

ATP Synthesis and Generation of Electrochemical Gradients of Protons in the Catecholamine Storage Organelle of the Adrenal Medulla

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The Authors Wish to Dedicate this Work to W. Hasselbach on the Occasion of His 60th Birthday

Catecholamine Storage Organelles, Membrane Ghosts, Mg-ADP Evoked $\Delta\mu\text{H}^+$, ATP Synthesis, ATP- P_i Exchange

The reaction rates of ATP formation from ADP and inorganic phosphate (P_i) by the catecholamine storage organelles isolated from bovine adrenal medulla accelerated 5–6 fold, when P_i was added at various times after addition of Mg-ADP, as compared to the rates observed when the reaction partners were added simultaneously. The increase of the rates of ATP- P_i exchange upon subsequent addition of P_i to Mg-ATP was less prominent. Mg-ADP induced a $\Delta\psi$ (60–70 mV, positive inside), almost equal that induced by Mg-ATP. In both cases $\Delta\psi$ was significantly higher in the absence of P_i than in its presence. At pH 7.4 of the medium the ΔpH was 1.4 units indicating an internal pH of 6. It was stable during the reaction time in the presence of Mg-ATP, while in the presence of Mg-ADP the internal pH increased gradually by 0.2 units. Both the ATP forming reactions as well as the nucleotide induced $\Delta\psi$ were uncoupler-sensitive (CCCP). Though inhibiting ATP formation from ADP + P_i by 50%, 0.1 mM AP_5A (an inhibitor of adenylate kinase) did neither affect $\Delta\psi$ nor ΔpH , hence ruling out the possibility that $\Delta\mu\text{H}^+$ induced by Mg-ADP would be actually due to ATP formed from ADP. Membrane ghosts were not able of ATP synthesis (in the absence of valinomycin), the ATP- P_i exchange was only 10–20% of that of intact organelles, due to the low $\Delta\mu\text{H}^+$ in the absence of intravesicular soluble constituents. It is proposed that the energy for ATP synthesis is furnished by the high gradients of protonated soluble constituents in the intact organelles.

Introduction

Recently we have shown that the H^+ translocating ATPase of the catecholamine storage organelle is “reversible”, *i.e.* capable of ATP formation from ADP + P_i . This function is confined to the intact storage organelle suspended in media of low ionic strength [1]. Membrane ghosts containing the complete ATPase apparatus are in principle likewise able to synthesize ATP, provided they are supplied with an energy source, which was achieved by a valinomycin induced K^+ gradient dependent diffusion potential [2].

Unlike the H^+ transferring systems of chloroplasts, bacteria and mitochondria, the catecholamine storage organelle does not dispose of an external energy

source to support the energy requiring reaction of ATP formation from ADP + P_i . Under the conditions of ATP formation the energetic state of the storage organelle, represented in $\Delta\mu\text{H}^+$, was studied using the methods of methylamine and thiocyanate distribution [3, 4] as measures of ΔpH and $\Delta\psi$ across the membrane.

Methods

Catecholamine storage vesicles from bovine adrenal medulla were isolated in 0.3 M unbuffered sucrose at 4 °C [5]. The catecholamine content of the purified preparation was 2.56 (2.2–3.4) $\mu\text{mol} \times \text{mg protein}^{-1}$. Membrane ghosts were formed from storage vesicles sedimented in 1.6 M sucrose according to [6] (KCl-ghosts). Alternatively, sodium isethionate was used to form membrane ghosts in a KCl-free medium (IE-ghosts). Resealing of the membrane fragments to closed vesicles was tested by the ATP-dependent accumulation of catecholamines [6].

The standard conditions were 0.3 M sucrose, buffered to pH 7.4 with 10 mM tris maleate, 1 mM

Abbreviations: [^{14}C]SCN, Potassium [^{14}C]thiocyanate; [^{14}C]- CH_3NH_3 , Methylamine hydrochloride; TCA, Trichloroacetic acid; CCCP, Carbonylcyanid-*m*-chlorophenyl hydrazone; DCCD, Dicyclohexylcarbodiimide; AP_5A , $\text{P}^i\text{P}^{\text{e}}$ -di(adenosine-5'-)pentaphosphate.

