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MITOCHONDRIAL MEMBRANE POTENTIAL, TRANSMEMBRANE DIFFERENCE IN THE NAD$^+$ REDOX POTENTIAL AND THE EQUILIBRIUM OF THE GLUTAMATE-ASPARTATE TRANSLOCASE IN THE ISOLATED PERFUSED RAT HEART

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The distribution of glutamate and aspartate and the mitochondrial membrane potential ($\Delta\psi$) were studied in isolated rat heart mitochondria and in the intact perfused rat heart. The diffusion potential imposed by the glutamate-aspartate exchange through mediation of the electrogenic glutamate-aspartate translocator attained a value close to the mitochondrial $\Delta\psi$ measured from the distribution of triphenylmethylphosphonium ion (TPMP$^+$) both in isolated mitochondria and in intact myocardium. Distributions of the $\Delta\psi$ probe and metabolites were determined by subcellular fractionation of the heart muscle in a non-aqueous medium. The results indicate that the glutamate-aspartate translocator is in near equilibrium in the myocardium. The diffusion potential of the glutamate-aspartate exchange, and the mitochondrial/cytosolic difference in the redox potentials of the free NAD$^+$/NADH pools are equal allowing for experimental error. These data obtained from intact tissue can therefore be interpreted as supporting the notion of the transmembrane uphill transport of reducing equivalent from the cytosolic free NAD$^+$/NADH pool being driven by the malate-aspartate cycle energized by the mitochondrial $\Delta\psi$.

Introduction

Glutamate and aspartate are translocated across the inner membrane of liver and heart mitochondria by an electrogenic antiporter [1], and hence their distribution is dependent on the membrane potential. The transmembrane gradients of glutamate and aspartate have been measured in intact hepatocytes [2,3] and isolated perfused livers [4], but the results are not in accordance with the predictions based on the characteristics of this translocation in isolated mitochondria. In effect, the results indicate that an equilibrium between the membrane potential and the gradients of these metabolites is not achieved. In the literature only one report [5] exists giving indirect evidence of an equilibrium between the glutamate-aspartate translocation and membrane potential in suspensions of isolated rat liver mitochondria supplemented with enzymes of the malate-aspartate cycle but corresponding data on heart mitochondria are lacking. There are indications that this cycle is the main way of transferring reducing equivalents into mitochondria in the myocardium [6].

The discrepancy between data obtained with suspensions of mitochondria on one hand, and with isolated cells and organs on the other, might be due to shortcomings in the experimental methods. However, as demonstrated by comparison of the regulation of the adenylate translocation in

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Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; TPMP$^+$, triphenylmethylphosphonium ion; $\Delta\psi$, membrane potential (negative inside).

suspensions of isolated mitochondria [7] on one hand and intact tissue on the other [8–10], the physiological poise of a transport mechanism can only be tested under conditions of physiological fluxes and concentrations of metabolites.

The deductions concerning the characteristics of the glutamate-aspartate translocator are mainly based on the kinetics of transport [11,12], and equilibrium data are scarce [5].

The present study was undertaken to test the relationship between the transmembrane gradients of glutamate and aspartate and the mitochondrial membrane potential in intact myocardium. Since the results deviated from those obtained with hepatocytes or isolated livers, experiments were also conducted with isolated heart mitochondria. The results show that a near equilibrium prevails between the mitochondrial membrane potential and glutamate-aspartate translocation both in intact myocardium and in suspensions of heart mitochondria.

Materials and Methods

Chemicals

Aminooxyacetic acid and *N*-ethylmaleimide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Silicone oils used for rapid separation of mitochondria were from Wacker-Chemie, GmbH, Munich, F.R.G. Triphenyl[^{3}H]methylphosphonium ($TPMP^+$) iodide and [U-^{14}C]sucrose were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. and tritiated water from New England Nuclear Chemicals, GmbH, Dreieich, F.R.G. The enzymes were obtained from Boehringer-Mannheim, GmbH, F.R.G. Safranine, non-labelled triphenylmethylphosphonium bromide and other chemicals were at least of reagent grade and obtained from E. Merck, AG, Darmstadt, F.R.G. Alkaline *Bacillus subtilis* proteinase (Alkalase) was from Novo Industri A/S, Copenhagen, Denmark.

Animals and tissue preparations

Male Sprague-Dawley rats weighing 250–330 g, obtained from the Department's own stock, were used. No fasting period preceded the experiments.

Heart mitochondria were isolated according to the *B. subtilis* proteinase digestion method of Tyler and Gonze [13] and suspended in 225 mM mannitol, 75 mM sucrose, 0.5 mM EDTA, pH 7.4 (medium A).

