BBA 3928

FURTHER STUDIES ON THE REQUIREMENT FOR PHOSPHATE FOR THE OXIDATION OF GLUTAMATE BY RAT-LIVER MITOCHONDRIA IN THE PRESENCE OF DINITROPHENOL

R. CHARLES, J. M. TAGER AND E. C. SLATER

Laboratory of Physiological Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

(Received October 29th, 1962)

SUMMARY

1. The oxidation of glutamate by rat-liver mitochondria in the presence of dinitrophenol and absence of added phosphate acceptor has been studied.

2. When the concentration of added inorganic phosphate was 50 μM or lower, the oxidation declined rapidly after about 10–15 min. Respiration could be restored by adding inorganic phosphate.

3. In the absence of added phosphate, the inorganic phosphate initially present in the mitochondrial suspension disappeared slowly and at an almost constant rate. The P:O ratio was 0.01–0.015.

4. In the absence of added phosphate, the oxidation of glutamate was further inhibited by AMP, but not by ADP. AMP had no effect in the presence of a sufficiently high concentration of added phosphate, and the inhibition by AMP could be relieved by the subsequent addition of phosphate.

5. It is concluded that, in addition to the reaction of inorganic phosphate with endogenous ADP, catalysed by the substrate-linked phosphorylation reaction, and the hydrolysis of the ATP formed by the dinitrophenol-induced ATPase, there is a side reaction which slowly leads to the disappearance of inorganic phosphate.

INTRODUCTION

TEPLY[1] and JUDAH[2] first demonstrated the stimulation by inorganic phosphate of the rate of oxidation of glutamate by isolated mitochondria, in the presence of ADP and dinitrophenol. This was confirmed by BORST AND SLATER[3,4] and explained by the discovery that glutamate is not oxidized by mitochondria primarily by way of glutamate dehydrogenase, but by way of transamination with oxaloacetate, followed by oxidation of the α-ketoglutarate formed to oxaloacetate[5,6] (see also refs. 7–9). Thus the oxidation of α-ketoglutarate to succinate, which includes the dinitrophenol-insensitive[2,10] substrate-linked phosphorylation, is normally involved in the oxidation of glutamate. In those mitochondria which contain glutamate dehydrogenase, such as rat-liver mitochondria, a slower alternative pathway for the oxidation of glutamate is open when the oxidation of α-ketoglutarate to oxaloacetate is inhibited in such a way that α-ketoglutarate does not accumulate. This is the case when malonate

is used as inhibitor[6]. When, however, the oxidation of α-ketoglutarate to succinate is inhibited, *e.g.* by removal of phosphate, the alternative pathway is also slowed down owing to inhibition of the glutamate dehydrogenase by α-ketoglutarate[4].

BORST AND SLATER[4] showed that rat-liver mitochondria as normally prepared contain appreciable amounts of inorganic phosphate, and that maximum stimulation by added phosphate could only be obtained after removal of this endogenous phosphate, preferably by a pre-incubation with substrate and phosphate acceptor. The concentration of endogenous phosphate is sufficient for a considerable respiration in the presence of dinitrophenol, and since in the presence of this uncoupler any ATP formed by the substrate-linked phosphorylation step in α-ketoglutarate oxidation would be expected rapidly to be hydrolysed by the dinitrophenol-induced ATPase[11], it would be anticipated that a steady-state concentration of phosphate would be maintained by the two reactions

$$ADP + P_i \longrightarrow ATP + H_2O \text{ (oxidative phosphorylation)} \quad (1)$$

$$ATP + H_2O \xrightarrow{\text{(dinitrophenol)}} ADP + P_i \text{ (dinitrophenol-induced ATPase)} \quad (2)$$

AZZONE AND ERNSTER[12] have recently shown, however, that the rate of respiration in the presence of dinitrophenol, and absence of added phosphate acceptor, is not maintained, but that there is a rapid decline after 15 min at 30°. Similar results were obtained with low concentrations (up to 0.1 mM) of added phosphate. A constant and high rate of O_2 uptake was found with added phosphate between 0.1 and 5 mM.

