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A RE-EVALUATION OF CONDITIONS REQUIRED FOR AN ACCURATE ESTIMATION OF THE EXTRAMITOCHONDRIAL ATP/ADP RATIO IN ISOLATED RAT-LIVER MITOCHONDRIA

RONALD J.A. WANDERS, GERARD B. VAN DEN BERG and JOSEPH M. TAGER

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

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The values reported in the literature for the extramitochondrial ATP/ADP ratio in resting rat-liver mitochondria (State 4) vary widely. The conditions required for an accurate determination of this parameter were therefore investigated. (1) In experiments with rat-liver mitochondria incubated under State-4 conditions, it was found that the extramitochondrial ATP/ADP ratio, as calculated from the values measured in neutralised perchloric acid extracts, was lower than that estimated from the concentrations of creatine and creatine phosphate, using the metabolite indicator method. The discrepancy is due to hydrolysis of ATP occurring in the presence of perchloric acid. (2) Conditions are described for minimising ATP hydrolysis in the presence of perchloric acid, and include the use of low concentrations of perchloric acid, short times of exposure to the acid before neutralisation, low temperatures and the presence of excess EDTA. Under these conditions, the values obtained for the extramitochondrial ATP/ADP ratio agreed with those calculated by the metabolite indicator method, provided ratios do not exceed the value of 100. (3) In cases where the extramitochondrial ATP/ADP does exceed 100, phenol/chloroform/isoamyl alcohol must be used to quench the reactions, as described by Slater et al. (Slater, E.C., Rosing, J. and Mol, A. (1973) Biochim. Biophys. Acta 292, 534–553). With this method, the extramitochondrial ATP/ADP ratio was found to have a value of more than 1000 in rat-liver mitochondria incubated with succinate + rotenone in the resting state (pH 7.0; $T = 37°C$), in agreement with Slater et al.

Introduction

Adenine nucleotides play a central role in intermediary metabolism, acting as the link between energy yielding and energy consuming reactions in the cell. The close coupling between oxygen uptake and synthesis of ATP was recognised in early studies in the 1930's (for a review see Ref. 1). Although an impressive number of papers has appeared in the literature since then, no consensus of opinion has been reached on the cardinal question of how mitochondrial respiration is controlled. For instance, opinions differ with regard to the question of whether respiration is controlled by the extramitochondrial ATP/ADP ratio or by the phosphorylation potential, defined as ATP/ADP · P_i [2].

Most experiments on the control of mitochondrial respiration have been carried out with isolated mitochondria. In these experiments,

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 4-morpholinepropanesulphonic acid.

reactions are usually terminated by addition of acid [3,4], followed by centrifugation to remove denatured protein, and neutralisation. Subsequently, ATP, ADP and AMP can be measured enzymically in the neutralised, protein-free extracts. Slater et al. [5] and Brawand and Walter [6] have pointed out that, when the concentration of ATP is high relative to that of ADP, the value of the ATP/ADP ratio might by underestimated if perchloric acid is used to terminate reactions, due to the known instability of ATP under acid conditions [7,8]. Therefore, Slater et al. [5] recommended the use of an organic solvent mixture, whereas Brawand and Walter [6] suggested low perchloric acid concentrations. Nevertheless, perchloric acid is still almost universally used as a quenching reagent in studies on mitochondrial oxidative phosphorylation (see Table I). Since an accurate estimation of the extramitochondrial ATP/ADP ratio is of great importance in such studies, we have re-examined the conditions required for an accurate estimation of this parameter.

Materials and Methods

Isolation of mitochondria. Rat-liver mitochondria were isolated from male Wistar rats (200–250 g) by the method of Hogeboom [9], as described by Myers and Slater [10], using 250 mM mannitol/5 mM Tris-HCl/0.5 mM EGTA as the isolation medium (final pH 7.4). The final pellet was suspended in 3–4 ml 250 mM mannitol and kept on ice until use.