Heart perfusions

The rats were anaesthetized with pentobarbital (Mebunat^R, Orion Pharmaceutical Co., Helsinki, Finland) given intraperitoneally (60 mg/kg body weight), 500 I.U. of heparin were injected 1 min before the excision of the heart. The isolated hearts were perfused without recirculation by the Langendorff procedure with Krebs-Ringer bicarbonate solution [14] containing 2.5 mM $CaCl_2$ and 10 mM glucose and in equilibrium with O_2/CO_2 (19 : 1). The perfusion pressure was 7.85 kPa (80 cm water). The perfusion time was 25 min. In the other experiments (arrested hearts) the perfusion was, after an equilibriation period of 10 min with the medium described above, switched to a medium containing 18 mM KCl, the NaCl concentration being decreased accordingly to keep then osmolarity constant. At the end of the perfusion, the hearts were quick frozen with aluminium clamps cooled with liquid nitrogen and lyophilized at −55°C.

Tissue fractionation

The lyophilized tissue was fractionated in a non-aqueous medium by the method of Elbers et al. [15] modified as described previously [8].

Analytical Methods

The marker enzymes for the subcellular compartments, citrate synthase and phosphoglycerate kinase, were assayed as described in Refs. 16 and 17. Protein was determined by the method of Lowry et al. [18] in connexion with the tissue fractionation and with the Biuret method [19] in experiments with isolated mitochondria. Glutamate [20] and aspartate [21] were measured by conventional enzymatic methods employing spectrophotometry and fluorometry, respectively. In the tissue fractionated in the non-aqueous medium, aspartate was measured by fluorometry taking advantage of the alkali-induced fluorescence of NAD^+ [22], the product of the coupled reactions of aspartate aminotransferase and malate dehydrogenase. When measuring aspartate in solutions containing aminooxyacetate, 5 mM acetaldehyde

was added to inactivate the inhibitor of the aminotransferase reaction.

Experiments with isolated mitochondria

Mitochondria were incubated in 100 mM KCl, 50 mM Tris-HCl, 5 mM potassium phosphate, 5 mM $MgCl_2$, and 0.5 mM EDTA, pH 7.4.

The mitochondria were loaded with glutamate in medium A by incubating in the presence of 100 mM potassium glutamate and 3 mM aminooxyacetate at 0°C for 4 min, after which 100 nmol *N*-ethylmaleimide per mg protein was added and the mitochondria were washed in medium A by centrifugation. Aspartate-loaded mitochondria were obtained by the addition of 5 mM oxaloacetate into mitochondria loaded with glutamate as above, but in the absence of aminooxyacetate and incubation for a further 2 min at room temperature after which 3 mM aminooxyacetate was added [23]. Although aminooxyacetate reacts with 2-oxo acids, this does not interfere with the interpretation of the data because their metabolism is blocked by *N*-ethylmaleimide and the retention of intramitochondrial glutamate and aspartate was determined in each experiment.

The mitochondrial membrane potential was monitored by recording the spectrum changes of safranine in an Aminco DW-2 dual-wavelength spectrophotometer using the wavelength pair 511 and 533 nm [24]. To calibrate this spectroscopic method, the mitochondrial membrane potential was also measured by determining the distribution of $TPMP^+$.

The metabolite and probe distributions were determined by rapid separation of the mitochondria from the suspension medium through a layer of silicone oil into 12% $HClO_4$ or 0.5 M sucrose containing 0.1% Lubrol PX in an Eppendorff centrifuge spinning at $8000 \times g$ for 30 s,

The concentrations of glutamate and aspartate in neutralized $HClO_4$ extracts of the silicone oil supernatant and infranatant fluids were determined enzymatically as described above for the tissue fractions.

In separate experiments the mitochondrial water space and content of extra-matrix water in the mitochondrial sediment were determined by incubating mitochondria in the presence of ^3H_2O (spec. act. 1900 dpm/μl) and [U-^{14}C]sucrose (250 dpm/μl). The average matrix water was 1.1 ± 0.2 μl/mg mitochondrial protein (mean $\pm$ S.E., $n = 4$) and extra-matrix water of miitochondria separated through silicone oil was 4.0 ± 0.7 μl/mg mitochondrial protein (mean $\pm$ S.E., $n = 4$).

The membrane potential was calculated from the $TPMP^+$ distribution by using the Nernst equation:

$$\Delta\psi = \frac{RT}{F} \ln \frac{C_i}{C_o} \quad (1)$$

where R is the gas constant, T the absolute temperature, F the Faraday constant and C_o and C_i are the outer and matrix concentrations of the cation, respectively.

Statistical methods

The significance of the differences of the means were calculated using the Student's *t*-test.