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[^{32}P]orthophosphate ($3\text{--}5\ \mu\text{Ci} \times \text{mmol}^{-1}$), adjusted to pH 7.4, ATP-Mg 5 mM or ADP-Mg 1 mM, 31°C and constant shaking. All experiments were started by the addition of the vesicular suspension. The protein concentrations varied in the different series of experiments and are given in the legends.

The ATP forming reactions were terminated by adding 10% (w/v) TCA. P_i was separated from the nucleotides according to [7], with the modification that isobutanol:toluene (1:1) was used for the extraction of P_i . ATPase activity in the presence of P_i was determined with [^{32}P]- γ -ATP (specific activity $0.1\text{--}0.4\ \mu\text{Ci} \times \text{mmol}^{-1}$). The $^{32}\text{P}_i$ was separated from [^{32}P]ATP by the charcoal method [1].

$\Delta\psi$ and ΔpH were measured by the distribution of [^{14}C]SCN $^-$ and [^{14}C]CH $_3\text{NH}_3^+$ between the medium and vesicular interior (v_i), which was determined as difference between $^3\text{H}_2\text{O}$ - and [^{14}C]dextran spaces [3, 4]. The reactions were stopped by sedimentation of the vesicles in a cooled Beckman-airfuge at $160\,000 \times g$ for 2 min. ^3H and ^{14}C radioactivities were extracted from the sediment with 10% (w/v) TCA.

Protein was determined by the Kjeldahl method, catecholamines according to [8].

Materials

ATP and ADP (Pharma Waldhof, Mannheim, Bundesrepublik Deutschland); [^{32}P]orthophosphate, Potassium [^{14}C]thiocyanate, Tritium (Hydrogen-3) H_2O (The Radiochemical Centre, Amersham, GB); Methylamine hydrochloride [^{14}C], Dextran-Carboxyl [carboxyl- ^{14}C] (NEN Chemicals GmbH, Dreieichenhain, Bundesrepublik Deutschland); ^{32}P - γ -ATP was synthesized according to [16]; CCCP, DCCD (Serva, Heidelberg, Bundesrepublik Deutschland); AP_5A (Boehringer, Mannheim, Bundesrepublik Deutschland); All chemicals were of reagent purity.

Results

Intact catecholamine storage organelles, suspended in iso-osmotic medium of low ionic strength are capable – in a reverse reaction of ATP hydrolysis – to form ATP from ADP + P_i , and to exchange the γ -phosphate group of ATP with P_i . As shown by the time courses, both the ATP forming reactions accelerated with time, while the velocity of ATP hydrolysis slowed down concomitantly (Fig. 1).

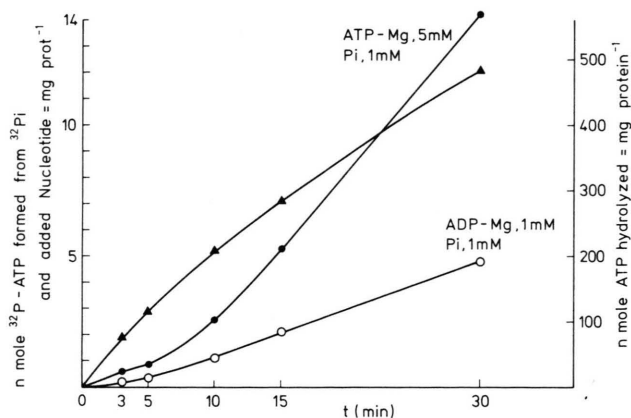


Fig. 1. Time course of the ATP forming reactions and of ATP hydrolysis. Concentration of vesicle protein: $0.3\text{--}0.5\ \text{mg} \times \text{ml}^{-1}$, pH 7.4 (10 mM Tris maleate), 31°C . Symbols: $\circ$ — $\circ$ ATP formation from ADP + P_i , $\bullet$ — $\bullet$ ATP- P_i exchange, $\blacktriangle$ — $\blacktriangle$ ATP hydrolysis. Left ordinate nmol $^{32}\text{P}_i$ incorporated into adenosine nucleotides $\times \text{mg protein}^{-1}$, right ordinate nmol ATP hydrolyzed $\times \text{mg protein}^{-1}$. Each point represents the mean of 8 experiments, the SEM were between 12 and 18%.