Incubation conditions. Except for the experiment of Table II, mitochondria (0.5–0.8 mg protein/ml) were incubated in a standard reaction medium containing the following components: 100 mM KCl, 50 mM Tris-HCl, 10 mM succinate, 1 mM malate, 10 mM potassium phosphate, 1 mM EGTA, 10 mM $MgCl_2$, 1 μg rotenone/ml and either 2 or 4 mM ATP. The final pH was 7.40. Further additions were as indicated in the legends to the figures and tables.

Denaturation procedures. Two procedures were used to terminate reactions. In method A, reactions were stopped with perchloric acid in the following way. An 0.6 ml sample was taken from the incubation mixture and added to an Eppendorf tube (1.6 ml) which was kept in ice/water and contained 0.6 ml 7% (w/v) perchloric acid with or without 50 mM EDTA. After 60 s in ice/water, samples were centrifuged in the cold (12000 × *g*, 60 s). An 0.9 ml sample of the supernatant was neutralised with cold 2 M KOH/0.2 M Mops to pH 6.8–7.2 and frozen immediately afterwards in liquid nitrogen.

In method B, an 0.6 ml sample was taken from the reaction medium and added to a glass tube containing 2 ml phenol/chloroform/isoamyl alcohol (38 : 24 : 1, v/v/v) plus 0.6 ml 50 mM EDTA (pH 7.4) with vigorous agitation in a Vortex mixer. Mixing was continued for 90 s, the tubes were put on ice and the phases were allowed to separate. Subsequently, a 1.0 ml sample was taken from the upper phase and centrifuged in a cooled Eppendorf centrifuge (12000 × *g*, 90 s). An 0.8 ml aliquot was taken from the supernatant and frozen in liquid nitrogen.

For the determination of intramitochondrial ATP and ADP, the silicone oil centrifugation technique was used to separate mitochondria from the suspending medium. In order to decrease hydrolysis of ATP to ADP in acid, the usual 14% (w/v) perchloric acid layer was replaced by a solution comprising 7% (w/v) perchloric acid/0.5 M mannitol/20 mM EDTA. The procedure used was exactly as described in detail for the extraction of mitochondrial carbamoyl phosphate [11]. In order to determine the extramitochondrial ATP/ADP ratios, the measured amounts of ATP and ADP in total extracts were corrected for the amounts found in the mitochondria after silicone oil centrifugation.

Metabolite determinations. ATP and ADP were measured spectrophotometrically or fluorimetrically according to standard procedures [3]. For the determination of ADP in samples obtained via the phenol/chloroform/isoamyl alcohol method, the amount of pyruvate kinase added had to be increased 2–3-fold due to the sluggishness of the assay in the presence of the organic solvents. Creatine and creatine phosphate were determined as described by Lawson and Veech [12].

Protein determination. Protein was determined as described by Cleland and Slater [13] using egg albumin as a standard.

Enzymes and reagents. Creatine kinase was obtained from Sigma (St. Louis, MO, U.S.A.) as a

lyophilised powder. Other enzymes and nucleotides were obtained from Boehringer (Mannheim, F.R.G.). All other reagents were of analytical grade.

Results and Discussion

Values reported in the literature for the magnitude of the extramitochondrial ATP/ADP ratio in isolated rat-liver mitochondria incubated under State-4 conditions vary considerably (Table I). Most groups have used perchloric acid as a quenching agent in concentrations ranging from 0.15 to 0.95 M (Table I). In order to avoid the potential problem of ATP hydrolysis to ADP, Slater et al. [5] terminated reactions with the aid of a phenol/chloroform/isoamyl alcohol mixture. The data collected in Table I do not allow conclusions to be drawn about the superiority of either denaturation method, since values for the extramitochondrial ATP/ADP ratio in State-4 mitochondria were determined under various conditions with differences in pH, p*Mg*, temperature, respiratory substrate and concentration of phosphate. Furthermore, the accuracy of the values for the ATP/ADP ratio measured with either denaturation method was not checked via an independent method. We therefore decided to compare the ability of the different denaturation procedures to provide an accurate estimation of the extramitochondrial ATP/ADP ratio under different conditions.