To explain this rapid decline, AZZONE AND ERNSTER postulate that the α-ketoglutarate-linked substrate-level phosphorylation operates with "compartmentalized ADP" as phosphate acceptor, yielding ATP which is not cleaved directly by the dinitrophenol-induced ATPase. Some inorganic phosphate is thus removed from the medium by Eqn. 1 and is not returned by Eqn. 2. When the amount of inorganic phosphate initially present in the reaction medium is less than that which is removed by Eqn. 1, this phosphate accumulates as "compartmentalized ATP", and the respiration declines owing to lack of inorganic phosphate.

In the present paper, we confirm by direct analysis, and by the stimulation of respiration brought about by the subsequent addition of phosphate after the decline has set in, that the decline is caused by the exhaustion of the inorganic phosphate. Our findings do not, however, support the view that the ATP formed by the substrate-linked phosphorylation is not accessible to the dinitrophenol-induced ATPase.

METHODS

Rat-liver mitochondria were isolated by the method of HOGEBOOM[13] exactly as described by MYERS AND SLATER[14].

O_2 uptakes were measured with differential manometers with a narrow capillary and small reaction flasks (gas volume, 6–8 ml). The standard reaction mixture contained 50 mM KCl, 25 mM Tris–HCl buffer (pH 7.5), 10 mM L-glutamate, 8 mM $MgCl_2$, 12.5 μM cytochrome *c*, 75 mM sucrose (derived from the mitochondrial suspension), 0.1 mM 2,4-dinitrophenol, and other additions as indicated in a final volume (after addition of the mitochondria) of 1 ml. The centre well was provided with 2 N

KOH and a roll of filter paper. At zero time 0.3 ml mitochondrial suspension was added and the flask attached to the manometer which was placed in a bath at 25°. The O_2 uptakes given on the figures have not been corrected for the uptake during the temperature-equilibrium period. For calculation of the P:O ratios this was calculated by extrapolation and added to the measured O_2 uptakes.

Mitochondrial protein was determined by the biuret method described by CLELAND AND SLATER[15].

Inorganic phosphate was determined on trichloroacetic acid (5 %) filtrates by LINDBERG AND ERNSTER'S[16] modification of the BERENBLUM AND CHAIN[17] method.

ADP, AMP and Tris were obtained from Sigma Chemical Co., cytochrome *c* was prepared by the method of MARGOLIASH[18], Amytal was purchased from the Amsterdam Quinine Factory. Other chemicals were obtained from British Drug Houses.

RESULTS

Fig. 1 illustrates the decline of respiration which sets in after 10–15 min when glutamate is oxidized by rat-liver mitochondria in the absence of added phosphate acceptor, in the presence of dinitrophenol and low concentrations of inorganic phos-

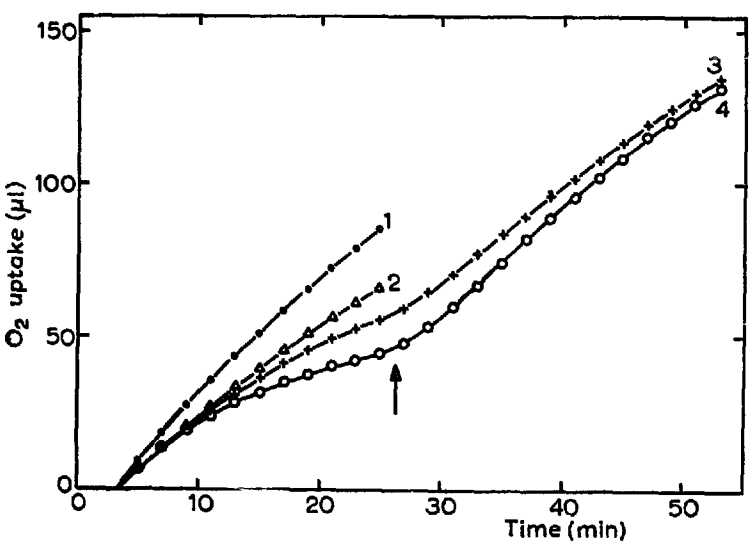

Fig. 1. Effect of P_i on oxidation of glutamate by rat-liver mitochondria in the presence of dinitrophenol. Standard reaction mixture contained 4.1 mg mitochondrial protein. Curve 1, 10 mM P_i initially present; curve 2, 100 µM P_i initially present; curve 3, 50 µM P_i initially present, 10 mM P_i added at arrow; curve 4, initially no added P_i, 10 mM P_i added at arrow.

