It seems, however, that if the discrepancy found in the present investigation is confirmed and extended, it will be necessary to develop a more sophisticated elaboration of this concept

Acknlowledgements

We would like to thank Drs. H. Westerhoff and J. Duszynski for helpful discussions, Mr. L. Pregnolato and Mr. M. Santato for expert technical assistance and Mrs. Franca Schiavon Mazzari for typing the manuscript. We are particularly grateful to Professor B A. Melandri for help in setting up the TPMP electrode.

References

1 Padan, E and Rottenberg, H (1973) Eur J Biochem 40, 431–437
2 Johnson, R N and Hansford, R G (1977) Biochem J 164, 305–322
3 Azzone, G F, Pozzan, T, Massari, S and Bragadin, M (1978) Biochim Biophys Acta 501, 296–306
4 Van Dam, K Westerhoff, H V, Krab, K, Van der Meer, R and Arents, J C (1980) Biochim Biophys Acta 591, 240–250
5 Kuster, U, Letko, G, Kunz, W, Duszynski, J, Bogucka, K and Wojtzak, L (1981) Biochim Biophys Acta 636, 32–38
6 Zoratti, M, Pietrobon, D and Azzone, G F (1982) Eur J Biochem 126, 443–451
7 Yaguzhinskij, L S, Krasinskaya, I P, Oragunova, S F, Zinchenko, V P and Yevtodiyenko, Yu V (1980) Biophysics 24, 1130–1133
8 Nicholls, D G (1974) Eur J Biochem 50, 306–315
9 Klingenberg, M (1980) J Membrane Biol 56, 97–105
10 Duszynski, J., Bogucka, K, Letko, G, Kuster, U, Kunz, W and Wojtczak, L (1981) Biochim Biophys Acta 637, 217–223
11 Hinkle, P C and Yu, M L (1979) J Biol Chem 254, 2450–2455
12 Rottenberg, H and Gutman, M (1977) Biochemistry 16, 3220–3227
13 Sorgato, M C, Ferguson, S J, Kell, D B and John, P (1978) Biochem J 174, 237–256
14 Portis, A R and McCarty, R E (1974) J Biol Chem 249, 6250–6254
15 Leiser, M and Gromet-Elhanan, Z (1977) Arch Biochem Biophys 178, 79–88
16 Cirillo, V P and Gromet-Elhanan, Z (1981) Biochim Biophys Acta 636, 244–253 (1981)
17 Kell, D B, Ferguson, S J and John, P (1978) Biochim Biophys Acta 502, 111–126
18 Kell, D B, John, P and Ferguson, S J (1978) Biochem J 174, 257–266
19 Williamson, J R, Steinman, R, Coll, K and Rich, T (1981) J Biol Chem 256, 7287–7297 (1975)
20 Lehninger, A L Biochemistry, 2nd edn, ch 17, Worth Publishers, New York
21 Hunter, F E, Jr and Hixon, W J (1949) J Biol Chem 181, 67–79
22 Hoek, J B, Ernster, L, De Haan, E J and Tager, J M (1974) Biochim Biophys Acta 333, 546–549
23 De Haan, E J and Tager, J M (1968) Biochim Biophys Acta 153, 98–112
24 Crane, R K and Lipmann, F (1953) J Biol Chem 201, 235–243
25 Bruni, A and Azzone, G F (1964) Biochim Biophys Acta 93, 462–474
26 Warburg, O and Christian, W (1939) Biochem Z 303, 40–68
27 Moore, S A and Gresser, M J (1982) Fed Proc 41, 749, Abstr 2795
28 Lehninger, A L (1975) Biochemistry, 2nd edn., ch 21, Worth Publishers, New York
29 Wanders, J A, Van Woerkam, G M, Nooteboom, R F., Meijter, A J and Tager, J M (1981) Eur J Biochem 113, 295–302
30 Massari, S, Balboni, E and Azzone, G F (1972) Biochim Biophys Acta 283, 16–22
31 Estabrook, R W, Williamson, J R, Frenkel, R and Maitra, P K (1967) Methods Enzymol 10, 474–482
32 Azzone, G F, Pozzan, T, Bragadin, M and Dell'Antone, P (1977) Biochim Biophys Acta 459, 96–103
33 Addanki, S, Cahill, F D and Sotos, J F (1968) J Biol Chem 243, 2337–2348
34 Nicholls, D G (1974) Eur J Biochem 50, 305–315
35 Addanki, S, Cahill, F D and Sotos, J F (1967) Science 155, 1678–1679
36 Pietrobon, D, Zoratti, M., Azzone, G F, Stucki, J.W and Walz, D (1982) Eur J Biochem 127, 483–494
37 Wilson, D F and Forman, N G (1982) Biochemistry 21, 1438–1444
38 Holian, A and Wilson, D F (1980) Biochemistry 19, 4213–4221

39 Casadio, R, Venturoli, G and Melandri, B A (1981) Photobiochem Photobiophys 2, 245–253
40 Rottenberg, H (1979) Methods Enzymol 55, 547–569
41 Tedeschi, H (1965) J Cell Biol 25, 229–242
42 Halestrap, A P (1978) Biochem J 172, 389–398
43 Fisher, R A and Yates, F (1963) Statistical Tables for Biological, Agricultural and Medical Research, 6th edn, Oliver and Boyd, London
44 Cain, R B (1972) Elementary Statistical Concepts, W B Saunders, Philadelphia
45 Colquhon, D (1971) Lectures on Biostatics, Clarendon Press, Oxford
46 Ter Welle, H F and Slater, E.C (1967) Biochim Biophys Acta 143, 1–17
47 Mitchell, R A, Chang, B F, Huang, C N and De Master, E G (1971) Biochemistry 10, 2049–2054
48 Laris, P C (1977) Biochim Biophys Acta 459, 110–118
49 Gamble, J G and Lehninger, A L (1973) J Biol Chem 248, 610–618
50 McGivan, J D, Bradford, N M and Beavis, A D (1977) Biochem J 162, 147–156
51 Bradford, N M and McGivan, J D (1982) FEBS Lett 140, 270–272
52 Brink, T and Konings, W N (1980) Eur J Biochem 111, 59–66
53 Zoratti, M, Pietrobon, D, Conover, T and Azzone, G F (1981) in Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F, Quagliariello, E, Siliprandi, N and Slater, E C, eds), pp 331–338, Elsevier/North-Holland, Amsterdam
54 Westerhoff, H V, Simonetti, A L M and Van Dam, K (1981) Biochem J 200, 193–202
55 Azzone, G F, Pozzan, T and Massari, S (1978) Biochim Biophys Acta 501, 307–316