An independent way of measuring the magnitude of the extramitochondrial ATP/ADP ratio is to use the metabolite indicator method. The creatine kinase reaction was chosen for this purpose; the apparent equilibrium constant of the reaction (K_{app}) at 37 °C and the dependence of K_{app} on the free Mg^{2+} concentration have recently been documented in detail by Lawson and Veech [12]. Mitochondria were incubated at 37 °C with an excess of creatine kinase plus either 2 mM creatine or 2 mM creatine phosphate, thereby approaching equilibrium from either side. In order to avoid large corrections for intramitochondrial adenine nucleotides, high concentrations of ATP (4 mM) were chosen together with low concentrations of mitochondria (0.5 mg/ml). Under these conditions, equilibrium was reached from either side within 10 min (not shown).

In the experiment of Table II, different rates of oxidative phosphorylation were induced in mitochondria oxidising succinate (plus rotenone) by addition of limiting amounts of hexokinase in the presence of glucose. ATP/ADP ratios were determined after termination of the reactions either with perchloric acid (method A) or with the phenol/chloroform/isoamyl alcohol method (method B), as described in Materials and Methods. Furthermore, extramitochondrial ATP/ADP ratios were calculated from the measured creatine/creatine phosphate ratios. The data of Table II show good agreement between the ex-

TABLE I

VALUES FOR THE EXTRAMITOCHONDRIAL ATP/ADP RATIO IN STATE-4 MITOCHONDRIA

Ref.	Substrate	$[P_i]_{out}$ (mM)	pH	$[Mg^{2+}]_{tot}$ (mM)	$[HClO_4]$ (M)	$[ATP/ADP]_{out}$
14	succinate	0.5	7.2	1.7	0.17	29
5	succinate	5.0	7.7	0	0 [a]	1144
5	succinate	5.0	7.4	0	0 [a]	631
15	glutamate + malate	2.0	7.4	2.0	0.60	40–70
16	succinate	5.0	7.4	5.0	0.95	100
17	glutamate + malate	3.0	7.4	4.0	0.60(?)	180
17	glutamate + malate	27.0	7.4	4.0	0.60(?)	220
18	glutamate + malate	12.0	7.4	2.5	0.15	245
19	glutamate + malate	1.5	7.4	0	0.52	300–520
20	succinate	10.0	7.4	10.0	0.35	500
21	succinate	5.0	7.2	11.0	0.40	150

[a] Phenol/chloroform/isoamyl alcohol method used.

TABLE II

MEASURED AND CALCULATED VALUES FOR THE EXTRAMITOCHONDRIAL ATP/ADP RATIO

Rat-liver mitochondria (0.5 mg protein/ml) were incubated at 37 °C in a medium of the following composition: 200 mM mannitol, 50 mM Tris-HCl, 4 mM ATP, 10 mM succinate, 1 mM malate, 5 mM $MgCl_2$, 10 mM potassium phosphate, 1 mM EGTA, 20 mM glucose, 50 U/ml creatine kinase, 1 μg rotenone/ml and either 2 mM creatine or 2 mM creatine phosphate. The final pH was 7.00. Different amounts of hexokinase were used to obtain different rates of respiration. After 10 min, reactions were terminated with perchloric acid (method A, EDTA present), or with the phenol/chloroform/isoamyl alcohol method (method B). ATP, ADP, creatine and creatine phosphate were determined subsequently. Data are presented as means ± S.D. for triplicate measurements. Extramitochondrial (exmt.) ATP/ADP ratios were calculated from the total ATP/ADP ratios, using the silicone oil centrifugation technique to determine the intramitochondrial levels of ATP and ADP. Corrections were only significant in State 4. For instance in experiment I, the total measured ATP/ADP ratio under State 4 conditions was 1175. Intramitochondrial ATP and ADP amounted to 13.6 and 1.45 nmol/mg protein, respectively; correction for these values resulted in an extramitochondrial ATP/ADP ratio of 1502 under these conditions. The free Mg^{2+} concentration under these conditions was calculated as described by Lawson and Veech [12] using the ionisation constants and stability constants described in Ref. 12. At the calculated free Mg^{2+} concentration (0.64 mM), K_{app} for creatine kinase is 150 [12]. This value was used to calculate the extramitochondrial ATP/ADP ratios from the measured creatine phosphate/creatine ratios. Abbreviations: Cr, creatine; Cr-P, creatine phosphate.