Accumulation of ADP as cause for the increasing velocity of the ATP- P_i exchange had been excluded [1]; the opposite changes in the velocities of the ATP-forming reactions on the one hand, and of ATP hydrolysis on the other hand, ought to indicate an altered state of the system itself, induced by ATP-Mg and also by ADP-Mg.

Addition of P_i , after the storage organelles had reacted for 3–30 min with ATP-Mg resulted in about a two fold acceleration of the reaction rates compared to that observed when ATP and P_i were added simultaneously (Fig. 2a). A dramatic acceleration of the velocity of ATP synthesis occurred, increasing progressively with time when P_i was added to the assays subsequent to ADP-Mg (Fig. 2b). The reaction rates obtained when P_i was added 3 to 30 min after ADP-Mg were about 5–6 times higher as those with the simultaneous addition of P_i . The rates even exceeded the rates of the ATP- P_i exchange obtained under similar conditions. In contrast, with simultaneous addition of P_i and the nucleotide, the ATP- P_i exchange reaction was 2–3 times faster than ATP synthesis from ADP + P_i (Fig. 2a and b). The rates of ATP hydrolysis were not affected by the subsequent addition of P_i (data not shown).

The protonophore, CCCP, added before or together with the nucleotide and P_i abolished both

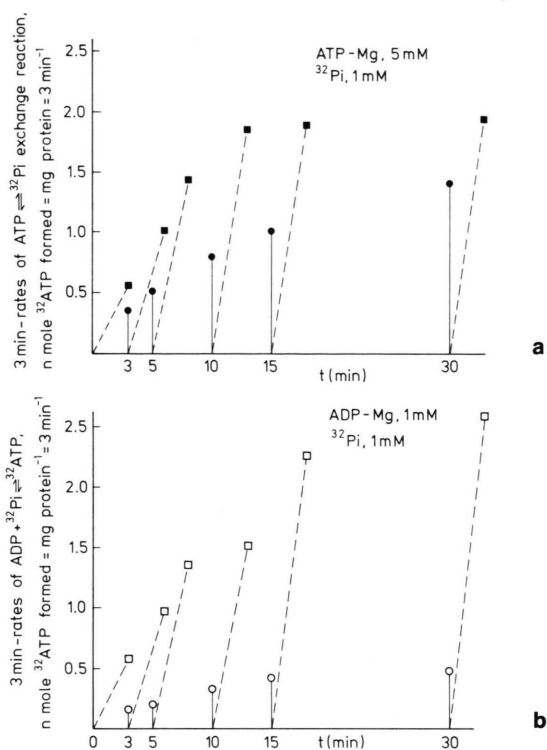


Fig. 2. Comparison of velocities of the ATP forming reactions with simultaneous and subsequent addition of P $_i$. 2a: ATP-P $_i$ exchange, 2b: ATP formation from ADP + P $_i$. Concentration of vesicle protein 0.25–0.4 mg $\times$ ml $^{-1}$, pH 7.4 (10 mM Tris maleate), 31 °C, ATP-Mg 5 mM, ADP-Mg 1 mM, P $_i$ 1 mM. Symbols: ●—● addition of P $_i$ simultaneously with ATP-Mg, ■—■ addition of P $_i$ at time intervals indicated on the figure after ATP-Mg. ○—○ addition of P $_i$ simultaneously with ADP-Mg, □—□ addition of P $_i$ at time intervals indicated on the figure after ADP-Mg. The results are presented as reaction rates in nmol $\times$ mg protein $^{-1} \times 3$ min $^{-1}$. Mean of 6 experiments, the SEM were between 10 and 15%.

ATP-forming reactions completely (Table I) and it enhanced the ATPase activity 2–2 1/2 fold.

When the inhibitor of energy transduction, DCCD, was added 10 min before starting the reaction with the nucleotide, the ATP-P $_i$ exchange reaction was inhibited by 60%, ATP synthesis from ADP + P $_i$ by 75% (Table I), and ATPase activity was diminished by 25%. Added simultaneously with the nucleotides, DCCD displayed only minor effects.

Since the catecholamine storage vesicle preparation contains adenylate kinase activity [1, 9] AP $_5$ A, a potent inhibitor of this enzyme [10] was used at a high concentration (0.1 mM). As shown in Table I, AP $_5$ A inhibited ATP synthesis from ADP + P $_i$ by 50%, whereas the ATP-P $_i$ exchange reaction as well as the ATPase activity were only insignificantly affected.