Results

Isolated mitochondria

When aspartate was added to glutamate-loaded mitochondria in State 4 in the presence of aminooxyacetate, a 1 : 1 stoicheiometric exchange of glutamate with aspartate was observed. Glutamate-loaded mitochondria represent a relatively low-energy state, the initial membrane potential being 90 mV as measured from the $TPMP^+$ distribution.

When the membrane potential was monitored in similar experiments, it was found that aspartate addition to glutamate-loaded mitochondria caused a rapid increase in the membrane potential measured from the spectrum change of safranine (Fig. 1). This was confirmed by measurements of the membrane potential from the $TPMP^+$ distribution (Fig. 1).

If the glutamate-aspartate translocation were fully electrogenic the metabolite distribution should obey the equation:

$$\Delta\psi = \frac{RT}{F} \ln \frac{[Glu_i][Asp_o]}{[Glu_o][Asp_i]} \quad (2)$$

where the subscripts i and o refer to the intramitochondrial and extramitochondrial concentrations of the amino acids, respectively. Fig. 1 shows

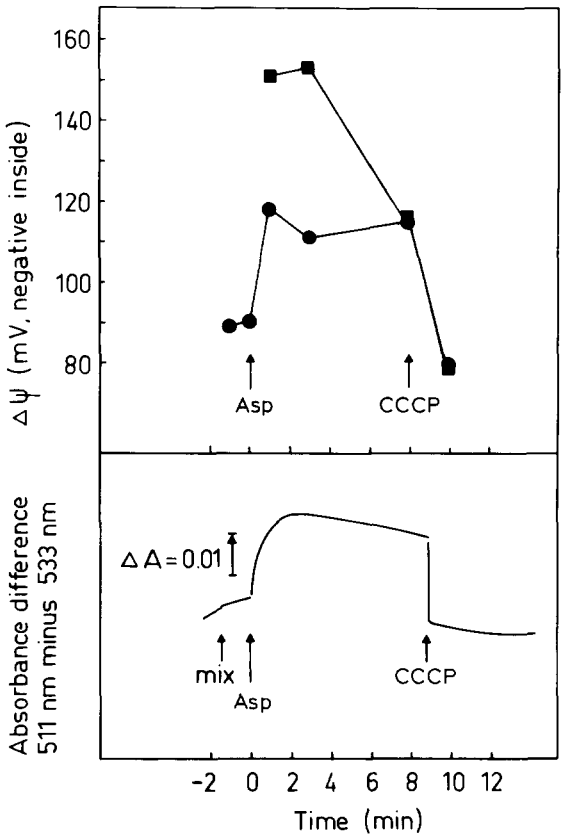

Fig. 1. Energization of the mitochondrial membrane by glutamate-aspartate exchange. Rat heart mitochondria were preloaded by glutamate and suspended in a KCl-based medium as described in Materials and Methods. 3 mM aspartate and 0.9 μM CCCP were added as indicated by arrows. Upper panel, conditions: 3 mM aminooxyacetate, 2.5 μM [^{3}H]TPMP$^+$ (spec. act. 42000 dpm/nmol), 2.7 mg mitochondrial protein per ml, total volume 2.5 ml. At the time points indicated, samples were take to separate mitochondria by centrifugation through silicone oil, and the distribution of the [^{3}H]TPMP$^+$ probe was determined. In duplicate samples the distributions of glutamate and aspartate were determined. The membrane potential was calculated from the TPMP$^+$ distribution and the glutamate and aspartate distribution as described in the text. (●) From TPMP$^+$ distribution, (■) from the glutamate and aspartate distribution. Lower panel, conditions: as in the upper panel but supplemented with 19 μM safranine, 0.8 mg mitochondrial protein per ml, total volume 1.1 ml. Upward deflection in the spectrophotometer trace indicates absorbance decrease at 533 nm.

that the theoretical $\Delta\psi$ imposed by the asymmetry of the glutamate and aspartate distribution is immediately after aspartate addition higher than the $\Delta\psi$ measured by TPMP$^+$ distribution and the amino acid-induced theoretical $\Delta\psi$ drifts to a value approximating the measured $\Delta\psi$. This drift was mainly due to an increase in the matrix aspartate concentration, but the stoicheiometry between glutamate and aspartate transport was close to 1 : 1.