Expt.	Relative rate of respiration (% of maximum)	Equilibrium approached from:	Measured $(ATP/ADP)_{exmt.}$		Cr-P/Cr	Calculated $(ATP/ADP)_{exmt.}$
			method A	method B		
I	17.5 [a]	Cr	501 ± 15	1502 ± 90	9.2 ± 0.61	1380 ± 92
		Cr-P	472 ± 27	1410 ± 71	9.3 ± 0.71	1395 ± 107
	60	Cr	96 ± 3	94 ± 3	0.62 ± 0.05	93 ± 8
		Cr-P	106 ± 2	103 ± 5	0.72 ± 0.04	108 ± 6
	78	Cr	57 ± 4	58 ± 1	0.37 ± 0.02	56 ± 3
		Cr-P	52 ± 3	53 ± 4	0.34 ± 0.02	51 ± 3
II	16 [a]	Cr	526 ± 37	1701 ± 78	11.9 ± 0.9	1785 ± 135
		Cr-P	501 ± 11	1580 ± 69	10.8 ± 0.4	1622 ± 60
	70	Cr	80 ± 2	83 ± 6	0.51 ± 0.04	77 ± 6
		Cr-P	75 ± 5	72 ± 3	0.50 ± 0.05	75 ± 8

[a] State 4.

tramitochondrial ATP/ADP ratios as calculated from the creatine/creatine phosphate ratios and as measured using method B. This was true at each respiratory rate. When perchloric acid was used as the quenching agent, good agreement between calculated and measured values was observed at ATP/ADP ratios of less than 100. However, substantial discrepancies were observed between calculated and measured values at high ATP/ADP ratios.

The factors responsible for the observed underestimation of the ATP/ADP ratio were investigated further. First, we studied the acid-catalysed hydrolysis of ATP to ADP (and AMP) as a function of the concentration of perchloric acid and the time of incubation under acid conditions. The standard incubation medium plus 2 mM ATP but without mitochondria was incubated at 25 °C. At the time intervals indicated in Fig. 1, 0.6-ml aliquots were taken and added to different concentrations of ice-cold perchloric acid with or without EDTA (final concentration after addition of sample 25 mM). Incubations were continued in an ice/water bath for different periods. After neutralisation with KOH-Mops and removal of $KClO_4$ by centrifugation in the cold, ATP and ADP were measured in the supernatant. The results are depicted in Fig. 1.

Due to contamination of commercial ATP with ADP, a substantial amount of ADP was routinely found in the reaction mixture. No increase in ADP concentration was found at neutral pH at 25 °C for at least 3 h. The results of Fig. 1 show that considerable amounts of ADP were formed as a

