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UNCOUPLING OF RESPIRATORY-CHAIN PHOSPHORYLATION BY ARSENATE

H. F. TER WELLE AND E. C. SLATER

Laboratory of Biochemistry, B. C. P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

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SUMMARY

1. The specific uncoupling by arsenate of oxidative phosphorylation in rat-liver mitochondria has been studied in EDTA-containing medium. The K_m for arsenate for stimulation of the oxidation of succinate in phosphate-free medium is about 1 mM.

2. Arsenate-stimulated respiration is inhibited by low concentrations of phosphate, in the absence of ADP. The effects of arsenate and phosphate are competitive. A phosphate:arsenate ratio of 0.1 inhibits completely. In the presence of ADP, an arsenate:phosphate ratio of 5–10 is necessary for a decrease of the P:O ratio by 50 %.

3. In the absence of phosphate, ADP stimulates the arsenate-induced respiration by 60 %. Atractyloside has no effect on the ADP-independent respiration, but stops the stimulation by ADP, whether added before or after the ADP.

4. Low concentrations of aurovertin inhibit the arsenate-induced respiration to the same extent as oligomycin. However, with 9 μg aurovertin per mg protein, in the presence of arsenate, the respiration is greater than with low concentrations. This extra respiration is inhibited by oligomycin or by phosphate.

5. The arsenate-induced ATPase amounts to only about 15 nmoles phosphate per mg protein per min (*cf.* 20 for endogenous ATPase, and 300 for 2,4-dinitrophenol-induced ATPase, in the same units). The arsenate-induced ATPase is inhibited by oligomycin, but not by azide.

6. It is concluded that stimulation by ADP of arsenate-induced respiration is not due to removal by ADP of endogenous phosphate. Nor is inhibition by phosphate due to removal of ADP.

7. It is concluded that the effects of arsenate provide strong evidence in favour of the existence of a $\sim$P intermediate of oxidative phosphorylation, either containing phosphate alone (X $\sim$P) or containing ADP as well as phosphate (ADP–X $\sim$P).

8. The different effects of oligomycin on dinitrophenol- and arsenate-induced respiration support the proposition that there are at least two non-phosphorylated high-energy intermediates (or one intermediate with two sites) in the sequence of energy-conserving reactions leading from the oxidoreduction reaction of the respiratory chain to ATP.

Abbreviation: PEP, phosphoenolpyruvate.

* Postal address: Plantage Muidergracht 12, Amsterdam.

INTRODUCTION

Because of its chemical resemblance to phosphate, arsenate has been widely used in biochemistry since HARDEN AND YOUNG[1] in 1911 showed that it stimulates alcoholic fermentation in yeast juice. With arsenate, unlike phosphate, the enhanced fermentation continued long after one equivalent of CO_2 had been evolved and arsenate was recovered unchanged at the end of the experiment. They examined but rejected the possibility that the effect of arsenate is due to the formation of an unstable arsenate ester, and proposed instead that arsenate stimulates the breakdown of hexose diphosphate. This was not supported by further work and in 1931 BRAUNSTEIN[2] revived the idea of the formation of an unstable arsenate ester intermediate of fermentation or glycolysis. More recently, arsenate has been shown to react in place of phosphate with a number of enzymes, such as glyceraldehydephosphate dehydrogenase (EC 1.2.1.12)[3,4], sucrose phosphorylase (EC 2.4.1.7)[5], α-glucan phosphorylase (EC 2.4.1.1)[6] and succinyl-CoA synthetase (EC 6.2.1.4)[7]. The organic arsenate compounds arising from all these reactions are very unstable in aqueous medium, so that the final result is an arsenolysis of the substrates reacting with these enzymes[8]. Thus arsenate is an effective uncoupler of the two so-called substrate-linked oxidative phosphorylations in animal tissues.

CRANE AND LIPMANN[9] showed in 1953 that arsenate is also an uncoupler of respiratory-chain phosphorylation. In particular, they showed that arsenate stimulated the respiration of a cyclophorase preparation in the absence of added P_i or phosphate acceptor. The same result was obtained by BORST AND SLATER[10] with rat-liver mitochondria depleted of endogenous P_i by prior incubation with substrate, ADP, glucose and hexokinase. Although these results show that, under the conditions of these experiments, arsenate acts as an uncoupler of oxidative phosphorylation, it has rather little effect on the P:O ratio when added to a reaction mixture containing ADP and P_i, at least in short-time experiments (*cf.* refs. 9, 11, 12), suggesting a competition between P_i and arsenate (*cf.* ref. 9) in oxidative phosphorylation. However, AZZONE AND ERNSTER[12] found no competition under their conditions. Conditions for demonstrating competition are examined in this paper. Competition between P_i and arsenate has also been clearly demonstrated by CHIGA AND PLAUT[13] for the ATP–P_i exchange enzyme isolated from pig-liver mitochondria, and by AVRON AND JAGENDORF[14] for light-induced phosphorylation in chloroplasts.

The uncoupling activity of arsenate on respiratory-chain phosphorylation became particularly interesting when ESTABROOK[15] and HUIJING AND SLATER[16] showed that arsenate-stimulated respiration, in contrast to that stimulated by dinitrophenol, is inhibited by oligomycin. Both groups (see also ref. 17) concluded in 1961 that dinitrophenol and arsenate (and, therefore, presumably phosphate) act at different sites in the sequence of energy-conserving reactions leading from the oxidoreduction reaction of the respiratory chain to ATP, *i.e.* that there are at least two high-energy non-phosphorylated intermediates in this sequence. This conclusion will also be examined in the light of the experiments reported here and in recent papers by other investigators.

Preliminary accounts of parts of this work have appeared[18,19].

METHODS

Rat-liver mitochondria were isolated according to the method of HOGEBOOM[20], exactly as described by MYERS AND SLATER[21]. O_2 uptake was measured polarographically with a vibrating oxygen electrode, using the Gilson Medical Electronics Oxygraph. The standard reaction mixture contained 15 mM KCl, 50 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA, 2.5 mM $MgCl_2$, 60 mM succinate and 0.1 μg rotenone, in a total volume of 2.0 ml. The reaction temperature was 25°.