The aspartate-induced energization of the membrane could only be demonstrated when the membrane potential was initially rather low. A prior addition of ATP which increased the membrane

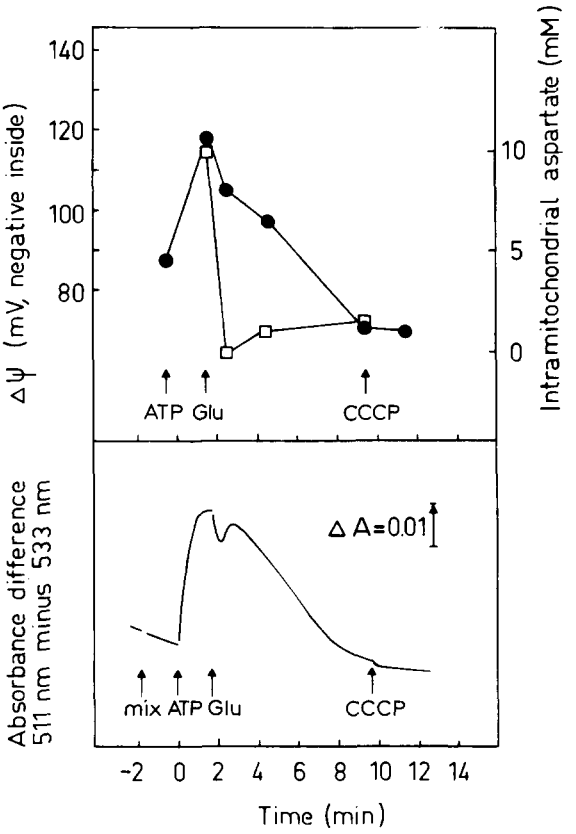

Fig. 2. An attempt to de-energize the mitochondrial membrane by aspartate-glutamate exchange. Rat heart mitochondria were preloaded with aspartate and suspended in a KCl-based medium as described in Materials and Methods. 30 μM ATP, 7 mM glutamate and 0.9 μM CCCP were added as indicated by the arrows. Upper panel, conditions: 3 mM aminooxyacetate, 2.5 μM [^{3}H]TPMP$^+$ (spec. act. 42000 dpm/nmol), 1.9 mg mitochondrial protein per ml, total volume 4.0 ml. The membrane potential was calculated and the matrix and extra-matrix water spaces determined as in Fig. 1. (●) $\Delta\psi$ calculated from the TPMP$^+$ distribution, (□) intramitochondrial aspartate. Lower panel, conditions: as in the upper panel but supplemented with 19 μM safranine, 0.6 mg mitochondrial protein per ml, total volume 1.1 ml. Upward deflection in the spectrophotometer trace indicates absorbance decrease at 533 nm.

potential to 180 mV abolished the aspartate effects on the membrane potential.

If the membrane can be energized by the exchange of internal glutamate to aspartate, an opposite exchange should lead to membrane de-energization. This was difficult to prove, because the matrix aspartate concentrations achieved by loading were rather low compared to the flux of aspartate-glutamate exchange. In aspartate-loaded mitochondria energized by ATP a rapid decrease in the membrane potential was observed (Fig. 2). Within 2 min the membrane potential returned towards the post-energization value and this time point correspond to the moment when the matrix aspartate concentration reached zero. This experiment also showed that the ATP synthetase reversal is able to develop a membrane potential which over-rides the de-energization induced by the amino acid translocation. The membrane polarization fades slowly as the ATP is consumed in hydrolysis (Fig. 2).

In the dual-wavelength method used in the experiments depicted in Figs. 1 and 2, the wavelengths are far enough from each other to confer on it, in principle, sensitivity to changes in light scattering, i.e., swelling-contraction of the mitochondria. This possibility was tested in the experiment given in Fig. 3, which shows that there is no detectable skewing of the baseline although an extensive bleaching occurs specifically at 525 nm. Therefore, the optical read-out is under these conditions specific for the safranine chromophore as previously demonstrated under some other conditions [24].

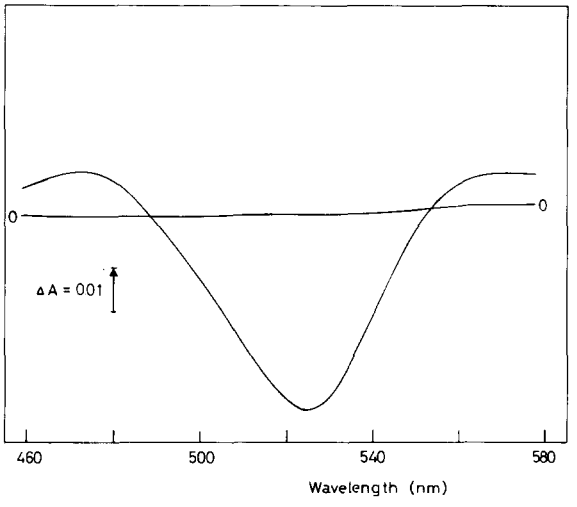

Fig. 3. Spectrum change of glutamate-loaded, safranine-stained rat heart mitochondria upon addition of aspartate. The conditions are as in Fig. 1, lower panel, 1.1 mg mitochondrial protein per ml. The tracing indicated by 0 represents the sample-versus-reference baseline. The spectrum scan was then repeated 3 min after the addition of 3 mM aspartate to the sample cuvette.