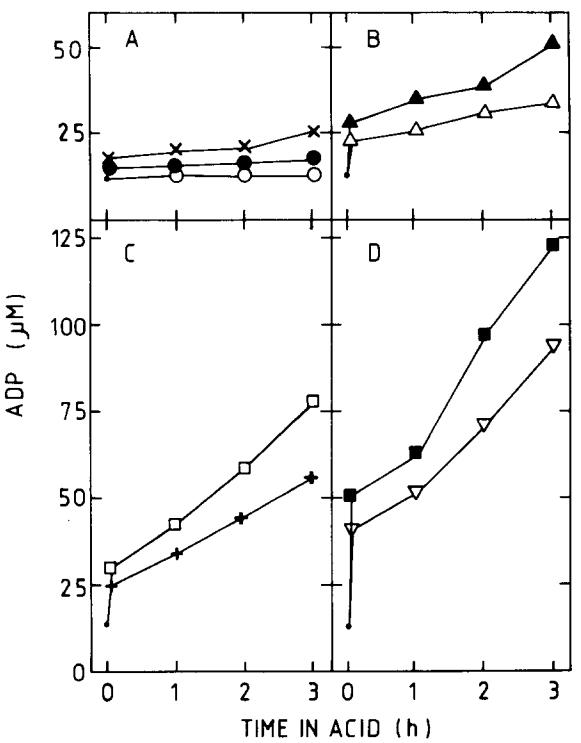

Fig. 1. Factors affecting the hydrolysis of ATP under acid conditions. A stock solution of ATP was prepared by dissolving the compound in water followed by neutralisation with solid Tris to pH 7.4. An aliquot of the stock solution of ATP (final concentration 2 mM) was added to the standard reaction medium described in Materials and Methods. At zero time, 0.6-ml samples were taken and added to Eppendorf tubes containing 0.6 ml ice-cold perchloric acid in the following final concentrations: 0.35 M (●, ×) (A); 0.7 M (▲, △) (B); 1.05 M (□, +) (C); and 1.40 M (■, ▽) (D). The following symbols denote incubations with EDTA present (final concentration 25 mM): ●, △, +, ▽. At the times indicated, tubes were removed from the ice/water bath and centrifuged in the cold for 60 s at 12000 × *g*. After immediate neutralisation of an 0.9 ml aliquot of the supernant with cold 2 M KOH/0.2 M Mops, samples were frozen in liquid nitrogen. ADP was measured fluorimetrically. Each value represents the mean of duplicate incubations and triplicate determinations. The ADP content of samples to which no acid was added is shown in panel A (○).

result of acid hydrolysis of ATP, the extent being dependent upon the time of incubation and the concentration of perchloric acid used. The presence of EDTA inhibited this process. AMP was also formed, but in much lower amounts (results not shown), in agreement with Hutchings et al. [8] who reported a 20-fold difference in the rate coefficients for the hydrolysis of ATP to ADP and that of ATP to AMP. The data of Fig. 1 show further that, depending on the perchloric acid concentration used, substantial amounts of ADP were already formed during the first short period of acid treatment (2–2.5 min).

When the experiment of Fig. 1 was modified by keeping the temperature of the incubation mixture at 0 °C instead of at 25 °C, the first rapid phase of ATP hydrolysis was greatly suppressed (not shown), suggesting that this phenomenon can at least partially be attributed to rapid hydrolysis of ATP at the elevated temperature during the first few minutes after addition of the sample (at 25 °C) to perchloric acid (at 0 °C). This explanation is strengthened further by the fact that decreasing the volume of added sample relative to that of the cold perchloric acid also led to a reduction in the extent of ADP formation during the first short time interval.

In the experiment of Fig. 2, hydrolysis of ATP to ADP under acid conditions was studied in the presence of mitochondria. For this purpose, rat-liver mitochondria were incubated for 5 min in the standard reaction medium together with succinate (plus rotenone) and 2 mM ATP. The ADP derived from the ATP solution (14.5 μM) was substantially lowered after the addition of mitochondria. This phenomenon reflects the ability of respiring mitochondria to phosphorylate ADP against high extramitochondrial phosphorylation potentials [5]. Indeed, oligomycin abolished the observed decrease in the concentration of ADP (not shown). As in the experiment of Fig. 1, 0.6-ml aliquots were taken from the reaction vessel and added to 0.6-ml ice-cold $HClO_4$ (0.35 M) with or without EDTA. Incubations were continued in ice-water for different periods of time. The following observations were made. Firstly, when the acidified reaction mixture was kept at 0 °C for increasing periods of time, there was a gradual increase in the amount of ADP subsequently found in the neutralised, protein-free extract. Secondly, EDTA decreased the rate of formation of ADP under acid conditions. Thirdly, when the samples were centrifuged for 1 min after acidification and immediately neutralised, the amount of ADP increased upon storage of the neutralised, protein-free extract at 0 °C. The effect of storage of neutralised extracts was less evident in samples kept in