TABLE I

DISAPPEARANCE OF INORGANIC PHOSPHATE DURING OXIDATION OF GLUTAMATE BY RAT-LIVER MITOCHONDRIA IN THE PRESENCE OF DINITROPHENOL

Same experiment as shown in Fig. 1.

Phosphate added (µM)	*Phosphate found (µM)*		*Disappearance of P_i (µM)*
	At zero time	*After 27 min*	
0	80	15	65
50	122	29	93
100	161	64	97

phate. The respiration was restored by tipping in phosphate after 27 min. The analyses given in Table I show that in the absence of added phosphate the mitochondria contributed about 80 μM phosphate to the medium (20 μmoles/g mitochondrial protein*). With no added phosphate or with 50 μM phosphate, the concentration

TABLE II

COURSE OF O_2 AND P_i UPTAKES DURING OXIDATION OF GLUTAMATE BY RAT-LIVER MITOCHONDRIA IN THE PRESENCE OF DINITROPHENOL

Standard reaction mixture. Replicate flasks were stopped at 0, 16, 30 and 46 min and the P_i contents determined on the trichloroacetic acid filtrates. 4.4 mg mitochondrial protein.

Time interval	*No added P_i*			*50 μM added P_i*		
	ΔO (μatoms)	*ΔP (μmoles)*	*P:O*	*ΔO (μatoms)*	*ΔP (μmoles)*	*P:O*
0–16 min	4.4	0.034	0.008	4.9	0.023	0.005
16–30 min	2.5	0.036	0.014	3.2	0.048	0.015
30–46 min	2.0	0.026	0.013	2.5	0.033	0.013

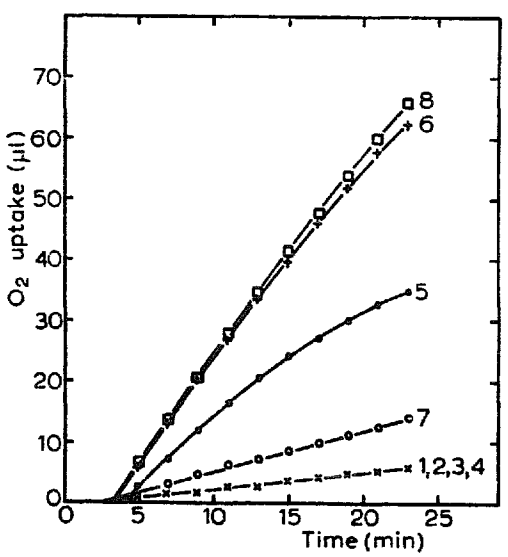

Fig. 2. The effect of AMP on the oxidation of glutamate by rat-liver mitochondria in the presence of dinitrophenol, with and without added phosphate. Standard reaction mixture without glutamate contained 3.7 mg mitochondrial protein. Curves 1–4, no glutamate; curves 5–8, 10 mM glutamate; curves 1 and 5, no further addition; curves 2 and 6, 0.5 mM P_i; curves 3 and 7, 1.0 mM AMP; curves 4 and 8, 0.5 mM P_i + 1.0 mM AMP. Curves 1–4 were practically identical; the mean is given.