Intact myocardium

Metabolite gradients. The verification of the transmembrane concentration gradients of aspartate in the myocardium proved to be difficult because of the extremely low concentration and absolute amount of intramitochondrial aspartate which was only 0.8–2% of the total myocardial aspartate. This necessitated a sensitization of the detection method by employing the alkali-induced fluorescence of NAD^+ which allowed the product of the indicator reaction to be measured, rather than the substrate NADH usually determined. Both the mitochondrial and total glutamate concentrations are high and easily amenable to analysis after tissue fractionation.

Myocardial glutamate showed a matrix/cytosol concentration gradient of 4 in the beating heart and 3.1 in the K^+-arrested heart (Table I). Assuming that glutamate carries one net dissociable proton these gradients are equivalent to transmembrane pH differences of 0.57 and 0.48 (alkaline inside), respectively. Those values are close to the ΔpH values previously observed by independent methods [9,10].

The concentration gradient of aspartate showed a polarity opposite to that of glutamate (Table I). The matrix/cytosol concentration ratio was 0.088 in the beating heart and 0.031 in the K^+-arrested heart.

The membrane potential imposed by the glutamate and aspartate gradients by mediation of a fully electrogenic glutamate-aspartate exchange would be 102 and 123 mV in the beating and K^+-arrested hearts, respectively.

Redox potential difference between the mitochondrial and cytosolic free NAD^+/NADH pools

Compartment-specific analysis of the reactants of the glutamate dehydrogenase system allows a

TABLE I

MITOCHONDRIAL AND CYTOSOLIC CONCENTRATIONS OF GLUTAMATE AND ASPARTATE IN THE ISOLATED PERFUSED RAT HEART

Experimental conditions are as described in Materials and Methods. The pH difference across the inner mitochondrial membrane is calculated from the equation $\Delta pH = \log[Glu_m]/[Glu_c]$ where $[Glu_m]$ and $[Glu_c]$ are the mitochondrial and cytosolic concentrations of glutamate, respectively. The mitochondrial membrane potential $\Delta\psi$ is calculated from the equation $\Delta\psi = RT/F \ln([glu_m][asp_c]/[glu_c][asp_m])$ where the subscripts m and c, refer to the mitochondrial and cytosolic concentrations, respectively. The values are means ± S.E. for the number of experiments given in parentheses.

Values	Beating heart	K^+-arrested heart
Glutamate (mM)		
mitochondrial	22.41 ±1.74 (8)	24.87 ±2.22 (6)
cytosolic	6.30 ±0.72 (8)	8.14 ±0.61 [a] (6)
$[Glu_m]/[Glu_c]$ (molar ratio)	4.0 ±0.6 (8)	3.1 ±0.2 (6)
ΔpH (pH units)	0.57 ±0.06 (8)	0.48 ±0.03 (6)
Aspartate (mM)		
mitochondrial	0.53 ±0.05 (6)	0.11 ±0.01 [b] (5)
cytosolic	6.10 ±0.46 (6)	3.44 ±0.22 [b] (5)
$[Asp_m]/[Asp_c]$ (molar ratio)	0.088 ±0.008 (6)	0.031 ±0.003 [b] (5)
$\Delta\psi$ (mV)	102.1	123.2

P (vs. beating heart, Student's *t*-test); [a] $P < 0.05$, [b] $P < 0.0005$.

more precise calculation of the mitochondrial $NAD^+/NADH$ ratio than was previously possible. The compartmentation of ammonia was not experimentally determined here, but its mitochondrial concentration can be estimated on the basis of previous knowledge of the mitochondrial transmembrane ΔpH [9], total matrix water [8], total intracellular water [8] and total ammonia concentration in the tissue [25]. These give a mitochondrial ammonia concentration of 0.034 mM in the beating heart and 0.041 mM in the K^+-arrested heart. The mitochondrial concentrations of glutamate and 2-oxoglutarate [9], and ammonia give a poise of the $NAD^+/NADH$ system at −303 mV in the beating heart and −296 mV in the arrested heart, a difference which is not statistically significant.