P:O ratios were measured by the method of CHANCE AND WILLIAMS[22], with AMP as phosphate acceptor. ATP (0.1 mM) was also added in order to abolish a slight time-lag. The AMP concentration was determined spectrophotometrically using a millimolar extinction coefficient at 260 mμ of 15.4. Protein was determined according to CLELAND AND SLATER[23].

Except where otherwise stated, ATPase activity was measured in a reaction mixture containing 75 mM KCl, 0.5 mM EDTA, 68 mM sucrose, 50 mM Tris–HCl buffer (pH 7.4) and 2 mM ATP. The final volume was 1.5 ml. The reaction temperature was 25°. Phosphate was determined by a slight modification of the method of LINDBERG AND ERNSTER[24]. In some experiments, the ATPase activity was followed by measuring the H^+ production with an automatic titrator (Radiometer), using a vessel closed to air. ATPase activity was calculated on the assumption that 0.89 mole H^+ is liberated for each mole of ATP hydrolysed (*cf.* ref. 25). The reaction mixture was the same as above, except that 112 mM sucrose was used.

ATPase activity was also measured in some experiments by the method of GATT AND RACKER[26], with 3.3 mM phosphoenolpyruvate (PEP), 2 mM ATP, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 75 mM KCl, 68 mM sucrose, 50 mM Tris–HCl buffer (pH 7.4) and 75 μg pyruvate kinase (EC 2.7.1.40).

Oligomycin, kindly supplied by Upjohn Chemical Co., was added in 96% ethanol. Atractyloside, kindly supplied by Professor V. SPRIO, was added in 50% ethanol. Aurovertin, kindly supplied by Professor H. A. LARDY, was added in 96% ethanol. Rotenone was obtained from S. B. Penick & Co.; sodium succinate and pyruvate kinase from Boehringer und Soehne; AMP, ADP, ATP and PEP from Sigma Chemical Co.; sodium arsenate and 2,4-dinitrophenol from British Drug Houses.

RESULTS

Effect of arsenate on rate of oxidation of succinate in phosphate-free medium

A typical experiment shown in Fig. 1 illustrates (i) stimulation by arsenate of respiration in a phosphate-free medium; (ii) further stimulation by ADP (*cf.* refs. 14, 27); (iii) complete sensitivity of the arsenate-stimulated respiration to oligomycin; (iv) relief of oligomycin inhibition by dinitrophenol. The stimulation of respiration by arsenate alone reaches its maximum within 30 sec. EDTA could be omitted from the reaction mixture when the order of addition was the same as that given in Fig. 1. However, if the mitochondrial preparation is pre-incubated with 40 mM arsenate for 12 min before addition of succinate, the respiration is insensitive to oligomycin, unless EDTA is present in the reaction mixture (Fig. 2). In some experiments, incubation with arsenate in the absence of EDTA caused considerable inactivation of succinate oxidation (*cf.* refs. 28, 29). The oligomycin-insensitive respiration in the

absence of EDTA is very likely due to structural damage to the mitochondria caused by the arsenate (*cf.* refs. 30, 31).

Fig. 3 shows the effect of different concentrations of arsenate, both in the presence and absence of ADP, on the rate of oxidation of succinate. The K_m is about 1 mM arsenate both in the presence and absence of ADP (*cf.* 2.0 mM in absence

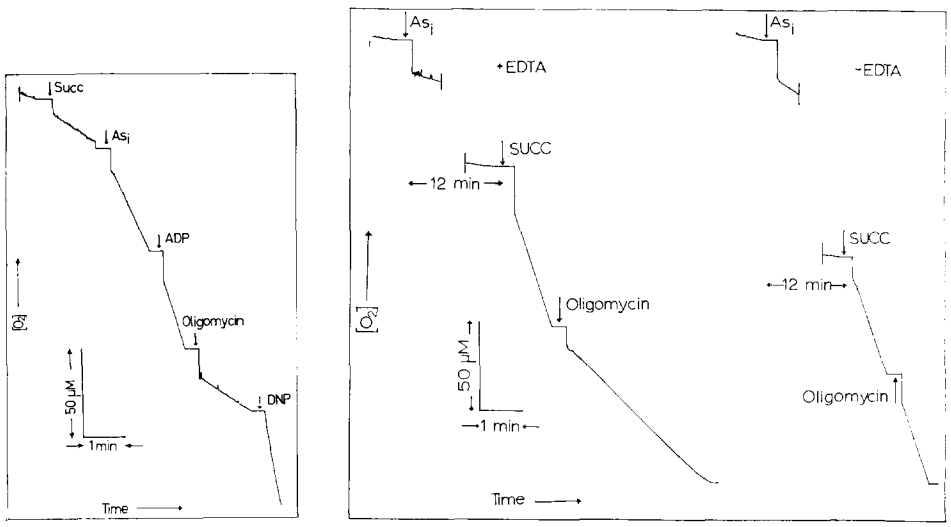


Fig. 1. Effect of ADP, oligomycin and 2,4-dinitrophenol on arsenate-induced respiration. Reaction medium as described under METHODS. Additions were: succinate (Succ), 60 mM; arsenate (As_i), 40 mM; ADP, 0.05 mM; oligomycin, 0.43 µg/mg protein; dinitrophenol (DNP), 0.1 mM. Mitochondrial protein, 1.13 mg/ml. The oxygen concentration was not registered while additions were made.

Fig. 2. Influence of pre-incubation with arsenate, with and without EDTA, on the oligomycin sensitivity of respiration. Reaction medium as described under METHODS. Additions were: arsenate (As_i), 40 mM; EDTA, 1 mM; succinate (Succ), 60 mM; oligomycin, 0.72 µg/mg protein. Mitochondrial protein, 1.4 mg/ml.

