Bioenergetics (1971) 2, 93–99

Mitochondrial ATP Contents During Phosphorylation

E. J. Harris

Department of Biophysics,
University College,
London, W.C.1

Date received: 30 November 1970

Abstract

Using the method of quick separation by centrifugation through a layer of silicone the contents of ATP in mitochondria during active phosphorylation of external ADP have been determined. The rate of phosphorylation is linearly related to the ATP content (in state 3) and this relation is independent of the substrate. The rate of phosphorylation and the associated internal ATP content were both diminished as incubations were carried out using the mitochondrial protein at increasing concentrations.

Introduction

When ADP is added to a suspension of rat liver mitochondria it exchanges with the internal ADP and ATP.[1] When phosphate and a substrate are present the internal ADP/ATP ratio is kept at a value depending on the phosphorylation process, so the exchange of the external ADP with the internal nucleotides ultimately leads to its replacement by a nearly stoichiometric amount of ATP. The aim of the present work was to establish the relation between the internal ATP content during phosphorylation and the rate of phosphorylation of the external ADP. According to the formulation of Heldt,[2] the proportion of ATP, exported in the total nucleotides emerging in exchange for entering ADP, is strictly proportional to the internal ATP; hence the phosphorylation flux should be related to the internal ATP by a factor which has to be evaluated.

In making the series of experiments described below it was noticed that the rate of phosphorylation per unit of mitochondrial protein was inversely related to the concentration at which the suspension was being used. This suggests that some inhibitory factor is present in the mitochondrial preparation.

Methods

Rat liver mitochondria were prepared by a variant of Schneider's[3] method using 0·5 mM EGTA and 0·05% bovine serum albumin (defatted and dialysed) in the 250 mM sucrose solution for homogenization and two subsequent washes. The wash media also contained 5 mM KCl which improved the yield of mitochondria without causing loss of cytochrome or extensive co-sedimentation of reticular membranes. The final stock suspension was made at about 50 mg/ml in 300 mM sucrose. Protein was measured by a biuret method.[4]

Rates of phosphorylation and the associated internal ATP contents were found by taking samples from incubations in an open jacketted vessel exposed to a stream either of oxygen for the tris-buffered media or to 95% oxygen + 5% carbon dioxide for the bicarbonate buffered media. A glucose + hexokinase trapping system was used in the incubation so that the phosphorylation rate could be found from the amount of glucose-6-phosphate formed in a given time. This method ensured that the extramitochondrial ATP was kept at a low concentration. This would not have been the case if conventional oxygen electrode—ADP—cycles were used. The medium contained KCl, 150 mM, mannitol 60 mM, $MgCl_2$ 0·7 mM, tris-phosphate 5 mM and either tris-chloride pH 7·4 20 mM or $NaHCO_3$ 20 mM. Substrates were added as tris salts at 5 mM unless otherwise stated. Most experiments were made at 20° but the temperature of the vessel could be adjusted to 10° or 30°.

For the phosphorylation reaction the stock mitochondrial suspension was diluted with medium to protein concentrations between 0·7 and 6 mg/ml. After 3–4 min incubation a 0·5 ml sample was withdrawn. This was transferred to a polypropylene centrifuge tube (5 cm bore length, 0·4 cm diameter) which had previously been prepared by loading with 0·1 ml 1·5 M perchloric acid beneath about 3 mm thickness of silicone (G. E. Versilube F.50). As each sample was taken it was centrifuged for 45 sec in a Coleman Microfuge which sufficed to bring the mitochondria into the acid beneath the oil. A specially constructed miniature swing-out head was used.

The analysis of the acid extract from the first sample provided a value for the content of ATP before phosphorylation was initiated. After the sample had been taken, additions of hexokinase (5 units, Sigma type F.300 dissolved in 5% albumin) and ADP to 120 μM were made. Following this, 5 successive 0·5 ml samples were withdrawn at intervals of about 40 sec. Each was centrifuged as described above and the supernatants were acidified promptly with 0·1 ml 1·5 M perchloric acid. Later, the supernatants were transferred from above the silicone layers by a Pasteur pipette into tubes cooled in ice. The precipitated protein was removed by centrifugation and the fluids were brought to pH about 5 by addition of 5 M KOH + 1 M K acetate mixture. The mitochondrial acid extracts were recovered from beneath the silicone in each tube and transferred to cooled tubes and also brought to pH 5 as before. The supernatants were assayed enzymatically for glucose-6-phosphate in a spectrophotometer and the mitochondrial extracts were analysed fluorimetrically for ATP. Methods for both have been described by Williamson and Corkey.[5]

Results

For measuring the rate of phosphorylation of glucose in presence of hexokinase and ADP tests were made to ensure that sufficient enzyme and ADP were present to stimulate the process maximally. At protein concentrations of about 1 mg/ml oxidative phosphorylation was half maximally stimulated with only 11 μM ADP (in presence of hexokinase and glucose) but 50–60 μM was required when the protein was at 4–6 mg/ml and rates of phosphorylation, rather than of respiration, were measured. Routinely 120 μM ADP was added. It was confirmed that with at least 1 unit hexokinase per 3 ml incubation medium the respiration was maximal but routinely 5 units were added.