With membrane ghosts formed and suspended in KCl, ATP-P $_i$ exchange and ATP synthesis were very low, amounting to 6–12% of the reaction velocities observed with intact storage organelles (Table II). Isethionate ghosts were only able to an ATP-P $_i$ exchange at rates decreasing from 20 to 10% with proceeding reaction time compared to those obtained with intact storage organelles. The synthesis of ATP from ADP + P $_i$ (*i.e.* in absence of any added energy source) was negligible, although the preparation contained the complete and unchanged ATPase apparatus [6]. In contrast to the acceleration of the reaction velocities observed with the intact storage organelle, the slow reactions of ghosts proceeded linearly with time. This data confirm the results of [2] who showed that the membrane of the emptied

Table I. Effect of agents on the ATP forming reactions and the components of $\Delta\mu\text{H}^\circ$. The experiments were performed at pH 7.4 (10 mM Tris maleate) and 31 °C. The reactions were started with the addition of the vesicle suspension giving a final concentration of 1.5–2.0 mg protein $\times$ ml $^{-1}$; ATP-Mg 5 mM, ADP-Mg 1 mM, P $_i$ 1 mM. The results are presented in percent of the untreated controls (mean $\pm$ SEM of n experiments).

	ATP $\rightleftharpoons$ P $_i$ (nmol $^{32}\text{P}_i$ incorporated into nucleotide $\times$ mg protein $^{-1} \times$ min $^{-1}$)	ADP + P $_i$ $\rightarrow$ ATP (nmol $^{32}\text{P}_i$ incorporated into nucleotide $\times$ mg protein $^{-1} \times$ min $^{-1}$)	ATP-Mg		ADP-Mg	
			$\Delta\psi$ [mV]	ΔpH [mV]	$\Delta\psi$ [mV]	ΔpH [mV]
control	0.33 $\pm$ 0.07 (n = 8)	0.12 $\pm$ 0.02 (n = 8)	64.7 $\pm$ 1.4 (n = 8)	76.1 $\pm$ 1.7 (n = 8)	67.9 $\pm$ 2.2 (n = 8)	67.3 $\pm$ 1.9 (n = 8)
percent of activity remaining after treatment with agents						
AP $_5$ A 0.1 mM	82.0 $\pm$ 7.1 (n = 6)	48.4 $\pm$ 6.2 (n = 7)	107.1 $\pm$ 2.0 (n = 3)	97.1 $\pm$ 2.3 (n = 3)	97.4 $\pm$ 2.2 (n = 3)	97.8 $\pm$ 2.6 (n = 3)
CCCP 0.015 mM	5.2 $\pm$ 1.4 (n = 4)	not measurable (n = 4)	11.6 $\pm$ 1.3 (n = 3)	76.7 $\pm$ 8.0 (n = 3)	not measurable (n = 3)	81.9 $\pm$ 5.6 (n = 3)
DCCD 0.02 mM	41.5 $\pm$ 6.6 (n = 4)	26.2 $\pm$ 4.1 (n = 4)	88.1 $\pm$ 2.2 (n = 3)	76.6 $\pm$ 7.4 (n = 3)	51.6 $\pm$ 6.2 (n = 3)	92.4 $\pm$ 1.5 (n = 3)