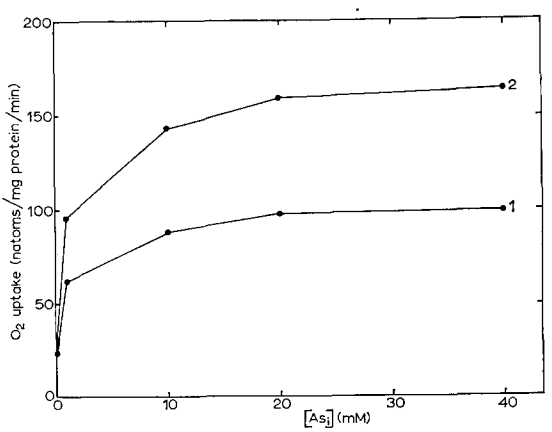


Fig. 3. Influence of ADP on the arsenate-induced respiration. Reaction medium as described under METHODS. Curve 1, no further additions; Curve 2, with 0.05 mM ADP. Mitochondrial protein, 0.94 mg/ml.

and presence of ADP, reported by ERNSTER, LEE AND JANDA[32], 0.3 mM in presence of ADP reported by ESTABROOK AND ITADA[27], and 0.7 mM in the absence of ADP reported by LEHNINGER AND GREGG[33] for digitonin particles). At all concentrations of arsenate, ADP stimulated about 60 %. A concentration of 25 μM ADP was sufficient for maximal effect. The maximum rate reached with 40 mM arsenate and ADP was about 80 % of that reached with P_i and ADP.

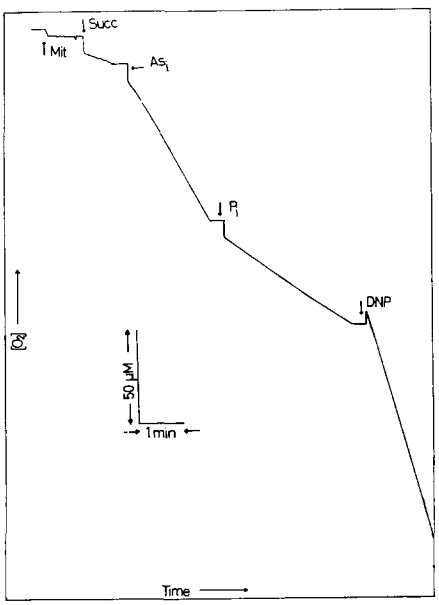


Fig. 4. Influence of phosphate on arsenate-induced respiration. Reaction medium as described under METHODS. Additions were: arsenate (As_i), 20 mM; P_i, 1 mM; dinitrophenol (DNP), 0.1 mM. Mitochondrial (Mit) protein, 0.86 mg/ml.

TABLE I

UNCOUPLING BY ARSENATE AND REVERSAL BY PHOSPHATE

Standard reaction mixture. Mitochondrial protein, 0.86 mg/ml.

As_i (*mM*)	P_i (*mM*)	$[P_i]:[As_i]$	$-\Delta O$ (*natoms/mg protein per min*)		
			P_i *alone*	As_i *alone*	$As_i + P_i$
0.5	1	2	29	47	30
0.5	20	40	(33)*	48	34
1	1	1	29	56	28
1	20	20	(33)*	51	34
10	1	0.1	29	86	32
20	1	0.05	29	93	37
40	0.5	0.0125	25	102	49
40	1	0.025	29	98	39
40	5	0.125	34	98	34
40	10	0.25	32	101	32

* Extrapolated from values obtained with 1–10 mM P_i.

Effect of phosphate on arsenate-induced respiration

The arsenate-stimulated respiration, measured in the absence of ADP, is inhibited by the subsequent addition of low concentrations of P_i (Fig. 4). Complete inhibition of stimulation by arsenate is obtained at $[P_i]$:[arsenate] ratios of about 0.1 or higher (Table I). A ratio as low as 1:80 gives an inhibition of more than 50 % (*cf.* ref. 18). ERNSTER, LEE AND JANDA[32] have reported similar data, and have calculated a K_m for arsenate of 2.2 mM and a K_i for P_i of 76 μM, *i.e.* in their experiments $[P_i]$:[arsenate] = 1:30 for 50 % inhibition of arsenate stimulation.

Effect of arsenate on oxidative phosphorylation

The effect of different concentrations of arsenate, at four different phosphate concentrations, on oxidative phosphorylation is shown in Fig. 5. (In this experiment, 20 mM P_i gave an appreciably higher P:O ratio in the absence of arsenate than 5 mM or 10 mM. This was not normally the case.) It is clear that uncoupling by arsenate is much greater at the low phosphate concentrations. An arsenate:P_i ratio of 5–10 is necessary for a decrease of the P:O ratio by 50 %. ERNSTER, LEE AND JANDA[32] have reported a K_m for arsenate uncoupling of 2.2 mM and a K_i for P_i of 1.2 mM in similar experiments, *i.e.* 50 % uncoupling would be obtained in their experiments with an [arsenate]:$[P_i]$ ratio of about 2.

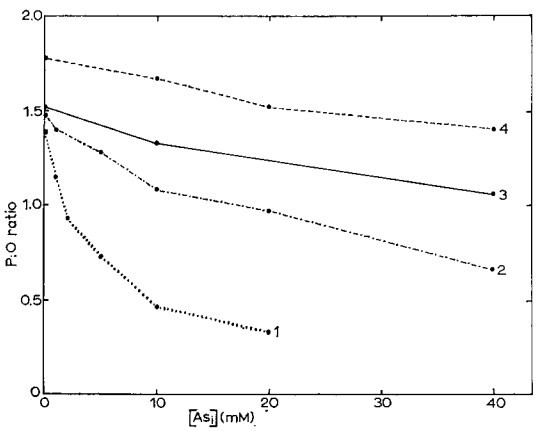


Fig. 5. Influence of arsenate on P:O ratios with succinate as substrate. Reaction medium as described under METHODS. Phosphate acceptor, 0.2 μmole ATP and 0.275 μmole AMP, except with 1 mM P_i, 0.1375 μmole AMP. Mitochondrial protein, 1.16 mg/ml. Curve 1, 1 mM P_i; Curve 2, 5 mM P_i; Curve 3, 10 mM P_i; Curve 4, 20 mM P_i.