When phosphorylation of the glucose was initiated by ADP + hexokinase the ATP

content of the mitochondria usually fell within the interval (30–50 sec) before the next sample was withdrawn (Fig. 1). Phosphorylation was rapid in this first interval but could not be referred to a properly weighted mean ATP content, because the ATP change was large and of unknown time course. After the initial ATP change, when there was one, the ATP either remained static or fell slowly over the remaining minutes of sampling. The rate of phosphorylation during this latter period found from the increment in glucose-

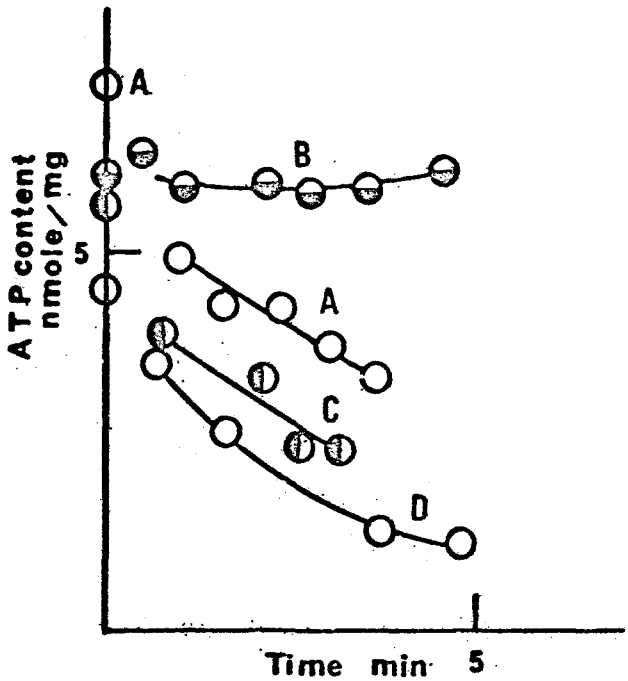

Figure 1. The time course of the ATP content of rat liver mitochondria after initiation of phosphorylation in presence of different substrates. The zero time value is the ATP content before the ADP addition. Phosphorylation was initiated by 120 μM ADP and 5 units hexokinase. Incubation conditions: Temp. 20°. Medium: KCl 150 mM, mannitol 60 mM, tris-chloride pH 7·4 20 mM, glucose 3·5 mM, $MgCl_2$ 0·7 mM, phosphate 5 mM. In (A) glutamate and malate at 5 mM were used with mitochondrial protein at 2·3 mg/ml, in (B) DL-isocitrate at 10 mM with malate at 1 mM were used with protein at 1·7 mg/ml, in (C) pyruavte at 5 mM was used with protein at 2·3 mg/ml and in (D) malate at 5 mM was used with protein at 3·9 mg/ml.

6-phosphate was plotted against the *mean* ATP content over the period. (When the ATP content changed significantly and nonlinearly with time, then a weighted mean was applied.) Figure 2 shows that the two quantities are linearly related irrespective of the substrate and mitochondrial preparation. For ease of analysis the earlier experiments of the series were set up using the mitochondrial protein at 4–6 mg/ml, these provided points corresponding to ATP contents between 1 and 3 μmole/g and low rates of phosphorylation. To obtain higher values, such as customarily are obtained in the oxygen polarograph, it was necessary to use the mitochondrial protein in more dilute suspension.

When results for a number of experiments with a given substrate were collected (Fig. 3) it was clear that there is an inverse relation between the rate of phosphorylation (per mg protein) and the protein concentration. In presence of serum albumin at 0·5% the inhibition remained sufficiently marked to discount the suggestion that it was arising from endogenous fatty acids.