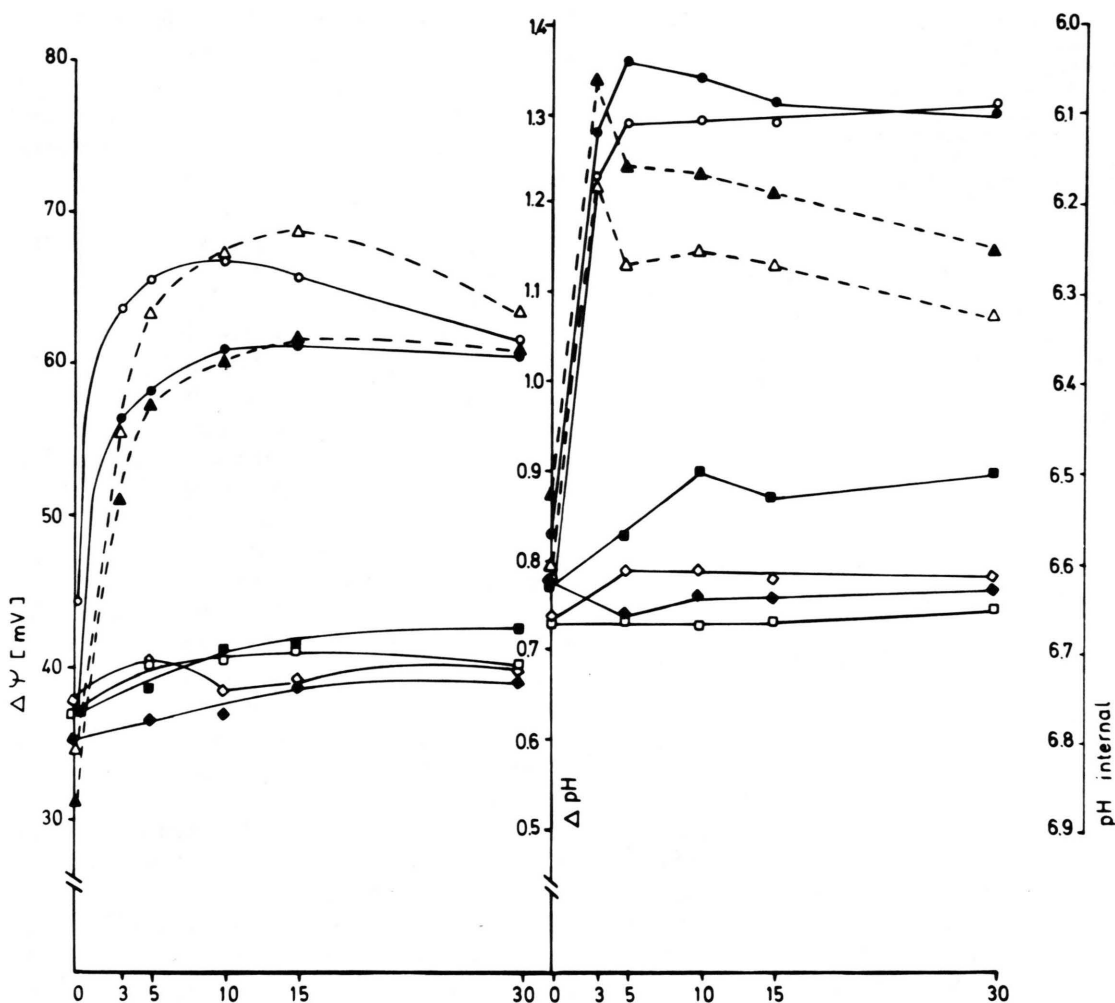


Fig. 3. Time courses of $\Delta\psi$ and ΔpH of intact storage vesicles and of membrane ghosts upon addition of ATP-Mg or ADP-Mg. 1.8–2.5 mg $\times$ ml⁻¹ of vesicle protein were suspended in 0.3 M sucrose, pH 7.4 (10 mM Tris maleate), 31 °C. 0.2–0.3 mg $\times$ ml⁻¹ of membrane protein were suspended in 0.16 M solutions of sodium isethionate or KCl. ATP-Mg 5 mM, ADP-Mg 1 mM, P_i, when present, 1 mM. $\Delta\psi$ and ΔpH were measured as described in "Methods". $\Delta\psi$ and ΔpH in presence of ATP-Mg or ADP-Mg were determined in parallel experiments of the same preparations. Symbols: ●—● $\Delta\psi$ and ΔpH induced by 5 mM ATP-Mg in the presence, ○—○ and in the absence of 1 mM P_i. ▲---▲ $\Delta\psi$ and ΔpH induced by 1 mM ADP-Mg in the presence, △---△ and in the absence of 1 mM P_i. $\Delta\psi$ and ΔpH induced by 5 mM ATP-Mg ■—■ or by 1 mM ADP-Mg □—□ across the membrane of ghosts formed in 15 mM KCl and suspended in 0.16 M KCl at pH 7.4. $\Delta\psi$ and ΔpH induced by 5 mM ATP-Mg ◆—◆ or 1 mM ADP-Mg ◇—◇ across the membrane of ghosts formed in 15 mM isethionate and suspended in 0.16 M isethionate at pH 7.4. Ordinates: left ordinate: $\Delta\psi$ (mV); center ordinate: ΔpH (pH units); right ordinate: pH in the vesicular interior. The results are means of 7 experiments (vesicle experiments) the SEM were between 2 and 4%. The membrane ghost experiments are means of 5 experiments, the SEM were between 8 and 10%.