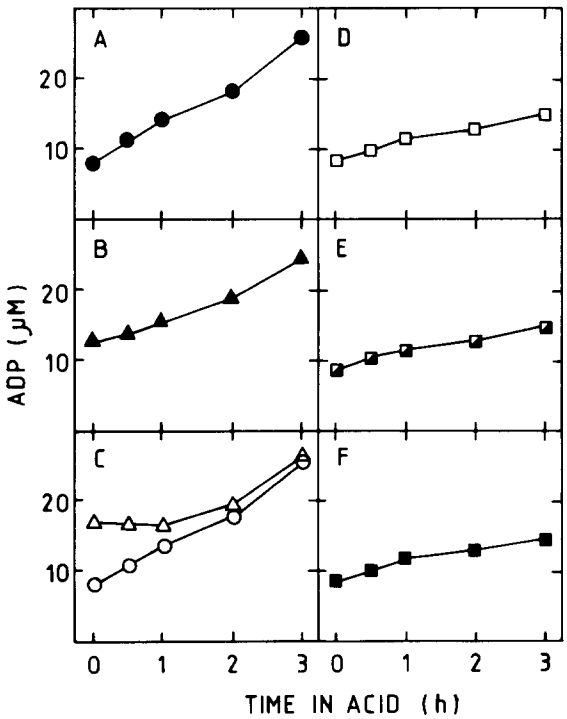

Fig. 2. The effect of EDTA and time of exposure to acid on the hydrolysis of ATP in acidified reaction mixtures containing ATP and mitochondria. Rat-liver mitochondria (1.0 mg protein/ml) were incubated in the standard reaction medium together with 2.0 mM ATP and 14.5 μM ADP (derived from the ATP solution). After 8 min, 0.6-ml samples were taken and added to Eppendorf tubes (1.6 ml) kept in ice/water and containing 0.6 ml 7% (w/v) $HClO_4$ (A–C). After 1, 30, 60, 120 and 180 min, tubes were withdrawn and subjected to centrifugation (12000 × *g*, 60 s) in the cold. After neutralisation, samples were frozen immediately in liquid nitrogen. ADP was determined in the neutralised perchloric acid extracts immediately after thawing in ice/water (A), or after being kept at 0 °C for 4 h (B). In C, data are shown for samples neutralised and kept at 0 °C for 8 h either in the absence (△), or in the presence (○) of 25 mM EDTA. In D, E and F, 25 mM EDTA was present in the acidification medium. Samples were kept in the acid medium for the times indicated and ADP was determined immediately after thawing (D), or after 4 h (E) or 8 h (F) at 0 °C. Each point represents the mean of triplicate determinations.

contact with acid for 1 h or longer. Fourthly, the presence of EDTA in the neutralised extracts prevented the time-dependent increase in ADP observed during storage at 0 °C.

Conclusions

In studies on the regulation of oxidative phosphorylation in rat-liver mitochondria, quenching with perchloric acid (0.15–0.95 M) is used almost universally to terminate reactions. Some authors immediately centrifuge the acidified sample and neutralise, others leave samples in acid for various periods of time. The latter procedure will lead to an underestimation of the true ATP/ADP ratio, especially when it is high, due to the time-dependent, acid concentration-dependent hydrolysis of ATP to ADP (Figs. 1 and 2). However, although a short acid treatment followed by rapid centrifugation and neutralisation is beneficial as far as acid-catalysed hydrolysis of ATP is concerned, care must be taken to avoid other artefacts: if the time of exposure to acid is too short, there is incomplete denaturation of kinases, as stressed by Brawand and Walter [6]. Indeed, adenylate kinase is known to be relatively stable to acid treatment [22]. As a consequence of this phenomenon, ADP is produced after thawing of the samples (Fig. 2). Brawand and Walter [6] suggested addition of pepsin to samples after acidification, whereas Jacobus et al. [21] suggested that acidified samples should be filtered. The results of Fig. 2 demonstrate that an excess of EDTA prevented the occurrence of this artefact very efficiently.

The results of Table II show that even under conditions of minimal hydrolysis of ATP (low perchloric acid concentrations, short periods of acid treatment, all manipulations in the cold, EDTA present), values obtained for the extramitochondrial ATP/ADP ratio using perchloric acid as quenching agent are accurate only if the ratios do not exceed values of 100–200. For accurate measurement of higher ratios, the phenol/chloroform/isoamyl alcohol method must be used.

In conclusion, the variation in the values reported in the literature for the extramitochondrial ATP/ADP ratio in rat-liver mitochondria incubated under State-4 conditions, is due in part to different extents of ATP hydrolysis. Indeed, values for the extramitochondrial ATP/ADP ratio in State-4 mitochondria obtained by Slater et al. [5] and by ourselves (this paper, Table II) are by far the highest reported in the literature.

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