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SIMULTANEOUS EVALUATION OF PHOSPHORYLATION EFFICIENCY OF THE THREE COUPLING SITES OF THE RESPIRATORY CHAIN IN ISOLATED MITOCHONDRIA

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SUMMARY

A polarographic method is described for the simultaneous evaluation of ADP/O and ADP/$2e^-$ ratios with intact rat-liver mitochondria. The method is based on the amount of O_2 taken up during the ADP-stimulated respiration in the presence and in the absence of a known amount of ferricyanide. The contribution of each coupling site to the overall P/O ratio may be estimated by a simple equation. The validity and the limits of the method are discussed in view of the previous work using abbreviated electron pathways for the study of oxidative phosphorylation.

INTRODUCTION

The study of oxidative phosphorylation has profited in the past by the use of abbreviated electron pathways. Cytochrome *c* has been used as terminal electron acceptor by SLATER[1] and LEHNINGER[2] for the study of the first two coupling sites. The region of the third has been investigated by feeding electrons to the chain at the level of cytochrome *c*, either directly[3–7] or *via* different dyes such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)[8–10] or phenazine methosulphate[11]. It has been possible specifically to by-pass coupling site II by means of TMPD in the presence of antimycin A (ref. 12). Phosphorylation taking place at coupling site I has been investigated in the past by means of different artificial electron acceptors such as phenazine methosulphate[13], fumarate[14,15] and coenzyme Q_1 (ref. 16).

Potassium ferricyanide, first introduced in the field by CROSS *et al.*[17], is known to interact with the respiratory chain preferentially at the level of cytochrome *c* (refs. 18, 19). The use of such an artificial electron acceptor in the presence of a terminal inhibitor of the respiratory chain has allowed the study of oxidative phosphorylation[17–25] involving either coupling sites I and II, during the oxidation of NADH-linked substrates, or only coupling site II during the oxidation of succinate.

All the above-mentioned methods make use of artificial electron acceptors or donors and often include the use of electron transport inhibitors. The experimental conditions, in addition, differ from one experiment to another.

In this paper we describe a method for the simultaneous estimation of ADP/O

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

and ADP/$2e^-$ ratios using intact rat-liver mitochondria oxidizing different substrates both by O_2 and ferricyanide, in the absence of terminal inhibitors of the respiratory chain. With this method it is possible to evaluate the contribution of the single coupling sites to the overall stoichiometry of the oxidative phosphorylation.

METHODS

Mitochondria were isolated from albino rat livers in 0.2 M mannitol according to JOHNSON AND LARDY[26].

ADP (Sigma Chem. Co.) was purified, when necessary, by ion-exchange chromatography as described by SCHMITZ[27]. It is essential that the amount of AMP present as a contaminant does not exceed 2–3 % of the total nucleotide present in the preparation, in order to avoid interference of the myokinase reaction in the estimation of ADP/O and ADP/$2e^-$ ratios. Standardization of ADP solutions was performed enzymically according to ADAM[28].

Ferricyanide (C. Erba) was purified by several recrystallizations from water to eliminate contaminating CN^- which may be present in old preparations of the reagent. Ferricyanide solutions prepared daily, protected from light, were standardized spectrophotometrically at 420 nm using the molar extinction coefficient of $1 \cdot 10^3$ cm^{-1}.

O_2 uptake was recorded at 30° using a Clark oxygen electrode essentially as described by ESTABROOK[29]. The incubation mixture consisted of: different amounts of mitochondrial protein; 200 mM mannitol; 50 mM KCl; 25 mM Tris–HCl buffer (pH 7.5); 10 mM phosphate buffer (pH 7.5); 5 mM $MgCl_2$. Further details are included in the legends to table and figures. The rate of back-diffusion of O_2 and its content in the reaction mixture was calibrated by means of sub-mitochondrial particles supplemented with limiting amounts of NADH as recommended by ESTABROOK[29]. It was found that, under the experimental conditions used, the diffusion rate of O_2 could be considered negligible.

Protein was determined according to GORNALL *et al.*[30].

Other chemicals were commercial products.