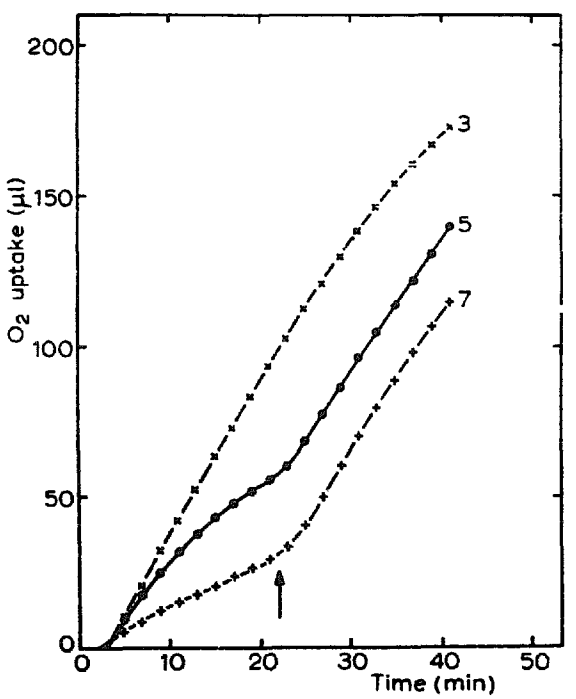

Fig. 3. Reversal by phosphate of inhibition by AMP of oxidation of glutamate by rat-liver mitochondria in the presence of dinitrophenol. Standard reaction mixture contained 5.3 mg mitochondrial protein. Curves 1–4, 10 mM P_i initially present; curves 5–8, no P_i initially present, 10 mM added at arrow; curves 1 and 5, no further addition; curves 2 and 6, 1 mM AMP; curves 3 and 7, 5 mM AMP; curves 4 and 8, 10 mM AMP. Curves 1–4 were practically identical and only 3 is shown. Curves 6–8 were practically identical and only 7 is shown.

* 4 preparations of freshly prepared mitochondria were found to contain 40–61 (mean 46) μmoles P_i/g protein. This was reduced to 3–5 (mean 4) μmoles P_i/g protein by pre-treatment with 2,4-dinitrophenol as described by AZZONE AND ERNSTER[12] (see also ref. 4).

declined to 15 μM and 29 μM, respectively, after 27 min. With 100 μM added phosphate, however, where there was little decline of respiration, there was still 64 μM present at the end of the experiment. These results indicate quite clearly that the decline of respiration is associated with the exhaustion of inorganic phosphate. Since the rate of O_2 uptake in this system was the same in the presence of 10 mM P_i*, or of 10 mM P_i + 5 mM ADP (see later) it is apparent that it is the concentration of phosphate rather than of phosphate acceptor which is rate-limiting.

The results shown in Table II indicate that the uptake of phosphate is not a rapid process confined to the initial stages of the reaction, but proceeds at an almost constant rate over 46 min (*cf.* Fig. 4 of ref. 12). Furthermore, the P:O ratio is of the order of 0.01–0.015, much lower than would be expected for the phosphorylation of ADP by the substrate-level phosphorylation of α-ketoglutarate oxidation, *viz.* 0.33**.

The addition of 1–10 mM AMP (Figs. 2 and 3) in the absence of added phosphate was found further to inhibit the oxidation of glutamate. In other experiments, similar results were obtained in the presence of 0.05 mM added P_i. Concentrations of AMP lower than 0.1 mM had little effect (Fig. 4). In the presence of 0.5–10 mM P_i, added AMP had no effect (Figs. 2 and 3). The inhibitory effect of AMP in the absence of phosphate was correlated with an increased disappearance of P_i as shown in Table III.

TABLE III

THE EFFECT OF AMP ON THE UPTAKE OF PHOSPHATE BY RAT-LIVER MITOCHONDRIA OXIDIZING GLUTAMATE IN THE PRESENCE OF DINITROPHENOL AND ON THE APPEARANCE OF PHOSPHATE IN THE ABSENCE OF SUBSTRATE

Standard reaction mixture except where glutamate was omitted as shown. 3.7 mg mitochondrial protein was used.

Added glutamate (mM)	*Added AMP (mM)*	*Added P_i (mM)*	*P_i found (μM)*		*ΔP_i (μM)*
			At zero time	*After 24 min*	
0	0	0	58	95	+37
0	0	0.5	580	650	
0	1	0	75	131	+56
0	1	0.5	650	670	
10	0	0	58	29	−29
10	0	0.5	600	610	
10	1	0	72	14	−57

It should be noted that incubation of the mitochondria with dinitrophenol in the absence of substrate leads to the formation of extra P_i, presumably by hydrolysis of endogenous organic phosphate compounds. This liberation of P_i was increased by the addition of AMP. It was not affected by the addition of 60 mM succinate + 1.7 mM Amytal (not shown in Table III (see AZZONE AND ERNSTER[12])).

Fig. 3 shows that the inhibition by AMP was completely relieved by the subsequent addition of phosphate.

* We did not observe the abrupt decline of respiration after about 25 min with 10 mM phosphate, reported by AZZONE AND ERNSTER[12].

** In the absence of hydrolytic side reactions, it would be expected that 1 molecule of ATP would be synthesized for each turn of the cycle represented by the sum reaction[5,6]

glutamate + 3 O → aspartate + CO_2.