Discussion

The electrogenic character of the glutamate-aspartate translocation seems to be firmly established [1,26]. However, mainly kinetic data are available on this system. Since reducing equivalents of cytosolic NADH are transported into the mitochondria, most probably by means of the malate-aspartate cycle [6] against a redox gradient, and the glutamate-aspartate translocator is involved in the mechanism, data on the energetics of the system are needed to assess its feasibility.

The present data suggest that near equilibrium prevails between the glutamate-aspartate translocation and the mitochondrial $\Delta\psi$ both in isolated heart mitochondria and in intact myocardium. The data presented in Fig. 1 indicate that Asp_o-Glu_i exchange occurs at a measurable rate also in the absence of an added energy drain, and the Asp/Glu asymmetry appears to be higher than expected from the measured $\Delta\psi$. The former finding could be due to a certain grade of membrane damage caused by the manipulations and use of inhibitors in the preparation of glutamate-loaded mitochondria and a resulting short-circuiting of charges. As shown in the lower panel of Fig. 1, the membrane capacitance is low enough to allow a build-up of the membrane potential by transport of trace amounts of aspartate. The reason for the higher than expected asymmetry of the aspartate and glutamate distribution during constant aspartate transport is not apparent from the data. However, the difficulty of measurement of low matrix aspartate concentration in the presence of high

extramitochondrial and intermembrane aspartate concentration is evident. In spite of this, the 1 : 1 stoicheiometry between aspartate and glutamate transport could be measured with high accuracy. The glutamate and aspartate distribution in intact myocardium (Table I), however, shows a good fit to the electrogenic hypothesis of transport of these two amino acids [5]. Although only one previous report exists indicating a near equilibrium of the electrogenic glutamate-aspartate translocation in isolated rat liver mitochondria [5], data on intact cells [2,3] and tissues [4] are at variance. Both possible polarities have thus been reported for the transmembrane gradient of aspartate in hepatocytes [2,3], and the glutamate gradients in isolated perfused rat livers [4,27]. Although these data have been considered as evidence for kinetic control of the translocator in vivo [12], in the case of the hepatocytes another explanation can be found in the analytical method used, viz., digitonin fractionation [28], which becomes unreliable when steep cytosol/matrix gradients (higher outside) are encountered. This is a consequence of the fact that physical separation of mitochondria and cytosol does not occur, and the cytosolic concentration is only estimated from the damage-induced leakage out of the cells.

The non-aqueous fractionation method [4,27] has provided data of both possible polarities of glutamate distribution in isolated perfused livers, and the reason for this behaviour of these substrates is difficult to explain. However, the apparent difference between the results obtained by the non-aqueous fractionation method in liver and heart muscle may be due to higher accuracy of this method in the myocardium because of a better resolution of the mitochondrial and cytosolic fractions, e.g., because of the structure given to the latter by the contractile proteins.

Although interpretation of the data on isolated mitochondria may be tempered with some reservations due to experimental difficulties, the data on intact myocardium as summarized in Table I provide a clear-cut picture of the steady-state conditions of glutamate-aspartate translocation. The results indicate that glutamate distribution is determined by the pH gradient across the mitochondrial membrane. This is in accord with the characteristics of the glutamate-aspartate translocase [11] and also with the properties of the glutamate-H^+ symporter [29]. The activity of the latter carrier is low in heart mitochondria [30] and the relative contribution of the two transport systems in establishing the glutamate gradient remains to be established. Functioning of the glutamate-H^+ symporter does not interfere with interpretation of the data on the glutamate-aspartate exchange. The proximity of the electrogenic glutamate-aspartate exchange to thermodynamic equilibrium can be assessed by comparison of the theoretical $\Delta\psi$ calculated from Eqn. 2 with the mitochondrial membrane potential under similar conditions. From the distribution of exogenous lipophilic cations in the beating isolated rat heart a mitochondrial $\Delta\psi$ of 125 mV has been calculated [10], and this is quite close to the $\Delta\psi$ imposed by the glutamate-aspartate exchange (Table I). In the potassium-arrested heart the $\Delta\psi$ imposed by the glutamate-aspartate exchange is higher than in the beating heart. This can also be reconciled with previous data related to the mitochondrial membrane potential in situ. Assuming electrogenic ATP-ADP translocation [31], the mitochondrial transmembrane ATP/ADP gradient indicates a $\Delta\psi$ of 168 mV [9] in the potassium-arrested heart and 125 mV in the beating heart under similar conditions. Thus, the $\Delta\psi$ imposed by the glutamate-aspartate exchange changes parallel with the $\Delta\psi$ during a metabolic perturbation caused by a change in the cellular energy consumption. This strengthens the interpretation of the tight connection between glutamate-aspartate exchange and mitochondrial membrane potential in intact tissues. That the $\Delta\psi$ estimated from the glutamate and aspartate gradients is always lower than the $\Delta\psi$ calculated on the basis of other endogenous or exogenous probes may be a problem related to the extremely low matrix aspartate concentration and its very steep gradient which thus becomes underestimated due to limitations of the methodology. Therefore, it appears justified to conclude that within experimental error, a near equilibrium exists between glutamate-aspartate exchange and mitochondrial $\Delta\psi$ in intact myocardium.

