The Effect of Atractylate and Oligomycin on the Behaviour of Mitochondria towards Adenine Nucleotides

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(Received 21 August 1964)

1. Investigation of a number of reactions involving both internal and externally added adenine nucleotides of isolated liver mitochondria has revealed that atractylate and oligomycin differ markedly in the site of their inhibitory action. 2. Both atractylate and oligomycin inhibited the respiratory-chain-level phosphorylation of added ADP. Neither compound inhibited the substrate-level phosphorylation of internal (endogenous) ADP or the respiration-dependent accumulation of bivalent metal ions (Ca^{2+} , Sr^{2+} or Mn^{2+}). 3. Atractylate, but not oligomycin, inhibited the substrate-level phosphorylation of externally added ADP, the ATP- and carnitine-dependent reduction of nicotinamide nucleotide by palmitate and the ATP-induced activation of succinate oxidation. 4. Oligomycin, but not atractylate, inhibited the respiratory-chain-linked phosphorylation of internal ADP, and the dephosphorylation of internal ATP that occurred on the addition of antimycin. 5. The enhancement of arsenate-stimulated respiration by ADP was prevented by atractylate added either before or after the ADP. Oligomycin abolished both the arsenate and ADP stimulation. 6. It is suggested that atractylate prevents the passage of adenine nucleotides across the mitochondrial membrane, whereas oligomycin interferes with the formation of a 'high-energy' phosphorylated intermediate.

Previous investigations on the effects of atractylate on respiratory-chain phosphorylation and on the partial reactions believed to be associated with this process, e.g. the 2,4-dinitrophenol-stimulated adenosine-triphosphatase activity, indicated that this inhibitor and oligomycin were similar in action (see, e.g., Bruni, Contessa & Luciani, 1962). Insofar as intact mitochondria, rather than submitochondrial particles, are concerned, both oligomycin and atractylate inhibit respiratory-chain-linked phosphorylation and the associated release of respiration, ATP-P_i exchange and 2,4-dinitrophenol-stimulated adenosine triphosphatase, but do not inhibit 2,4-dinitrophenol-stimulated respiration. However, oligomycin and atractylate differ in their action in a number of important points. Perhaps the most significant of these differences is that there is a competitive effect between ADP and atractylate in phosphorylation (Bruni *et al.* 1962), and that atractylate does not inhibit ATP synthesis in submitochondrial particles (Vignais, Vignais & Stanislas, 1962). It has been suggested by Löw, Vallin & Alm (1963) that there is in intact mitochondria an enzyme that confers ADP-specificity on the respira-

tory-chain phosphorylation system, since they found that with intact ox-heart mitochondria the system was atractylate-sensitive and ADP-specific, but that with submitochondrial particles atractylate was without effect, and nucleoside diphosphates other than ADP would act as phosphate acceptors.

In the present paper the effects of oligomycin and atractylate on a number of reactions involving both internal and externally added adenine nucleotides have been investigated. In general it has been found that atractylate does not inhibit reactions involving internal adenine nucleotides, but completely abolishes the utilization of external adenine nucleotides by mitochondria. The sole exception to this general statement was found to be adenylate kinase, but evidence is presented that this enzyme is probably located on the outside of the mitochondrion. It is particularly significant that atractylate inhibited the activation of palmitate by ATP, the reactivation by ATP of succinate oxidation that had been inhibited by preincubation with uncoupling agents, and phosphorylation of ADP at the substrate level, coupled to oxidative decarboxylation of α -oxoglutarate. Since oligomycin does not affect these three reactions, it is thought that none of them involves the normal sequence of reactions in true

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respiratory-chain phosphorylation. It appears therefore that atractylate does not act on the phosphorylation system directly, but on the penetration of the mitochondrial membrane by ADP or ATP.

METHODS

Most of the materials and methods used were as described by Chappell & Crofts (1965a). The fluorescence of mitochondrial reduced nicotinamide nucleotides was followed fluorimetrically by using the apparatus shown diagrammatically in Fig. 1.

Assay of ATP. Mitochondrial suspensions containing 2.5–3.5 mg. of protein/ml. were deproteinized by the addition of 0.05 ml. of 72% (w/w) HClO_4 /3.0 ml. of reaction medium. After cooling, the precipitate was removed by centrifugation, and the supernatant decanted into a tube containing 1.0 ml. of 0.6 N-KOH together with 0.5 M-tris-HCl, the pH of which was originally 7.2. After mixing and standing at 0° for at least 30 min., the pH of the neutralized solution was checked with an external indicator, and if necessary adjusted to