Table II. ATP forming reactions of membrane ghosts suspended in iso-osmotic solutions of sodium isethionate or KCl. Experimental conditions: 0.15–0.35 mg of membrane protein $\times$ ml⁻¹, pH 7.4 (10 mM Tris maleate), 31 °C. The results are presented as reaction rates obtained 10 min after the start of the reactions that proceeded linearly with time. Means $\pm$ SEM of *n* experiments. The corresponding values of $\Delta\psi$ and Δ pH obtained from the same preparations are shown in Fig. 3.

	nmol ³² P _i incorporated into nucleotide $\times$ mg membrane protein ⁻¹ $\times$ min ⁻¹	
	ATP $\rightleftharpoons$ P _i	ADP + P _i $\rightarrow$ ATP
Isethionate 0.16 M	0.0689 $\pm$ 0.0128 (<i>n</i> = 6)	0.0209 $\pm$ 0.0034 (<i>n</i> = 6)
KCl 0.16 M	0.0226 $\pm$ 0.0031 (<i>n</i> = 6)	0.0183 $\pm$ 0.0026 (<i>n</i> = 6)

catecholamine storage organelle is only capable of ATP synthesis when supplied with an energy source (which was artificially imposed in the form of a KCl concentration gradient induced by valinomycin).

Bioenergetic aspects of the ATP forming reactions

In the absence of added nucleotides, but in the presence of 5 mM Mg²⁺, the resting electrochemical potential of intact catecholamine storage organelles was 25–30 mV; this value was constant over 15 min at 31 °C. Under these conditions, the Δ pH was 1.45–1.55 pH units, indicating an internal vesicular pH of 5.9 with the medium at pH 7.4. Thus, under resting conditions, the $\Delta\mu$ H⁺ amounted to 110–120 mV to which the $\Delta\psi$ contributed only 20–25%.

As shown in Fig. 3, ATP-Mg induced a fast enhancement of $\Delta\psi$ to 60–70 mV (positive inside), which was completed within 3–5 min. Upon the addition of ATP-Mg the Δ pH was 1.35–1.4 pH units (Fig. 3). The small differences to that observed in the absence of nucleotides (0.1–0.15 pH units), however, never reached a level of significance. In the presence of ATP-Mg the Δ pH remained constant between 3 and 30 min, indicating that in a sucrose medium no ATP-Mg induced acidification of the vesicular interior took place. These data are in excellent agreement with the results of others [17], with the exception that the internal vesicular pH under our conditions was found to be between 5.8 and 6.1, in contrast to the value of 5.5 as described by [11]. The $\Delta\mu$ H⁺ was 135–145 mV to which the $\Delta\psi$ contributed about 40%.

With the addition of ADP-Mg (in absence of added ATP) an almost as high $\Delta\psi$ arised as with ATP, reaching its maximum between 10–15 min of reaction. As shown in Fig. 3, the Δ pH after the addition of ADP-Mg dropped in a fast initial reaction by 0.1 pH unit and gradually further decreased over 30 min, resulting in an increase of the internal vesicular pH to 6.3. The $\Delta\mu$ H⁺ was about 10 mV lower than that observed in the presence of ATP-Mg, the $\Delta\psi$ contributing to 50% to the $\Delta\mu$ H⁺.

P_i, added in order to study its effect on the both components of $\Delta\mu$ H⁺ under the conditions of both ATP forming reactions, significantly decreased the magnitudes of the $\Delta\psi$ compared to those observed in its absence. The Δ pH in the presence of ATP-Mg was not affected by P_i, while the ADP-Mg induced fall in Δ pH was slightly diminished by P_i (Fig. 3).