In the context of the transport of reducing equivalents into mitochondria the present results can be considered in relation to the mitochondrial and cytosolic redox potentials. When the redox

potential of myocardial cytosolic $NAD^+/NADH$ is taken as -215 mV from the data of Nuutinen [25], obtained under similar conditions, the transmembrane $NAD^+/NADH$ redox potential difference is 88 mV (positive outside). The mitochondrial membrane potential (125 mV) [9,10] is in excess of this and, moreover, is approached by the potential calculated from the poise of the glutamate-aspartate translocation in the myocardium in the study. Here, the total tissue NH_4^+ was taken to distribute across the mitochondrial membrane according to the ΔpH. The question of free NH_4^+ in tissues is subject to a certain amount of uncertainty, because some earlier data indicate that a large fraction of the total NH_4^+ is probably bound in liver cells and mitochondria and released during acid extraction [32,33]. However, the data on NH_4^+ binding must also be evaluated in light of the common finding that in isolated perfused livers the 3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase systems are in an apparent near equilibrium with the same free $NAD^+/NADH$ pool when total tissue NH_4^+ in acid extracts is taken to represent free ammonia [34]. In a recirculating perfusion of an isolated rat heart a continuous production of NH_4^+ proceeds [35] and the perfusate NH_4^+ concentration reaches 20 μM in 60 min in the beating heart and 4 μM in the potassium-arrested heart when the total tissue concentration of NH_4^+ is 2.1 and 1.8 mM, respectively. the use of the extracellular NH_4^+ concentration and the mitochondrial and plasma membrane pH gradients in the calculation of the intramitochondrial NH_4^+ concentration gives E_h values 40–60 mV more negative than the use of the total tissue NH_4^+ concentration and the measured mitochondrial ΔpH. In the once-through perfusion the use of extracellular NH_4^+ in the calculation becomes untenable. The data obtained by Takala et al. [35] suggest that the intra- and extracellular NH_4^+ concentrations may not be in equilibrium and the data on isolated perfused livers [34] suggest that the tissue concentrations are more appropriate to be used in calculations of the poise of the glutamate dehydrogenase reaction.

To summarize, the present data expand the concept of thermodynamic equilibria as driving forces and regulators in intact tissues. It has been previously observed that a near equilibrium exists between the respiratory chain-linked redox reactions and the cytosolic adenylate system [8,36,37]. This necessitates a near equilibrium both in the ATP synthetase and ATP-ADP translocase reactions. That the latter is in near equilibrium (with mitochondrial $\Delta\psi$) under physiological conditions is also indicated by the measured poise of the mitochondrial and cytosolic ATP/ADP ratios in comparison with the measured mitochondrial $\Delta\psi$ in the myocardium [8–10]. The glutamate-aspartate translocase in the myocardium is in near equilibrium with the mitochondrial $\Delta\psi$ and the latter is also practically equal to the matrix/cytosol difference of the redox potentials of the free $NAD^+/NADH$ pools. Thus, an equilibrium network in the intact cell extends from the cytosolic $NAD^+/NADH$ pool by mediation of the membrane potential to the substrate level terminus (mitochondrial free $NAD^+/NADH$ pool) of the respiratory chain and from the respiratory chain-linked redox reactions by mediation of the protonic and electric membrane potentials to the cytosolic adenylate system. It is interesting that compared to other tissues the myocardial mitochondrial respiratory chain operates at quite a low redox potential of the mitochondrial free $NAD^+/NADH$ pool [37] and a high cytosol-to-mitochondrion redox potential difference. In the liver, more positive values of the redox potential of the mitochondrial $NAD^+/NADH$ pool and more gently sloping transmembrane redox gradients [34] and also lower phosphorylation potentials [36] have been observed. The lower mitochondrial $NAD^+/NADH$ redox potential and higher cytosolic phosphorylation state of the adenine nucleotides in the heart [8] probably have a common denominator in the higher relative capacity of the heart mitochondria both in working and in resting muscle. Since the myocardial energy state and the mitochondrial transmembrane gradients are only moderately affected by the ATP consumption varied on a large scale, the persistence of the high energetic state may be a consequence of the intrinsic properties of heart mitochondria.