Effect of atractyloside, aurovertin and Dio-9 on the arsenate-stimulated respiration

As was to be expected, now that it is known that atractyloside has no effect on oxidative-phosphorylation reactions within the mitochondrion but affects the reaction of this system with added ADP (refs. 34–36), atractyloside (10 μmoles/g protein) had no effect on the arsenate-stimulated respiration, but prevented the further stimulation of the respiration by added ADP, and inhibited the ADP-stimulated respiration when added after the ADP (*cf.* refs. 36, 37). This was also the case when the mitochondria were pre-incubated with glucose, hexokinase and ADP to remove endogenous P_i (ref. 10; *cf.* ref. 37).

In agreement with BRUNI AND AZZONE[38], atractyloside (20 μmoles/g protein), in the absence of EDTA, caused a very rapid increase in the respiration in the presence of arsenate. Since this respiration was oligomycin-insensitive, it was probably caused by structural damage to the mitochondria. EDTA prevented this effect of atractyloside (*cf.* ref. 39).

Low concentrations of aurovertin inhibit the oxidation of succinate, in the presence of ADP and P_i ("State 3") or 20–40 mM arsenate, to the same extent as oligomycin*. With higher concentrations of aurovertin (8.7 μg/mg protein = 17.8 μmoles/g protein), however, the arsenate-induced respiration is much greater than with lower concentrations. This increased respiration is sensitive to oligomycin. State-3 respiration remains inhibited with high aurovertin concentrations.

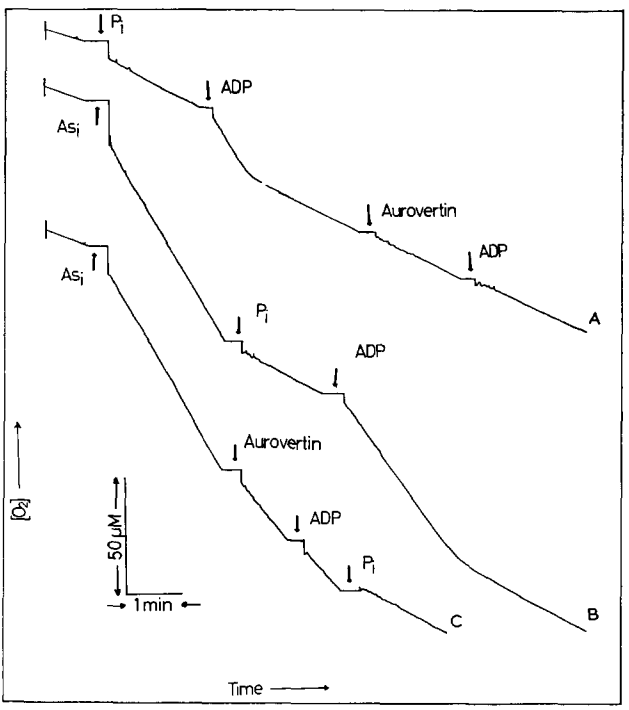


Fig. 6. Inhibition by phosphate of arsenate-induced respiration, in the presence of ADP. Reaction medium as described under METHODS. Curve A: P_i, 4 mM; ADP, 0.1 mM; aurovertin, 8.7 μg/mg protein. Curve B: arsenate (As_i), 20 mM; P_i, 4 mM; ADP, 0.1 mM. Curve C: arsenate (As_i), 20 mM; aurovertin, 8.7 μg/mg protein; ADP, 0.1 mM; P_i, 4 mM. Mitochondrial protein, 1.15 mg/ml protein.

The reason for the effect of high concentrations of aurovertin on arsenate-induced respiration is not known. It was much less marked with 2.5 mM arsenate. However, it provided the opportunity of showing that, under conditions in which ADP cannot be phosphorylated, phosphate still inhibits the arsenate-stimulated respiration. Fig. 6, Curve A, shows the normal stimulation of respiration by ADP and the inhibition of this stimulation by the prior addition of 8.7 μg aurovertin per

* The oligomycin-insensitive arsenate-induced respiration found in the absence of EDTA was also insensitive to low concentrations of aurovertin.

mg protein. Curve B shows the inhibition of arsenate-induced respiration by P_i([P_i]: [arsenate] = 0.2) and the stimulation by ADP. The amount of oxygen taken up during "State 3" respiration is 2.7 times that in Curve A, *i.e.* this concentration of arsenate has inhibited phosphorylation by 63 %. Curve C shows that 8.7 μg aurovertin per mg protein inhibits the arsenate-induced respiration only partially and the addition of ADP has no effect. However, the arsenate-induced respiration is completely inhibited by the subsequent addition of P_i.

Table II shows that aurovertin does not act by preventing the exchange of endogenous and added adenine nucleotide, as does atractyloside[40]. The fact that phosphate completely inhibits the arsenate-induced respiration, even in the presence of 8.7 μg aurovertin per mg protein, shows that aurovertin does not prevent the entry of phosphate into the mitochondria, as suggested by CHAPPELL AND CROFTS[41].

TABLE II

INFLUENCE OF AUROVERTIN AND ATRACTYLOSIDE ON THE EXCHANGE OF ENDOGENOUS ADENINE NUCLEOTIDES WITH ADDED ADP

Mitochondria (2.5 mg protein per ml) were incubated at 0° in 20 mM Tris–HCl buffer (pH 7.4), 1 mM EDTA, 125 mM sucrose and 2.7 μM [^{14}C]ADP (180000 disint./min). Final volume, 2 ml. After 5 min, the suspension was centrifuged at 12500 × *g* for 10 min, and the radioactivity of the supernatant determined in a liquid-scintillation counter (Nuclear-Chicago).