AP₅A inhibiting adenylate kinase which at a high concentration (0.1 mM) inhibited ATP formation from ADP + P_i by 50%, but which allowed an almost unimpaired ATP-P_i exchange reaction (Table I), was without effect upon $\Delta\psi$ and Δ pH under both experimental conditions. This result excludes the possibility that the $\Delta\psi$ induced by ADP-Mg might be due to ATP, produced by adenylate kinase, from ADP.

The protonophore, CCCP, abolished the ATP- as well as the ADP-induced $\Delta\psi$, and was without effect upon the Δ pH (Table I).

DCCD, which strongly inhibited ATP-P_i exchange and ATP formation from ADP + P_i, dissipated the ADP-Mg induced $\Delta\psi$ by 50% without affecting the Δ pH; however, it affected both the components of $\Delta\mu$ H⁺ occurring in the presence of ATP-Mg slightly (Table I). Membrane ghosts, suspended in iso-osmotic KCl or isethionate, displayed a very low Δ pH, indicating an internal pH of 6.6–6.7; which was only slightly acidified by ATP in the Cl⁻ containing medium. The $\Delta\psi$ of 35–40 mV observed under all conditions was almost unchanged with time of reaction (Fig. 3).

Discussion

The fact that the catecholamine storage organelle is able to form ATP from ADP + P_i without any added energy, suggests that it is equipped with an inherent energy source, which is made available by the addition of ADP. An alternative possibility is

that ATP, formed by adenylate kinase from the added ADP, serves as the energy source, and the product measured in the assays, ^{32}P -ATP, actually is the result of an ATP- P_i exchange. Both these possibilities were tested in the present study.

Although the membrane and soluble protein moiety of the catecholamine storage organelle contain adenylate kinase [9, 12] and AP_5A at high concentration inhibited 50% of ATP formation from $\text{ADP} + \text{P}_i$, it is highly improbable that the formed ATP is due to ATP- P_i exchange: 1. The ADP-Mg induced energetic state of the organelle is unaffected when ATP formation by adenylate kinase is prevented by AP_5A . Thus, the observed inhibition by AP_5A must take place at a later step of the ATP formation reaction sequence; it, however, does not interfere with the first fundamental reaction, by which ADP-Mg induces the $\Delta\psi$; 2. Considering that the pH optimum of adenylate kinase is at 6 [13], and the ATP- P_i exchange has a sharp optimum at pH 7 [1], if ATP formation would be due to an ATP- P_i exchange its pH optimum ought to be between 6 and 7. However, ATP formation from $\text{ADP} + \text{P}_i$ increases with increasing pH of the medium [1]. 3. Membrane ghosts (devoid of the large pool of small molecular constituents) which, however, contain adenylate kinase [9] are incapable of $\text{ADP} + \text{P}_i \rightarrow \text{ATP}$. With ATP as energy source, only a low ATP- P_i exchange takes place, even though the membrane – supplied with an artificially imposed energy

source – is capable of a highly effective ATP synthesis [2].

The difference of $\Delta\psi$ in the presence and absence of P_i , which when ADP-Mg was the inducing nucleotide was paralleled by a drop of ΔpH , obviously reflects a withdrawal of energy necessary for ATP formation. The higher magnitude of $\Delta\psi$ occurring in the absence of P_i may explain the increasing acceleration of the ATP forming reactions when P_i was added subsequent to the nucleotides. The low ATP induced $\Delta\mu\text{H}^+$ observed with membrane ghosts (exactly confirming results of [14]) indicates that the energy from ATP hydrolysis, carrying H^+ from the outside to the inside, contributed only a part of the large $\Delta\mu\text{H}^+$ that is obviously preformed in the intact storage organelle, and which is converted into an useable form by Mg-nucleotides.

Concentration gradients of Na^+ and K^+ stabilize – as has been shown by Skulachev [15] – the $\Delta\mu\text{H}^+$ of bacteria and algae. For the catecholamine storage organelle it is proposed that the concentration gradients of small molecular constituents between organelle and cytoplasm are used to establish the $\Delta\mu\text{H}^+$. Since 0.1 M catecholamine in the medium abolishes ATP synthesis almost completely (unpublished observation) it is most likely that the high catecholamine gradient across the membrane and disequilibrium of the Donnan-equilibrium by ADP, that evokes catecholamine efflux, represents the source of energy by which ATP is formed.

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