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References

1 LaNoue, K.F., Bryla, I. and Bassett, D.J.P. (1974) J. Biol. Chem. 249, 7514–7521
2 Siess, E.A., Brocks, D.G., Lattke, H.K. and Wieland, O.H. (1977) Biochem. J. 166, 225–235
3 Tischler, M.E., Hecht, P. and Williamson, J.R. (1977) Arch. Biochem. Biophys. 181, 278–292
4 Soboll, S., Elbers, R., Scholz, R. and Heldt, H.-W. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 69–76
5 Davis, E.J., Bremer, J. and Åkerman, K.E.O. (1980) J. Biol. Chem. 255, 2277–2283
6 Safer, B., Smith, C.M. and Williamson, J.R. (1971) J. Mol. Cell. Cardiol. 2, 111–124
7 Jacobus, W.E., Moreadith, R.W. and Vendegaar, K.M. (1982) J. Biol. Chem. 257, 2397–2402
8 Kauppinen, R.A., Hiltunen, J.K. and Hassinen, I.E. (1980) FEBS Lett. 112, 273–276
9 Kauppinen, R.A., Hiltunen, J.K. and Hassinen, I.E. (1982) Biochim. Biophys. Acta 681, 286–291
10 Kauppinen, R.A. (1983) Biochim. Biophys. Acta 725, 131–137
11 Tischler, M.E., Pachence, J., Williamson, J.R. and LaNoue, K.F. (1976) Arch. Biochem. Biophys. 173, 448–462
12 Murphy, E., Coll, K.E., Viale, R.O., Tischler, M.E. and Williamson, J.R. (1979) J. Biol. Chem. 254, 8369–8376
13 Tyler, D.D. and Gonze, J.C. (1969) Methods Enzymol. 10, 75–77
14 Krebs, H.A. and Henseleit, K. (1932) Hoppe-Seyler's Z. Physiol. Chem. 210, 33–36
15 Elbers, R., Heldt, H.-W., Schumucker, P., Soboll, S. and Wiese, H. (1974) Hoppe-Seyler's Z. Physiol. Chem. 355, 378–393
16 Shepherd, R. and Garland, P.G. (1969) Methods Enzymol. 13, 11–16
17 Bücher, T. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H.-U., ed.), p. 463, Verlag Chemie, Weinheim
18 Lowry, O.H., Roseborough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
19 Layne, E. (1957) Methods Enzymol. 3, 450–454
20 Bernt, E. and Bergmeyer, H.-U. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H.-U., ed.), pp. 1659–1664. Verlag Chemie, Weinheim
21 Bergmeyer, H.-U., Bernt, E., Möllering, H. and Pfleiderer, G. (1970) in Methoden der Enzymatischen Analyse (Bergmeyer, H.-U., ed.), pp. 1651–1653, Verlag-Chemie, Weinheim
22 Kaplan, N.O., Colowick, S.P. and Barnes, C.C. (1951) J. Biol. Chem. 191, 461
23 LaNoue, K.F., Meyer, A.J. and Brouwer, A. (1974) Arch. Biochem. Biophys. 161, 544–550
24 Åkerman, K.E.O. and Wikström, M.K.F. (1976) FEBS Lett. 68, 191–197
25 Nuutinen, E.M. (1983) Basic Res. Cardiol., in the press
26 LaNoue, K.F. and Tischler, M.E. (1974) J. Biol. Chem. 249, 7522–7528
27 Soboll, S., Elbers, R., Scholz, R. and Heldt, H.-W. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 69–76
28 Zuurendonk, P.F. and Tager, J.M. (1974) Biochim. Biophys. Acta 333, 393–399
29 Hoek, J.B. and Njogu, R.M. (1976) FEBS Lett. 71, 341–346
30 Julliard, J.H. and Gautheron, D.C. (1973) FEBS Lett. 37, 10–16
31 LaNoue, K.F., Mizani, S.M. and Klingenberg, M. (1978) J. Biol. Chem. 253, 191–198
32 Sainsbury, G.M. (1980) Biochim. Biophys. Acta 631, 305–316
33 Wanders, R.J.A., Hoek, J.B. and Tager, J.M. (1980) Eur. J. Biochem. 110, 197–202
34 Williamson, D.H., Lund, P. and Krebs, H.A. (1967) Biochem. J. 103, 514–526
35 Takala, T., Hiltunen, J.K. and Hassinen, I.E. (1980) Biochem. J. 192, 285–295
36 Wilson, D.F., Stubbs, M., Veech, R.L., Erecinska, M. and Krebs, H.A. (1974) Biochem. J. 140, 57–64
37 Hassinen, I.E. and Hiltunen, J.K. (1975) Biochim. Biophys. Acta 408, 319–330