RESULTS

The first polarographic trace shown in Fig. 1 shows the classical state IV–state III transition that occurs when limiting amounts of ADP are added to intact mitochondria supplemented with substrate[31,32]. The second and the third traces show that upon addition of ferricyanide to mitochondria respiring in state III a clear inhibition of the respiration rate occurs which is gradually reversed. During the course of the experiment ferricyanide added is progressively and totally reduced as visualized by the rapid decoloration of the content of the vessel or as followed spectrophotometrically at 420 nm. It may be noticed, in addition, that the inhibition increases with the amount of ferricyanide added. Moreover, for the same amount of ADP added, the absolute amount of O_2 taken up during the ADP-stimulated respiration is smaller in the presence than in the absence of ferricyanide. The explanation for these findings is that, under these experimental conditions, the substrate may be oxidized both by O_2 and by ferricyanide. It is expected therefore that the competition of the two

electron acceptors results in an inhibition of the rate of respiration. Since both processes are coupled to phosphorylation of ADP it is also expected that the extent of ADP-stimulated respiration will decrease on addition of ferricyanide since a portion of the nucleotide added is phosphorylated during the reduction of the complex-ion.

Since cytochrome *c* is known to be the site of preferential interaction of ferricyanide with the respiratory chain in intact mitochondria[18,19], we have investigated the effect of ferricyanide on the redox steady-state level of this cytochrome. The experiment reported in Fig. 2 shows that addition of succinate to mitochondria, in the presence of rotenone, causes a reduction of the cytochrome corresponding to state IV. Subsequent addition of ADP causes an oxidation of cytochrome *c* corresponding to state III, in accordance with the early finding of CHANCE AND WILLIAMS[33]. Ad-

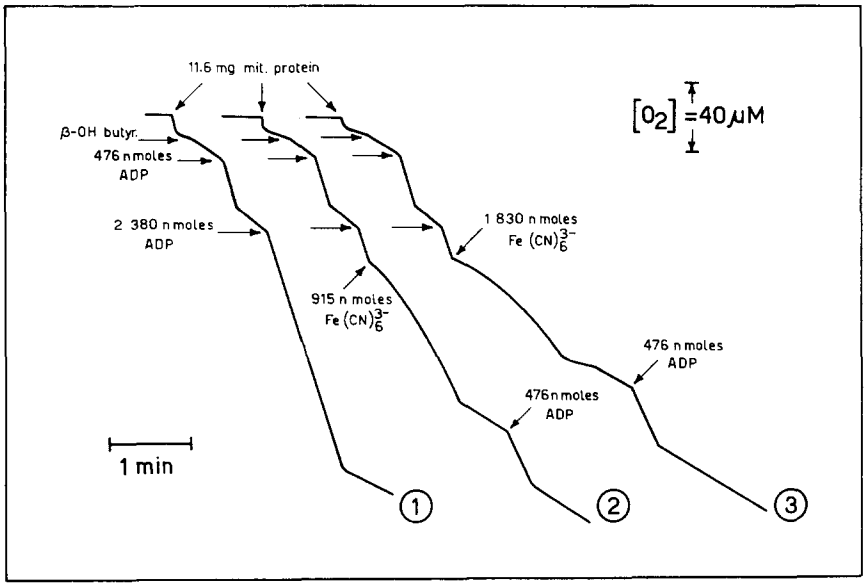

Fig. 1. Effect of ferricyanide on state III respiration rate of intact rat-liver mitochondria. Experimental conditions as described under METHODS. β-Hydroxybutyrate, 2.5 mM; final volume 3.2 ml.

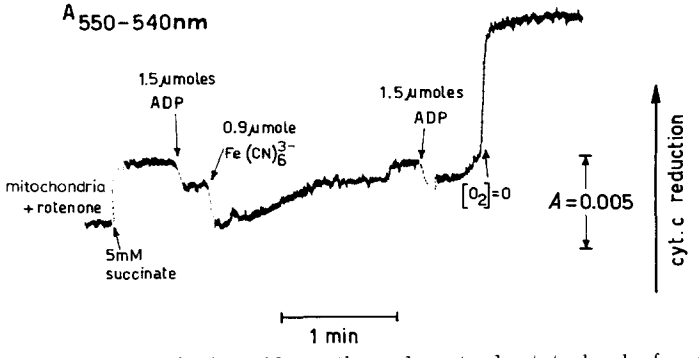

Fig. 2. Effect of ferricyanide on the redox steady-state level of cytochrome *c*. Phoenix dual-wavelength spectrophotometer recording at 20°. The reaction mixture as described under METHODS. In 3 ml 8 mg mitochondrial protein were added.