Conditions	*Total radioactivity in supernatant (disint./min)*
Zero time*	180000
Complete system	2030
Complete system + atractyloside (20 μg/mg protein)	171000
Complete system + aurovertin (0.4 μg/mg protein)	877
Complete system + aurovertin (12 μg/mg protein)	845

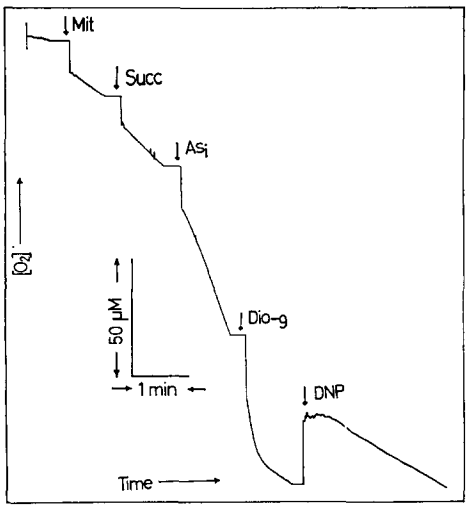
* ADP added after centrifugation.

GUILLORY[42] has shown that, in the presence of phosphate, the antibiotic Dio-9, after an initial stimulation of the respiration in an ADP-deficient medium, causes an inhibition of the respiration that is not relieved by dinitrophenol. In the absence of phosphate, only the stimulation was seen. Fig. 7 shows that arsenate can substitute for phosphate in this reaction (*cf.* Fig. 7 with Fig. 1 of GUILLORY[42]).

The arsenate-induced ATPase

A characteristic of uncouplers is that they induce an ATPase in mitochondria[43–45], and this has also been reported for arsenate by WADKINS[46], who found that 50 mM arsenate induced an ATPase in rat-liver mitochondria equal to 75 % of the activity obtained with 2,4-dinitrophenol. Similar results were obtained with digitonin fragments, with 10 mM arsenate in the presence of 2 mM $MgCl_2$. AZZONE AND ERNSTER[12] reported that a low concentration (3 mM) of arsenate caused an ATPase activity equal to 30 % of that obtained with dinitrophenol.

In preliminary experiments it was found that the degree of stimulation of the ATPase appeared to be markedly dependent on the protein concentration, being much greater with higher protein concentration (Fig. 8). This is because, in the absence of



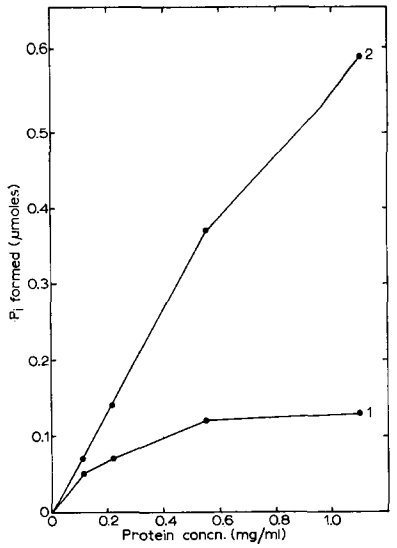


Fig. 7. Effect of Dio-9 on the arsenate-induced respiration. Additions were: mitochondria (Mit), 1.4 mg protein per ml; succinate (Succ), 60 mM; arsenate (As_i), 40 mM; Dio-9, 43 μg/mg protein; dinitrophenol (DNP), 0.1 mM. (The apparent increase in O_2 concentration after addition of dinitrophenol is an instrumental artefact.)

Fig. 8. Influence of protein concentration on the ATPase activity with and without arsenate. Curve 1, no further additions to reaction medium described under METHODS; Curve 2, with 5 mM arsenate. Reaction time, 15 min.

arsenate, the amount of P_i liberated from ATP is little affected by protein concentration between 0.1 and 1 mg/ml, whereas in the presence of arsenate the amount liberated is linear with protein concentration, at least up to 0.5 mg protein per ml. Similarly, the reaction was linear with time up to 15 min in the presence of arsenate (with up to 2.1 mg protein per ml), as measured by H^+ production, but the rate fell rapidly with time in the absence of arsenate. Clearly, the method of measuring the endogenous ATPase of mitochondria is unsatisfactory, since it takes no account of resynthesis of ATP brought about by the oxidation of endogenous substrate. In agreement with GATT AND RACKER[26], the endogenous ATPase was much greater when measured by the hydrolysis of PEP in the presence of pyruvate kinase and ADP as catalysts, and the activity was proportional to the protein concentration (between 0.18 and 0.92 mg/ml). With this system, 5 mM arsenate was found to stimulate the endogenous ATPase only slightly. Concomitant oxidative phosphorylation does not affect the ATPase measured by the method of GATT AND RACKER[26].

Oxidative phosphorylation may also be eliminated by carrying out the reaction in the presence of 1 mM cyanide. Fig. 9 shows an experiment carried out with the titrator. Cyanide stimulates H^+ production in the absence of arsenate, but has little effect in its presence. Curve 2 represents the true endogenous ATPase activity (initial rate 18 nmoles P_i per mg protein per min) and the difference between the rates in Curve 4 (32 nmoles P_i per mg protein per min) and Curve 2, *i.e.* 14 nmoles P_i per mg protein per min, represents the ATPase activity induced by 5 mM arsenate. In a similar experiment already reported in a preliminary note[18], the arsenate-induced ATPase equalled 28—22 = 6 nmoles P_i per mg protein per min.