Fig. 5 shows that ADP, unlike AMP, did not inhibit in the absence of P_i. On the contrary, the initial rate was maintained, probably due to the presence of small amounts of P_i in the ADP used, since the latter had no effect in the presence of added P_i. Thus, the concentration of endogenous phosphate acceptor is not a limiting factor for the oxidation of glutamate in our experiments.

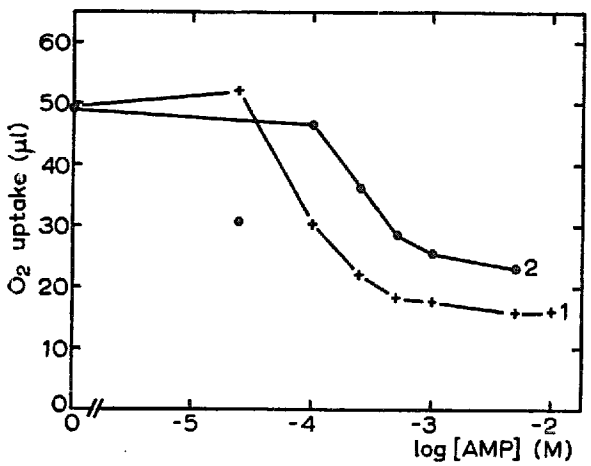

Fig. 4. The effect of various concentrations of AMP on the oxidation of glutamate by rat-liver mitochondria, in the presence of dinitrophenol. Two experiments are shown: Curve 1, 3.3 mg mitochondrial protein; curve 2, 3.5 mg mitochondrial protein. Standard reaction mixture; reaction time, 30 min.

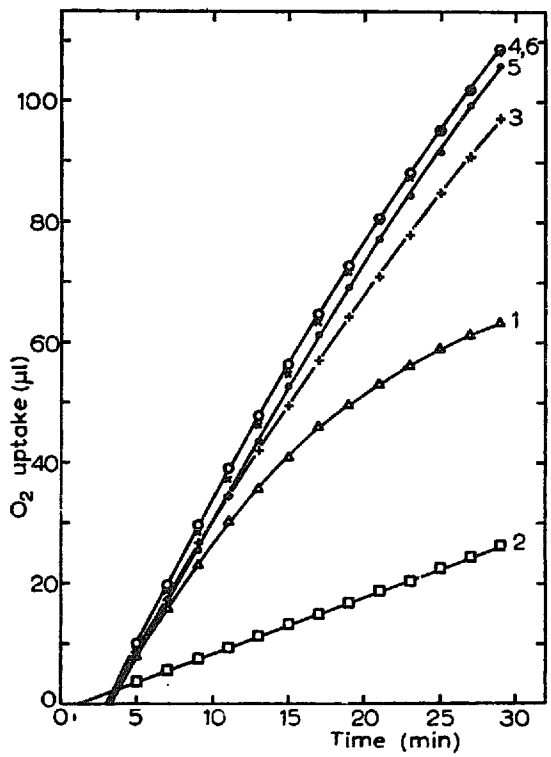

Fig. 5. Effect of ADP on oxidation of glutamate by rat-liver mitochondria in the presence of dinitrophenol. Standard reaction mixture contained 4.7 mg mitochondrial protein. Curve 1, no further addition; curve 2, 5 mM AMP; curve 3, 5 mM ADP; curve 4, 10 mM P_i; curve 5, 5 mM AMP + 10 mM P_i; curve 6, 5 mM ADP + 10 mM P_i.

DISCUSSION

We have confirmed by direct analysis AZZONE AND ERNSTER's[12] conclusion that the decline in the rate of oxidation of glutamate by rat-liver mitochondria in the presence of dinitrophenol and low concentrations of inorganic phosphate, and absence of phosphate acceptor, is due to the disappearance of phosphate. The nature of the phosphate compound formed remains to be discussed.

AZZONE AND ERNSTER suggest that it is the ATP synthesized by the substrate-linked phosphorylation. According to their explanation, this ATP is made in a compartment not directly accessible to the dinitrophenol-induced ATPase. In our view, there are two serious objections against this explanation: (a) The rate of depletion of inorganic phosphate should equal the rate of phosphorylation of ADP by the substrate-level phosphorylation of α-ketoglutarate oxidation. The data in Table III and in Fig. 4 of AZZONE AND ERNSTER show that it is much less. (b) If the formation of "compartmentalized ATP" leads to the depletion of P_i, in the presence of limiting amounts of P_i, it would be expected to lead to the depletion of ADP in the presence

of adequate amounts of P_i. Thus, one would expect to find the same decline of respiration at higher concentrations of P_i as found at lower. This was not the case in either AZZONE AND ERNSTER's experiments or our own.