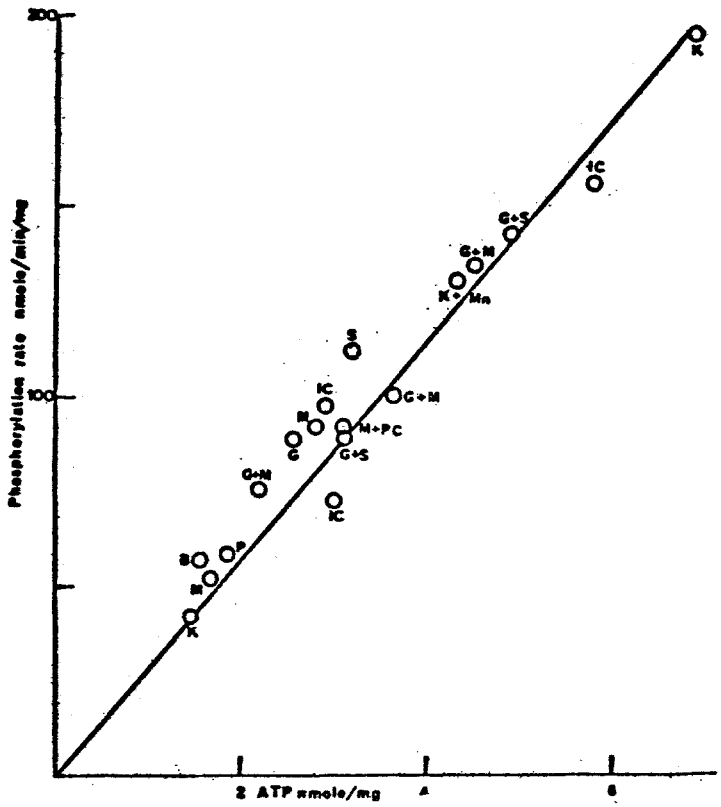

Figure 2. The rate of phosphorylation of external glucose in presence of 5 units hexokinase and 120 μM ADP and the internal ATP content of the mico-chondria. Each point is from a different incubation and most are from different mitochondrial preparations. The protein was used at between 0·7 and 4 mg/ml, higher rates are usually from lower protein concentrations. Various substrates were used at 5 mM. The points are marked with M ≡ malate, P ≡ pyruvate, G ≡ glutamate, K ≡ oxoglutarate, S ≡ succinate, B ≡ hydroxybutyrate, Mn ≡ malonate, IC ≡ DL-isocitrate (at 10 mM), Mn ≡ malonate, PC ≡ palmitoyl carnitine 10 μM in presence of 0·5% serum albumin. The medium was as described in "methods", no difference was noticed between the tris and bicarbonate buffers. Note that much of the isocitrate becomes converted to citrate,[6] and therefore the concentration of oxidizable substrates in this case is less than 5 mM.

Effect of Fatty Acid on Permeability

In absence of serum albumin the permeability to ATP is diminished by fatty acids.[7] This could be shown by the present technique by adding a fatty acid or a derivative during the phosphorylation process. Table 1 shows that oleate at 33 μM or palmitoyl

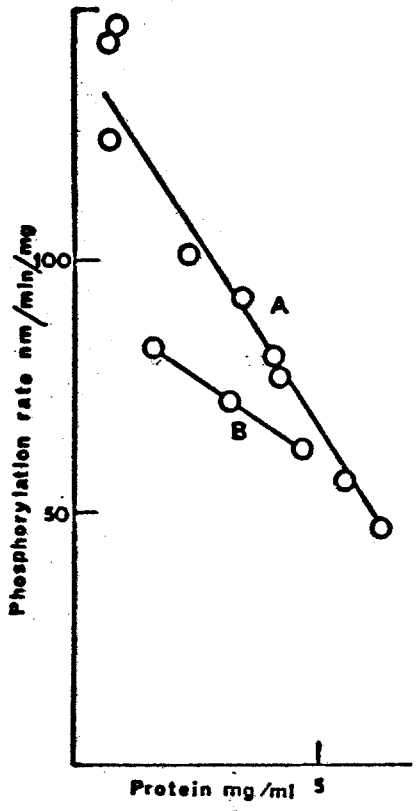

Figure 3. The relation between the rate of phosphorylation per unit of mitochondrial protein and the concentration of mitochondrial protein in the medium. Incubation conditions: Temp. 20°. Medium: KCl 150 mM, mannitol 60 mM, tris-phosphate 5 mM glucose 3·5 mM, hexokinase 5 units, $MgCl_2$ 0·7 mM, ADP 120 μM and in (A) DL-isocitrate 10 mM, malate 1 mM, in (B) glutamate and malate 5 mM each.

carnitine at 25 μM reduces the quotient: Phosphorylation flux/ATP by about 2/3. With serum albumin present at 0·2–0·5% the palmitoyl carnitine acted as a substrate and some points obtained with it in combination with other substrates are shown in Fig. 2. In conditions of production of oxalacetate from malate the addition of the palmitoyl carnitine (in presence of serum albumin) increased the ATP content from 1·5 to 3·0 μmole/g and there was a burst of more rapid phosphorylation.