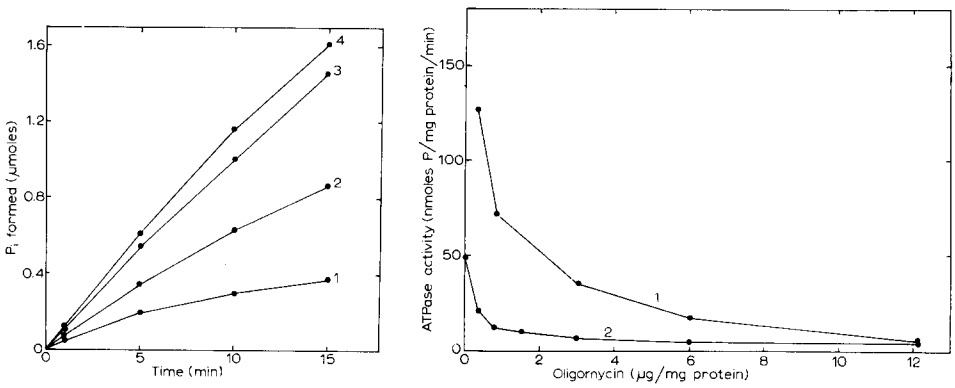


Fig. 9. Influence of KCN on ATPase activity, followed with automatic titrator. Reaction medium as described under METHODS. Curve 1, no additions; Curve 2, 1 mM KCN; Curve 3, 5 mM arsenate; Curve 4, 1 mM KCN + 5 mM arsenate. Mitochondrial protein, 1.93 mg/ml.

Fig. 10. Influence of oligomycin on the ATPase activity induced by arsenate or dinitrophenol. Reaction medium as described under METHODS. Curve 1, 0.1 mM dinitrophenol; Curve 2, 40 mM arsenate. Reaction time, 15 min. Mitochondrial protein, 0.43 mg/ml. The point with dinitrophenol and without oligomycin was omitted to avoid underestimation during a 15-min incubation.

These experiments were all carried out in the presence of 0.5 mM EDTA. The omission of EDTA had little effect with 5 mM arsenate, but with 40 mM it caused a decline in the initial activity and a stimulation (up to 3-fold) at 15 min.

Varying the ATP concentration between 2 and 8 mM ATP had little effect on the ATPase activity in the presence of arsenate. Addition of $MgCl_2$ to the assay medium also had little effect.

The arsenate-induced ATPase was inhibited by oligomycin to about the same extent as the dinitrophenol-induced ATPase (Fig. 10; *cf.* ref. 12). However, in our hands, azide, which induces an ATPase[47,48] and more slowly[48] inhibits the dinitrophenol-induced ATPase[47–50], had no effect on the arsenate-induced ATPase, except for a slight stimulation at 5 mM azide. This is in disagreement with the results reported by WADKINS[46] (60 % inhibition with 0.5 mM azide, 75 % inhibition with 5 mM azide).

DISCUSSION

Two effects of arsenate on oxidative phosphorylation and associated reactions must be distinguished: (i) a specific uncoupling of oxidative phosphorylation characterized by an oligomycin-sensitive stimulation of the oxidation of succinate in the absence of P_i, and a slow hydrolysis of added ATP, both reactions proceeding at a steady rate; (ii) a non-specific uncoupling of oxidative phosphorylation, characterized by an oligomycin-insensitive stimulation of the oxidation of succinate in the absence of P_i, and a more rapid hydrolysis of added ATP, both reactions increasing with time. Under our conditions, the second reaction could be prevented by the addition of 0.5–1 mM EDTA to the reaction medium. The oligomycin-insensitive respiration was greatly increased by the addition of atractyloside in the absence of EDTA. The subsequent discussion will be restricted to the specific uncoupling.

The most important features of this uncoupling by arsenate are: (i) that maxi-

mal stimulation of respiration by arsenate in the absence of phosphate requires the addition of ADP (refs. 14, 15); (ii) the reversal by phosphate of stimulation of respiration by arsenate[9]; (iii) the very low arsenate-induced ATPase in comparison with the dinitrophenol-induced ATPase and the respiratory rate in the presence of arsenate and ADP (Table III).

TABLE III

RELATIVE RATES OF SOME MITOCHONDRIAL REACTIONS

The rates refer to rat-liver mitochondria. Temperature, 25°.

Reaction	*Rate (μmoles ATP or μatoms O per mg protein per min)*
Respiratory rate*: ADP + P_i	0.18
: ADP + arsenate	0.15
Endogenous ATPase	0.02
Arsenate**-induced ATPase***	0.015
Dinitrophenol-induced ATPase***	0.30
ATP–ADP exchange§	0.04

* Succinate in the presence of rotenone.
** 5 mM.
*** Subtracting the endogenous ATPase.
§ Oligomycin-sensitive. Determined according to GUILLORY AND SLATER[51], except that 5 mM EDTA was used, instead of 10 mM.

The possibility that the first two features are interrelated should be considered. Indeed, BRUNI *et al.*[37] suggested that stimulation by ADP of the arsenate-induced respiration is due to removal by ADP of endogenous P_i. Alternatively, inhibition by P_i could conceivably be due to removal of ADP, that is required for uncoupling by arsenate[36]. That the stimulation by ADP is not due to removal of P_i was shown by an experiment of CHAPPELL AND CROFTS[36], in which atractyloside was found to reverse the stimulation by ADP when added after the ADP. We have confirmed this using larger concentrations of arsenate (10–40 mM) than those used by CHAPPELL AND CROFTS[36] (1.2 mM). Moreover, rat-liver mitochondria pre-incubated with glucose, ADP and hexokinase to remove endogenous P_i (ref. 10) showed the same responses to successive additions of arsenate, ADP and atractyloside as normally prepared mitochondria (*cf.* ref. 37).

That the inhibition by P_i is not due to removal of ADP is strongly suggested by the experiment given in Fig. 6, in which P_i still inhibits the arsenate-induced respiration when the phosphorylation of ADP is prevented by the addition of aurovertin. The demonstration by ERNSTER, LEE AND JANDA[32] that inhibition of arsenate-induced respiration by P_i is not associated with an appreciable phosphorylation of endogenous ADP points in the same direction.

It is now generally accepted that phosphate inhibits the arsenate-induced respiration by competing with arsenate for a site in the oxidative phosphorylation sequence[9,18,32]. The nature of this competition will now be discussed. For the purpose of this discussion, we may write oxidative phosphorylation as

$$P_i + ADP \xrightarrow{\text{(respiration)}} ATP \qquad (1)$$

Most theories of oxidative phosphorylation, including the chemiosmotic theory of MITCHELL[52] but not that of WILLIAMS[53,54], include a phosphorylated intermediate, often represented by X ~ P, in the sequence. A frequently used formulation is an oligomycin-sensitive phosphorylation (Eqn. 2) followed by an oligomycin-insensitive phosphotransferase (Eqn. 3).

$$X + P_i \xrightarrow{\text{(respiration)}} X \sim P \tag{2}$$

$$X \sim P + ADP \rightleftharpoons X + ATP \tag{3}$$

The sum of Eqns. 2 and 3 is Eqn. 1.