In our opinion, the results are better explained by postulating a side reaction which slowly leads to the disappearance of phosphate. The phosphate which is present in the mitochondrial preparations or is liberated by incubation with dinitrophenol would be expected to be taken up by Eqn. 1 utilizing endogenous ADP, with a P:O ratio approaching 0.33. Most of the P_i would be immediately liberated by the dinitrophenol-induced ATPase (Eqn. 2). Some of the ATP can, however, react with a compound X by the slow reaction.

$$ATP + X \longrightarrow ADP + XP \qquad (3)$$

which leads to the removal of P_i from the system.

X could be a phosphate acceptor in the mitochondria or it could conceivably be P_i itself, inorganic pyrophosphate being formed by the reaction sequence

$$2\ ADP + 2\ P_i \longrightarrow 2\ ATP + 2\ H_2O \qquad (1)$$
$$ATP + R\cdot COOH + CoA \longrightarrow AMP + PP_i + R\cdot CO\sim CoA \qquad (4)$$
$$R\cdot CO\sim CoA + H_2O \longrightarrow R\cdot COOH + CoA \qquad (5)$$
$$AMP + ATP \longrightarrow 2\ ADP \qquad (6)$$
$$\textit{Sum}\ \ 2\ P_i \longrightarrow PP_i + H_2O$$

That endogenous fatty acids can be activated by ATP formed by oxidative phosphorylation has been made probable by the work of HÜLSMANN[19]. CROSS *et al.*[20] reported the formation of inorganic pyrophosphate during the oxidation of glutamate and other substrates by kidney or liver cyclophorase preparations in the presence of AMP and P_i.

It is even possible that X is a "compartmentalized ADP" as suggested by AZZONE AND ERNSTER[12]. Thus, it is conceivable that most of the GTP formed by the reaction

$$\text{Succinyl-CoA} + GDP + P_i \rightleftharpoons \text{succinate} + CoA + GTP \qquad (7)$$

reacts with ADP to form ATP accessible to the dinitrophenol-induced ATPase, but that some reacts more slowly with "compartmentalized ADP" to form "compartmentalized ATP" not accessible to the ATPase. AZZONE AND ERNSTER[12] have reported that when $^{32}P_i$ (20–50 μM) was added to rat-liver mitochondria oxidizing glutamate in the presence of dinitrophenol, 94% of the ^{32}P was recovered as organic P, and that chromatographic assay revealed that over 95% of the incorporated ^{32}P was in the form of ATP.

The exhaustion of P_i by AMP can be readily explained by the reaction sequence

$$ADP + P_i \longrightarrow ATP + H_2O \qquad (1)$$
$$ATP + AMP \longrightarrow 2\ ADP \qquad (6)$$
$$\textit{Sum}\ AMP + P_i \longrightarrow ADP + H_2O$$

In one respect, our results differ from those of AZZONE AND ERNSTER, who found a marked stimulation of the oxidation of glutamate by concentrations of AMP between 0.01 mM and 1 mM. Above 1 mM AMP, lower rates of oxidation were obtained, but even with 10 mM AMP the rate was still greater than in the absence of AMP. We found only a slight stimulation by low concentrations of AMP (see Fig. 4) even when low concentrations of P_i (0.01–0.03 mM, see Table VI of AZZONE AND ERNSTER) were also added, and a marked inhibition by higher concentrations of AMP. We are unable to explain this discrepancy.

AZZONE AND ERNSTER give no explanation for their finding that 10 mM AMP gave substantially lower rates of oxidation than 1 mM. They explain the stimulation by lower concentrations as "due to a cleaving of mitochondrial ATP, via the myokinase reaction, thus transferring ATP from one mitochondrial compartment to another, and thereby rendering it more accessible to hydrolytic breakdown". A large stimulation by AMP would indeed be more easily explained by AZZONE AND ERNSTER's mechanism than by our own, but despite repeated attempts we have been unable to find this stimulation.