dition of ferricyanide to mitochondria respiring in state III causes a further oxidation of the steady-state level of cytochrome *c* which is gradually reversed to the original state III level as the added ferricyanide is progressively reduced. When all the ADP has been phosphorylated, a state III–state IV transition occurs and a new steady-state level is established which corresponds precisely to the previous state IV level. The cycle may be repeated until the preparation goes to anaerobiosis. This experiment confirms the early finding of ESTABROOK[19] that ferricyanide causes a rapid oxidation of cytochrome *c*. In contrast to the experiment of ESTABROOK[19], however, no terminal inhibitor of the respiratory chain was added to our sample. Under these conditions, the addition of ADP to mitochondria respiring in state IV causes an oxidation of the cytochrome. In the presence of a terminal inhibitor of the respiratory chain[19,34], on the contrary, addition of ADP to mitochondria respiring in state IV is followed by a reduction of cytochrome *c*. In our experiment a second addition of ADP after the complete reduction of ferricyanide is followed by an oxidation of cytochrome *c* which indicates that no CN^- was added as contaminant of ferricyanide.

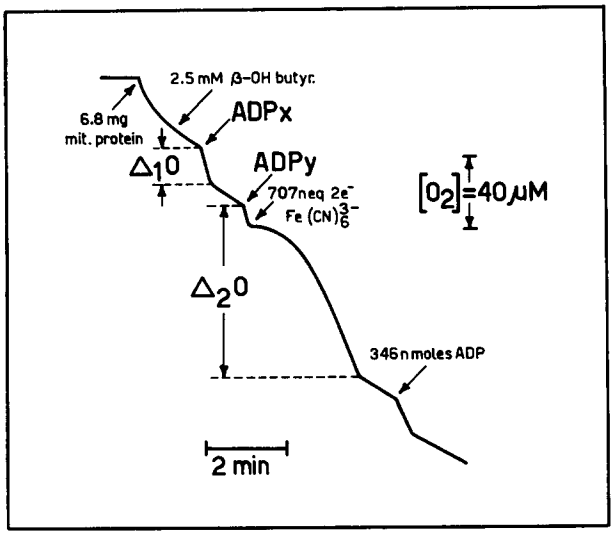

Fig. 3. Polarographic measurement of ADP/O and ADP/$2e^-$ ratios with intact rat liver mitochondria. Experimental conditions as for Fig. 1. Final volume 3.1 ml. ADP_x = 346 nmoles; ADP_y = 2.780 nmoles; Δ_1O = 0.138 μmole AO_2; Δ_2O = 0.633 μmole AO_2.

The experiments reported suggest the possibility of estimating simultaneously the stoichiometry of phosphorylation coupled to oxidation of substrates both by O_2 and ferricyanide. This is illustrated by the experiment reported in Fig. 3. As first described by CHANCE AND WILLIAMS[31,32], from the ratio ADP_x/Δ_1O a value ADP/O, equivalent to P/O, can be calculated. Addition of a further amount of ADP (ADP_y) is followed by a state III respiration which is transiently inhibited by subsequent addition of ferricyanide, as described above. The overall ADP-stimulated respiration has been denoted in the figure as Δ_2O. A portion of the ADP_y added (ADP_h) is phosphorylated during the aerobic oxidation of the substrate whereas another portion (ADP_k) is phosphorylated during the reduction of added ferricyanide. If we assume

that the phosphorylation efficiency of the system is not altered by the addition of ferricyanide, the amount of ADP phosphorylated aerobically should be:

$$ADP_h = \Delta_2O \cdot ADP_x/\Delta_1O \quad (1)$$

By difference then the amount of ADP phosphorylated during reduction of ferricyanide will be:

$$ADP_k = ADP_y - ADP_h \quad (2)$$

and by combining Eqns. 1 and 2

$$ADP_k = ADP_y - \Delta_2O \cdot ADP_x/\Delta_1O \quad (3)$$

The amount of ferricyanide added and the value of ADP_k phosphorylated during reduction of ferricyanide being known an $ADP/2e^-$ ratio can be derived by the following equation:

$$ADP/2e^- = \frac{ADP_y - \Delta_2O \cdot ADP_x/\Delta_1O}{\text{Ferricyanide}} \quad (4)$$

where ADP_y and ADP_x are expressed as nmoles and ferricyanide as nequiv $2e^-$. In the trace shown in Fig. 3 a further addition of ADP allows a second calculation of ADP/O and a check of the respiratory control after utilization of ferricyanide.