In order to explain COHN AND DRYSDALE'S[55] finding that mitochondria catalyse a more rapid exchange of ^{18}O from $H_2{}^{18}O$ into ATP than into phosphate, one of us[56] suggested in 1958 that Eqn. 3 might have to be split into two reactions

$$X \sim P + ADP \rightleftharpoons X \sim P \cdot ADP + H_2O \tag{4}$$

$$X \sim P \cdot ADP + H_2O \rightleftharpoons X + ATP \tag{5}$$

The reaction given by Eqn. 6 was proposed to explain the exchange of oxygen between water and P_i,

$$\begin{array}{l} X \sim I + P\text{–}OH \rightleftharpoons X \sim P + I\text{–}OH \\ \qquad\qquad\qquad\qquad\quad \Downarrow\Uparrow \\ \qquad\qquad\qquad\qquad\quad I^+ + OH^- \end{array} \tag{6}$$

X ~ I having been formed as an intermediate in the reaction given by Eqn. 1. COOPER[57] has shown that ADP is necessary for this exchange reaction and he and CHAPPELL AND CROFTS[36] have suggested that X ~ P is not formed in the absence of ADP, *e.g.* Eqns. 2 and 4 are replaced by the concerted reaction given by Eqn. 7.

$$X + P_i + ADP \xrightarrow{\text{(respiration)}} ADP \cdot X \sim P \tag{7}$$

CHAPPELL AND CROFTS have suggested further that ADP ~ As is made in the presence of arsenate (As_i), thus

$$X + As_i + ADP \xrightarrow{\text{(respiration)}} ADP\text{–}X \sim As \tag{8}$$

$$ADP\text{–}X \sim As \rightleftharpoons X\text{–}ADP \sim As \tag{9}$$

$$X\text{–}ADP \sim As \rightleftharpoons X + ADP \sim As \tag{10}$$

followed by hydrolysis of ADP ~ As (*cf.* refs. 14, 58)

$$ADP \sim As + H_2O \longrightarrow ADP + As_i \tag{11}$$

According to this explanation, added ADP will stimulate the arsenate-induced respiration because endogenous ADP is the rate-limiting factor.

In our preliminary note[18], we adopted the simplest form of the X ~ P theory, in which arsenate may replace P_i in Eqn. 2, thus

$$X + As_i \xrightarrow{\text{(respiration)}} X \sim As \tag{12}$$

followed by hydrolysis of X ~ As

$$X \sim As + H_2O \longrightarrow X + As_i \tag{13}$$

As ERNSTER, LEE AND JANDA[32] have pointed out, this mechanism does not explain the stimulation of the arsenate-induced respiration by ADP. The explanation given by ESTABROOK AND ITADA[27] and VAN DEN BERGH[11], namely that ADP liberates X from X ~ P, is now made unlikely by the experiments with atractyloside, especially those in the presence of glucose + hexokinase. The inhibition of the respiratory rate induced by atractyloside added after the ADP suggests that the latter is continuously needed[36].

Like CHAPPELL AND CROFTS[36], ERNSTER, LEE AND JANDA[32] suggest that the unstable arsenylated product is ADP ~ As. They differ from CHAPPELL AND CROFTS by proposing that it is formed from X ~ As

$$X \sim As + ADP \rightleftharpoons X + ADP \sim As \qquad (14)$$

instead of by the concerted reaction given by Eqn. 8, and that this reaction takes place only with added ADP. We now agree that the most likely explanation of the stimulation by ADP is the formation of the unstable ADP ~ As intermediate. The 1958 extension of the X ~ P theory, when applied to arsenate, provides a possible explanation of the fact that the $As_I \rightleftharpoons H_2O$ exchange is much faster than the arsenate-induced ATPase[59].

We have no evidence that allows us to choose between CHAPPELL AND CROFTS'[36] view that ADP is absolutely essential for the arsenolysis of X ~ I and the suggestion of ERNSTER, LEE AND JANDA[32] that X–As can be broken down slowly in the absence of ADP.

As pointed out above, the inhibition of the arsenate-induced respiration by P_I is not caused by removal of endogenous ADP. All the mechanisms discussed can explain this inhibition if it is assumed that P_I has a much greater affinity for X than arsenate. Thus, in the presence of P_I, X will accumulate as X ~ P or ADP–X ~ P $\rightleftharpoons$ X + ATP. Added ADP will relieve the inhibition by reacting with X ~ P or with ATP formed within the mitochondrion.

According to the X ~ P theory the arsenate-induced ATPase is explained by the sequence

$$ATP + X \rightleftharpoons X \sim P + ADP \qquad (3) = (5) + (4)$$

$$X \sim P + I \rightleftharpoons X \sim I + P_I \qquad (15)$$

$$X \sim I + As_I \rightleftharpoons X \sim As + I \qquad (16)$$

$$X \sim As + ADP \rightleftharpoons ADP\text{–}X \sim As + H_2O \qquad (17)$$

$$ADP\text{–}X \sim As + H_2O \longrightarrow X + ADP + As_I \qquad (18)$$

A similar sequence follows from CHAPPELL AND CROFTS' mechanism[36]

$$ATP + X \rightleftharpoons ADP\text{–}X \sim P + H_2O \qquad (5)$$

$$ADP\text{–}X \sim P + I \rightleftharpoons X \sim I + ADP + P_I \qquad (19)$$

$$X \sim I + As_I + ADP \rightleftharpoons ADP\text{–}X \sim As + I \qquad (20)$$