TABLE I. Effects of oleate or palmitoyl carnitine on the phosphorylation flux without added serum albumin. Results obtained in presence of albumin are included on Fig. 2

Substrate	Agent added	Flux/ATP before addition, min^{-1}	Flux/ATP after addition, min^{-1}
Succinate	Palmitoyl carnitine 33 μM	115/3·2 = 36	46/2·2 = 21
Hydroxybutyrate	Oleate 25 μM	83/2·6 = 32	30/1·6 = 19

Incubation conditions: Temp. 20°, medium KCl 150 mM, mannitol 60 M, phosphate 5 mM, tris-chloride 20 mM, substrate 5 mM, protein at 4 mg/ml.

Effect of Temperature on Permeability

Phosphorylation rates were measured at 10°, 20° and 30° with either glutamate + malate or with isocitrate as added substrate. The quotients of the phosphorylation

flux/ATP content increase by a higher factor between 10° and 20° than between 20° and 30° (Table II).

TABLE II. Temperature dependence of phosphorylation flux and internal ATP contents. Results are for two different substrate mixtures (IC and GS) defined below

Temp °C	10		20		30	
	IC	GS	IC	GS	IC	GS
Flux (nmole per mg per min)	38	14	72	80	175	180
ATP content nmole/mg	2·8	2·0	2·1	2·6	2·2	2·7
Permeability quotient, min^{-1}	14	7	36	31	78	67

Incubation conditions: Medium KCl 150 mM, mannitol 60 mM, $MgCl_2$ 0·7 mM, glucose 3·5 mM, ADP 120 μM and for (IC) tris-chloride 20 mM, isocitrate 10 mM, malate 1 mM; for (GS) $NaHCO_3$ 20 mM, glutamate and malate 5 mM each. Protein concentrations: for (IC) 0·94 mg/ml, for (GS) 4·1 mg/ml.

Discussion

The results presented in Fig. 2 show that the export of ATP from rat liver mitochondria exposed to excess external ADP takes place at a rate proportional to the ATP content. At 20° the factor is about 28 × the content per min. To obtain a high phosphorylation rate the internal nucleotides have to be kept in the form of ATP by the internal phosphorylation reactions. This means that during rapid phosphorylation, with its high associated ATP content, there is still the possibility of ATP-requiring reactions such as pyruvate carboxylation. On the other hand enzymes inhibited by ATP, such as citrate synthase[8] may still be inhibited. In this respect respiratory stimulation by ADP can be expected to give different results from those obtained with an uncoupling agent since the latter removes internal ATP.

It is not possible at present to explain the inhibitory effect of high mitochondrial protein concentrations on the rate of phosphorylation. That it is not merely a consequence of slow release of endogenous fatty acid is suggested by the effect persisting in presence of serum albumin and by the fact that the internal ATP content is low. As seen in Table I, when fatty acid reduces the flux, the quotient Flux/ATP is lessened; this is not the case in the results obtained with high mitochondrial protein concentrations.

The dependence of the Flux/ATP content on the temperature (Table II) may include a limitation of the flux by diffusion when the phosphorylation is sufficiently rapid. The variability of the permeability quotient at 10° with different substrates requires further investigation.

Acknowledgements

This work was supported by the Muscular Dystrophy Assocn of America, Inc., the Medical Research Council and the Wellcome Trust. The centrifuge head and the tubes were kindly made for us by Mr. L. Ward.

References

1. M. Klingenberg and E. Pfaff, in: *Regulation of Metabolic Processes in Mitochondria*, J. M. Tager *et al.* (eds.), Elsevier, London, 1966, p. 180.
2. H. W. Heldt, in: *Mitochondria, Structure and Function*, L. Ernster and Z. Drahota (eds.), Academic Press, London, 1969, p. 93.
3. W. S. Schneider, *J. biol. Chem.*, **176** (1952) 259.
4. E. Layne, *Meth. Enzym.*, **3** (1957) 45.
5. J. R. Williamson and B. E. Corkey, *Meth. Enzym.*, **13** (1969) 434.
6. E. J. Harris, *F.E.B.S. Letters*, **2** (1969) 259.
7. L. Wojtczak and H. Zaluska, *Biochim. Biophys. Acta*, **189** (1969) 455.
8. P. Garland, in: *Metabolic Roles of Citrate*, T. W. Goodwin (ed.), Academic Press, London, 1968, p. 41.