The trace reported in Fig. 4 illustrates an experiment carried out using succinate as substrate and also reports the calculated ADP/O ratios before and after reduction of ferricyanide as well as the $ADP/2e^-$ ratio obtained. It may be seen that the ADP/O calculated before and after reduction of ferricyanide is the same, indicating that the presence in the reaction mixture of 944 nmoles of ferrocyanide does not modify the phosphorylating efficiency of the mitochondria. This conclusion is reinforced by the

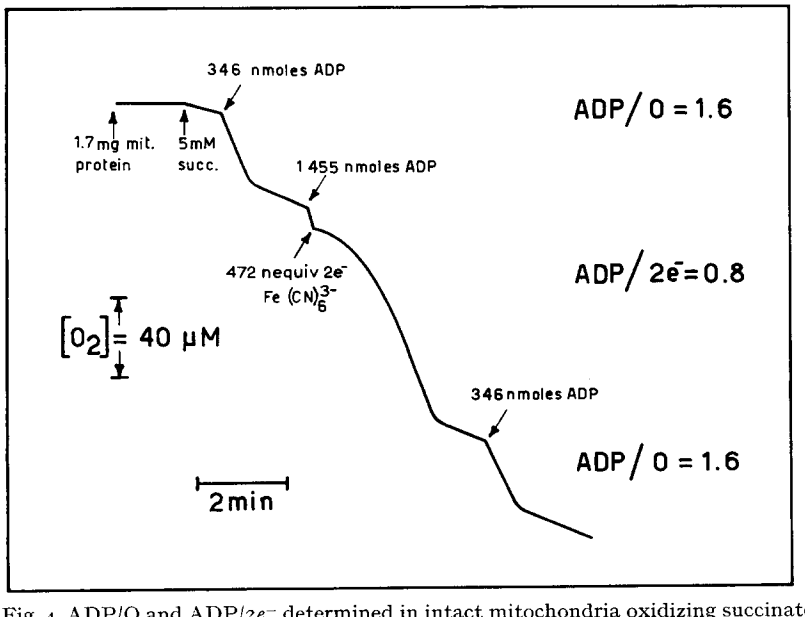

Fig. 4. ADP/O and $ADP/2e^-$ determined in intact mitochondria oxidizing succinate. Experimental conditions as described under METHODS; 1.5 μM rotenone was added to a final volume of 3.05 ml.

observation that the state III respiration rate is virtually the same throughout the whole experiment and that essentially the same respiratory control ratio may be measured at the beginning and at the end of the experiment. These findings were expected on the basis of the results obtained in the previous experiment where the same state III and state IV steady-state levels of cytochrome *c* were found before and after addition of ferricyanide. It is most striking that the ADP/$2e^-$ ratio calculated is exactly one-half of the corresponding ADP/O.

Fig. 5 illustrates the linear relationship between the amount of ferricyanide added and the amount of ADP phosphorylated during its reduction either with β-hydroxybutyrate or succinate as substrates. It may be seen that, above 0.6 μequiv $2e^-$ ferricyanide, the amount of ADP phosphorylated is lower than one would predict. Such a phenomenon disappears, however, if the amount of mitochondrial protein present in the sample is doubled (see point denoted by a cross on the line of succinate in Fig. 5). Apparently the linear relationship is maintained over a rather large range of ferricyanide concentrations, provided that a sufficient amount of mitochondrial protein is present in the assay vessel. This conclusion is supported by the experiment with β-hydroxybutyrate where the protein concentration was 4 times higher than with

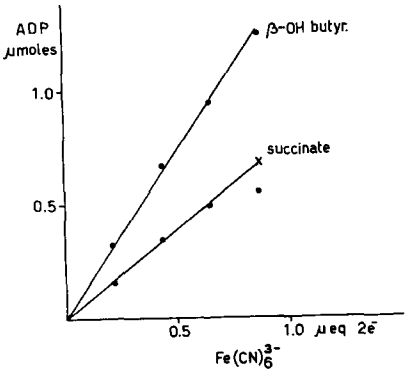

Fig. 5. Relationship between ADP phosphorylated and amount of ferricyanide added. Experimental conditions as for previous figures. The amount of mitochondrial protein was 16 mg for β-hydroxybutyrate and 4 mg for succinate. The value denoted by the cross (×) refers to a sample containing 8 mg mitochondrial protein. 1.5 μM rotenone was added in the sample oxidizing succinate.