$$ADP\text{–}X \sim As + H_2O \longrightarrow X + ADP + As_I \qquad (18)$$

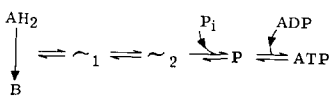
In Table III, the rates of respiration, in the presence of ADP + P_I, or of ADP + arsenate, are compared with the rates of the various ATPase reactions. The fact that the rate of respiration in the presence of arsenate and ADP is about 80 % of

that in the presence of P_i and ADP suggests that the rate-limiting step in arsenate-induced respiration is that given by Eqns. 16, 17 or 18. Since the rate of the arsenate-induced ATPase is much lower than that of the arsenate-induced respiration, another factor must be coming into play in the former reaction. In disagreement with ERNSTER, LEE AND JANDA[32] we do not believe that the formation of $X \sim P$ (Eqn. 3) can be rate-limiting, since the dinitrophenol-induced ATPase, that must include this reaction, is the most rapid of all the reactions given in Table III. The factor limiting the arsenate-induced ATPase is very likely the back reaction of Eqn. 15, which ensures that, in the steady state, little $X \sim I$ is present. In other words, as already emphasized, $X \sim I$ has a much greater affinity for P_i than for arsenate. The dinitrophenol-induced ATPase, on the other hand, is promoted by the rapid removal of $X \sim I$ by subsequent reactions.

The concerted mechanism represented by Eqn. 7 provides a ready explanation for the finding of KULKA AND COOPER[60] that the rate of the ADP–ATP exchange reaction in digitonin particles is the same as the rate of the P_i–ATP exchange reaction, since both exchange reactions require the participation of the two reactions given by Eqns. 5 and 19. On this basis, it is also understandable why the ATP–ADP exchange reaction is relatively slow (of the order of the ATPase activity in the presence of arsenate) and that arsenate inhibits the exchange activity[51]. The explanation of the slow exchange activity is the same as that given for the slow arsenate-induced ATPase.

ERNSTER, LEE AND JANDA[32] have brought forward in favour of their mechanism the observation that increasing the arsenate concentration beyond 10 mM does not cause an appreciable increase in the ATPase activity. Actually, if we subtract the activity in the absence of arsenate from that in its presence, we find activities at 20 mM and 40 mM equal to about 40 % and 95 % higher than at 10 mM. These data yield an activity at infinite arsenate concentration about 3 times that at 10 mM with a K_m of about 30 mM. This is much greater than the K_m and K_i values calculated from the effects of arsenate on respiration or the P:O ratio. It is also much greater than the value of 0.7–1.4 mM found for the K_i for arsenate for photosynthetic phosphorylation[14]. It is true that an ATPase activity equal to only 3 times that measured with 10 mM arsenate is less than the rate of the respiration in the presence of arsenate and ADP, and according to the mechanism proposed one must expect the rate of the ATPase at infinite arsenate concentration (when the back reaction of Eqn. 15 will play no role) to be equal to the respiration in the presence of arsenate and ADP. However, it is doubtful whether it is justifiable to use the data at these very high arsenate concentrations to extrapolate to infinite arsenate concentration. It is possible that other non-specific inhibitory reactions of arsenate play a role.

As already mentioned in INTRODUCTION, ESTABROOK[15] and we[16,17] concluded from the different effects of oligomycin on the dinitrophenol- and arsenate-uncoupled respiration that there are at least two non-phosphorylated high-energy intermediates in the sequence of energy-conserving reactions leading from the oxidoreduction reaction of the respiratory chain to ATP, thus



where $\sim_1$ is the dinitrophenol-sensitive site and $\sim_2$ the oligomycin-sensitive. $\sim$P is excluded as the oligomycin-sensitive site by the demonstration that oligomycin affects phosphate-free systems[61] and non-phosphorylating particle preparations[62,63]. In terms of chemical equations this mechanism may be written

$$AH_2 + B + I \rightleftharpoons A \sim I + BH_2 \quad (21)$$

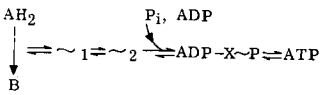
$$A \sim I + X \rightleftharpoons A + X \sim I \quad (22)$$

$$X \sim I + P_i \rightleftharpoons X \sim P + I \quad (15)$$

$$X \sim P + ADP \rightleftharpoons X + ATP \quad (3)$$

where either X or X $\sim$ I (assuming that the concentration of all forms of I exceeds the concentration of all forms of X) is the site of action of oligomycin.

The only modification necessary in this scheme to accommodate the suggestion[36,57] that ADP is necessary for the formation of the $\sim$P compound is to replace the $\sim$P compound by ADP–X $\sim$P, thus



The conclusion that there are two $\sim$ intermediates is not affected by the newer data on the mechanism of uncoupling by arsenate.

An alternative formulation is that there is only one non-phosphorylated high-energy intermediate, and it has two sites, a dinitrophenol-sensitive and an oligomycin. There is, in fact, little difference between the two formulations. I and X may well represent "active sites" on an enzyme or enzymes.

The most important conclusion to be drawn from this study is that arsenate uncouples respiratory-chain phosphorylation by competition with phosphate, just as in substrate-linked phosphorylation. However, respiratory-chain phosphorylation is relatively much more specific for phosphate than substrate-linked phosphorylation, and much higher $[As_i]:[P_i]$ ratios are needed for uncoupling. The effects of arsenate provide strong evidence in favour of the existence of a $\sim$P intermediate of oxidative phosphorylation, either containing phosphate alone (X $\sim$ P) or containing ADP as well as phosphate (ADP–X $\sim$ P). We are at present unable definitely to decide between these two possibilities although CHAPPELL AND CROFTS' mechanism provides a better explanation of the properties of the ADP–ATP exchange reaction. The experiments do seem to eliminate concerted mechanisms of the type

$$X \sim I + P_i + ADP \rightleftharpoons X + I + ATP \quad (23)$$

in which no intermediate $\sim$P compounds are formed. Concerted mechanisms do not explain the inhibition by P_i of arsenate-induced respiration or the slowness of the arsenate-induced ATPase.

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