In conclusion, our results do not support the interpretation of the decline of the rate of oxidation of glutamate in terms of compartmentation of the ATP formed in the substrate-linked phosphorylation reaction, as proposed by AZZONE AND ERNSTER[12]. Our results do not, of course, disprove the general concept of compartmentation of mitochondrial ATP, which has received experimental support by, for example, SIEKEVITZ[21] and HEMKER[22].

NOTE ADDED IN PROOF

The experiments of CHAPPELL AND GREVILLE[8] have also provided important evidence that the ATP formed in the substrate-linked phosphorylation can be hydrolysed by the dinitrophenol-induced ATPase. It was shown that ADP is necessary for maximal oxidation of glutamate in the presence of P_i and dinitrophenol when oligomycin was also added, but not when oligomycin was absent. Since oligomycin inhibits the dinitrophenol-induced ATPase[23], CHAPPELL AND GREVILLE concluded that "in absence of oligomycin, dinitrophenol ensures a supply of ADP from the endogenous adenine nucleotide adequate for the needs of the succinyl coenzyme A synthetase". We have obtained similar results under the conditions of our experiments. Using our standard reaction mixture and 4.6 mg mitochondrial protein, the O_2 uptake in 30 min was 67 μl with no further additions, 93 μl with 10 mM P_i, 66 μl with 0.1 mM ADP, glucose and hexokinase, and 98 ml with P_i, ADP, glucose and hexokinase. When 10 μg oligomycin was also present, the O_2 uptakes were 41, 48, 34 and 94 μl, respectively. Thus, in the presence of oligomycin and dinitrophenol, both P_i and ADP are necessary for maximal oxidation.

Received January 22nd, 1963

ACKNOWLEDGEMENT

This work was supported in part by the Life Insurance Medical Research Fund.

REFERENCES

[1] L. J. TEPLY, *Arch. Biochem. Biophys.*, 24 (1949) 383.
[2] J. D. JUDAH, *Biochem. J.*, 49 (1951) 271.
[3] P. BORST AND E. C. SLATER, *Nature*, 184 (1959) 1396; 185 (1960) 537.
[4] P. BORST AND E. C. SLATER, *Biochim. Biophys. Acta*, 48 (1961) 362.
[5] P. BORST AND E. C. SLATER, *Biochim. Biophys. Acta*, 41 (1960) 170.
[6] P. BORST, *Biochim. Biophys. Acta*, 57 (1962) 256.
[7] H. A. KREBS AND D. BELLAMY, *Biochem. J.*, 75 (1960) 523.
[8] J. B. CHAPPELL AND G. D. GREVILLE, *Nature*, 190 (1961) 502.
[9] E. A. JONES AND H. GUTFREUND, *Biochem. J.*, 79 (1961) 608.
[10] F. E. HUNTER, in W. D. MCELROY AND B. GLASS, *Symp. on Phosphorus Metabolism*, Vol. 1, Johns Hopkins Press, Baltimore, 1951, p. 297.
[11] H. A. LARDY AND H. WELLMAN, *J. Biol. Chem.*, 195 (1952) 215.
[12] G. F. AZZONE AND L. ERNSTER, *J. Biol. Chem.*, 236 (1961) 1501.
[13] G. H. HOGEBOOM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, Inc., New York, 1955, p. 16.
[14] D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
[15] K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
[16] O. LINDBERG AND L. ERNSTER, *Methods Biochem. Anal.*, 3 (1956) 8.
[17] I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
[18] E. MARGOLIASH, *Biochem. J.*, 56 (1954) 529.
[19] E. C. SLATER AND W. C. HÜLSMANN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1109.
[20] R. J. CROSS, J. V. TAGGART, G. A. COVO AND D. E. GREEN, *J. Biol. Chem.*, 177 (1949) 655.
[21] P. SIEKEVITZ, *Ciba Foundation Symp. on the Regulation of Cell Metabolism, 1958*, Churchill, London, 1959, p. 129.
[22] H. C. HEMKER, *Het mechanisme van de werking van onthoppelende fenolen op de ademhalings-ketenfosforylering, M.D. thesis*, Klein Offset Drukkerij, Amsterdam, 1962.
[23] H. A. LARDY, D. JOHNSON AND W. MCMURRAY, *Arch. Biochem. Biophys.*, 78 (1958) 587.