TABLE I

ADP/$2e^-$ AND ADP/O RATIOS DETERMINED SIMULTANEOUSLY WITH RAT-LIVER MITOCHONDRIA

Experimental conditions as described under METHODS. Mitochondrial protein were 6.6 and 13.2 mg for succinate and other substrates, respectively. Substrate concentrations were 5 and 2.5 mM for succinate and other substrates, respectively. Final volume was 3 ml.

Substrate	*ADP (μmoles)*	*Ferricyanide (μequiv $2e^-$)*	*ADP/$2e^-$*	*ADP/O*
Succinate + rotenone (1.5 μM)	0.400	0.465	0.86	1.65
β-Hydroxybutyrate	0.788	0.465	1.69	2.62
Pyruvate + malate	0.788	0.465	1.69	2.62
α-Ketoglutarate + malonate (1 mM)	1.210	0.465	2.60	3.50

succinate and no apparent deflection from linearity was detected. This finding is in agreement with our previous experience using much higher concentrations of ferricyanide in the presence of CN^- and determining separately the amount of ferricyanide reduced and phosphate esterified. Under these conditions, maximal $P/2e^-$ ratios were obtained only at very high protein concentrations.

Table I collects data of a typical experiment in which ADP/O and $ADP/2e^-$ were simultaneously determined using different substrates. It may be noticed that the $ADP/2e^-$ ratio (0.86) obtained with succinate is almost exactly one-half of the corresponding ADP/O (1.65). The latter value is virtually the same as the $ADP/2e^-$ (1.69), obtained with either β-hydroxybutyrate or pyruvate + malate, which in turn is almost two-thirds of the corresponding ADP/O (2.62). The $ADP/2e^-$ ratio (2.60)

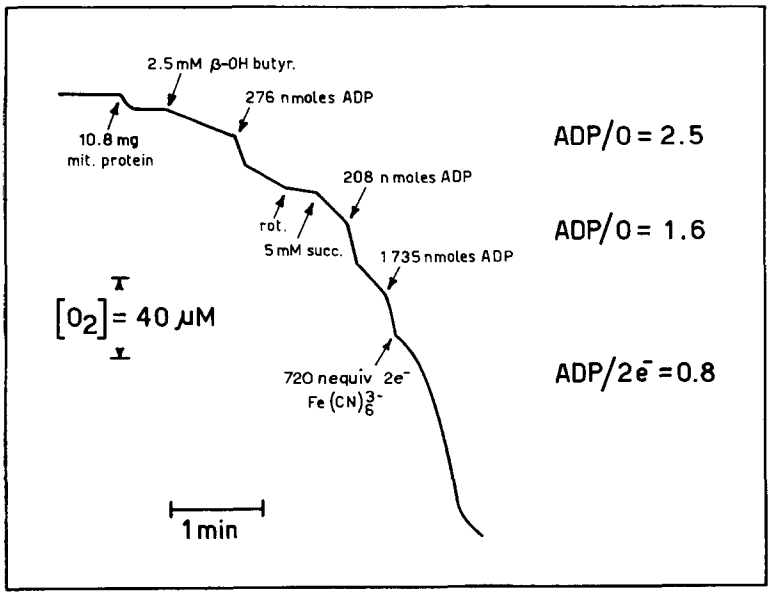

Fig. 6. Simultaneous evaluation of phosphorylating efficiency of the three coupling sites of the respiratory chain. For experimental conditions see previous figures. The final volume was 3.2 ml. Rotenone concentration was 1.5 μM.

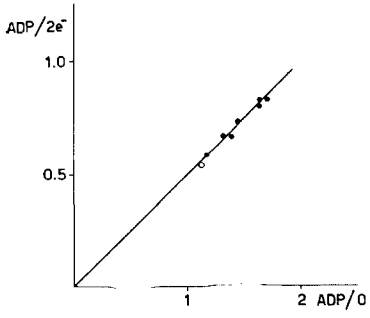

Fig. 7. Correlation between $ADP/2e^-$ and ADP/O in rat-liver mitochondria oxidizing succinate. Experimental conditions as for previous figures. The decrease of ADP/O ratios was obtained either by ageing (●) the mitochondria in ice up to 7 h or by adding $1 \cdot 10^{-5}$ M 2,4-dinitrophenol (○). Rotenone concentration 1.5 μM. Final volume 3.05 ml.

found for α-ketoglutarate corresponds precisely to the ADP/O measured with both β-hydroxybutyrate and pyruvate + malate (2.62) and represents three-quarters of the corresponding ADP/O ratio (3.50).

To attempt the simultaneous evaluation of the phosphorylation efficiency at the three coupling sites of the respiratory chain, the experiment reported in Fig. 6 was carried out. Intact rat-liver mitochondria in isotonic medium were allowed to oxidize β-hydroxybutyrate aerobically first in the absence and then in the presence of a limiting amount of ADP. This allows the calculation of an ADP/O ratio of 2.5. The oxidation of β-hydroxybutyrate was then blocked by addition of rotenone, and succinate was added as substrate. Upon addition of a limiting amount of ADP, the corresponding ADP/O ratio was found to be 1.6. Subsequent addition of ADP and ferricyanide permits the calculation of the corresponding ADP/$2e^-$ ratio in the span of the respiratory chain between succinate and cytochrome *c* (0.8). The contribution of sites I and III to the overall P/O ratio may be estimated by difference.

Results so far obtained seem to indicate that the calculated stoichiometry for each coupling site is always the same. We have investigated whether this conclusion holds true regardless of the overall P/O ratio of the mitochondria. Mitochondria either fresh or aged for different times were assayed for ADP/O and ADP/$2e^-$ in the presence of succinate as substrate. For comparison a sample was also assayed in the presence of $1 \cdot 10^{-5}$ M 2,4-dinitrophenol. The results of this experiment, reported in Fig. 7, clearly indicate that the ADP/$2e^-$ ratios measured are always one-half of the corresponding ADP/O regardless of the original degree of coupling. Similarly, experiments carried out using NADH-linked substrates always give ADP/$2e^-$ two-thirds of the corresponding ADP/O.

Table II summarizes the results of a number of experiments in which the phosphorylation efficiency has been calculated for the individual coupling sites. The differences among the data reported have no statistical significance, in agreement with the previous findings.

TABLE II

STOICHIOMETRY OF OXIDATIVE PHOSPHORYLATION AT THE SINGLE COUPLING SITES

Values (± S.D.) are calculated as described in the text. In parentheses the number of experiments.

	Site I	*Site II*	*Site III*
P/$2e^-$	0.82 ± 0.12 (9)	0.78 ± 0.07 (15)	0.79 ± 0.09 (15)

DISCUSSION

Similarly to other methods based on the use of ferricyanide, the technique described allows the calculation of the ADP/$2e^-$ ratio in the span of the respiratory chain involving either coupling sites I and II or coupling site II alone. In contrast, however, this method involves the simultaneous estimation of the overall ADP/O ratio. This permits the evaluation by difference of the contribution of each coupling site separately and within the same experiment. The efficiency of the three coupling sites seems always to be the same regardless of the overall P/O ratio of the single

preparations. We have noticed, for instance, that in aged preparations of mitochondria, where P/O ratios for NADH-linked substrates were 2 or less, the corresponding $P/2e^-$ ratio was proportionally decreased as if the degree of uncoupling were the same at all three coupling sites. Apparent discrepancies between ADP/O and $ADP/2e^-$ ratios have occasionally been met in preparations of mitochondria where no special care had been taken to avoid contamination of mitochondria by submitochondrial particles. The simplest explanation for this finding is that the contaminant may react with ferricyanide also at the level of flavoproteins in a non-phosphorylating rotenone-, amytal- and antimycin A-insensitive manner. Such a phenomenon does not occur in intact mitochondria, presumably because of the barrier to the entry of ferricyanide represented by the "inside-in" inner mitochondrial membrane[35]. These findings limit the use of the method described to intact mitochondria displaying good respiratory control ratios.

A source of error in the estimation of the $ADP/2e^-$ ratio may arise from an inadequate proportion between ADP and ferricyanide added. Obviously a prerequisite for a correct calculation is that all ferricyanide is reduced during the course of the experiment. This is indicated by the re-establishment of the state III respiration rate after the addition of ferricyanide, indicating that an excess ADP is still present when all ferricyanide has been reduced. Conditions must therefore be chosen so that the amount of ADP added is high enough to fulfill the above-mentioned condition but not to exceed the limits imposed by the O_2 content of the vessel.

The method described in this paper offers for the first time the possibility of evaluating the efficiency of the three coupling sites of the respiratory chain within the same experiment. The values are obtained by difference as first proposed for coupling site I by GREEN *et al.*[36]. The criticism that "the difference between two ratios is usually too inaccurate to be of any real value"[16] is minimized when applied to measures obtained within the same sample.

In conclusion it seems to us that the method described represents a simple and reliable tool for future investigations of oxidative phosphorylation.

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