pH 7.2 with KOH or HCl. Then 2.0 ml. samples of the supernatant after centrifugation were added to a cuvette of 1.0 cm. light-path, containing 0.5 ml. of a solution with the following composition: glucose (25 mM), MgSO_4 (5 mM), ethylene glycol bis(aminoethyl)tetra-acetate (1 mM), tris-HCl, pH 7.2 (0.5 M), and NADP (1.0 mg./ml.). The final pH was 7.2–7.5. After warming the contents of the cuvette to 30°, the reduction of NADP, equivalent to the amount of ATP present (Kornberg, 1950), was followed fluorimetrically after the addition of 100 μg . of hexokinase and 50 μg . of glucose 6-phosphate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). The system was calibrated with standard ATP solution, the strength of which was determined spectrophotometrically by using the method of and the materials supplied by C. F. Boehringer und Soehne G.m.b.H., by following the oxidation of reduced NAD that occurred when ATP was added to a solution containing 3-phosphoglycerate, Mg^{2+} , 3-phosphoglycerate kinase and 3-phosphoglycerate dehydrogenase.

Adenylate-kinase assay. This activity was assayed by the method outlined below:

Overall reaction:

Fig. 1. Fluorimeter used for determination of both free and mitochondrial reduced nicotinamide nucleotide. A, 36 w tungsten lamp; B, Beckman DU lamp-house; C, Wratten 18A filter (peak transmission at 360 μm); D, thermostatically controlled cell holder; E, Wratten 2B filter (excludes radiation < 420 μm); F, end-window photomultiplier, 6255 B (E.M.I. Electronics Ltd., Hayes, Middlesex); G, compartment containing dynode resistors, sensitivity control etc. The high-tension supply was provided by extra-high-tension unit type 532/D (Isotope Developments Ltd., Aldermaston, Berks.). The photomultiplier signal was measured without amplification by a Honeywell-Brown recorder (1 mv full-scale, 1 sec. response time). Under these conditions the changes in the amount of fluorescence are mainly derived from changes in the redox state of nicotinamide nucleotides (see Chance & Baltscheffsky, 1958).

In the presence of excess of hexokinase and glucose 6-phosphate dehydrogenase, adenylate-kinase activity was rate-limiting. The experimental procedure was as follows. Mitochondria (1–2 mg. of protein) were added to 3.0 ml. of a reaction medium containing KCl (80 mM), tris-HCl buffer, pH 7.2 (20 mM), MgSO_4 (1 mM), glucose (10 mM), glucose 6-phosphate dehydrogenase (25 μg .), hexokinase (50 μg .), NADP (2.5 mg.) and antimycin (2 μg .). The temperature was 30°. The reaction was started by the addition of ADP (0.8 mM), and the reduction of NADP was followed fluorimetrically in the apparatus shown in Fig. 1. Increasing the amounts of hexokinase or glucose 6-phosphate dehydrogenase did not affect the rate of the reaction. This rate was proportional to the concentration of mitochondria over the range 0.5–3.0 mg. of mitochondrial protein/3.0 ml. The progress curve was linear after 15 sec. for a period of 90–120 sec. Antimycin was added to prevent changes in the fluorescence of mitochondrial nicotinamide nucleotides due to changes in redox state, and to prevent any possible reoxidation of reduced NADP. When the mitochondria were treated with Triton X-100 or frozen-thawed and HCN (1 mM) was used in place of antimycin, no difference in result was observed.

Rapid addition of reactants. So that the kinetics of phosphorylation of internal ADP at 30° could be followed, an apparatus for the rapid addition of reactants was constructed. Reactants were loaded into micro-pipettes connected by short tubes to 20 ml. glass syringes. The reactants were ejected into the rapidly stirred reaction medium by pushing the plungers of the syringes by means of solenoids whose operation was timed electronically.

Potassium atractylate was a generous gift from Professor

E. C. Slater. We also thank Dr P. K. Tubbs for samples of palmitoylcarnitine and palmitoyl-CoA.

RESULTS

Arsenate-stimulated respiration. Estabrook (1961) showed that ADP caused a twofold increase in the rate of arsenate-stimulated respiration. He also showed that oligomycin inhibited completely the stimulation by arsenate. Bruni, Luciani, Contessa & Azzone (1964b) studied the effect of atractylate on arsenate-stimulated succinate oxidation by mitochondria that had been incubated with ADP, glucose and hexokinase; atractylate caused a 30% inhibition. Bruni *et al.* (1964b) considered this inhibition to be insignificant. However, this partial inhibition may be extremely important in revealing the mechanism of action of both arsenate and atractylate on respiratory-chain phosphorylation. In Fig. 2 the effects of oligomycin and atractylate on arsenate-stimulated respiration are shown. Atractylate abolished the enhancement of arsenate-stimulated respiration by ADP, whereas oligomycin prevented the effect of both arsenate and ADP, as reported initially by Estabrook (1961). Dinitrophenol-stimulated respiration was not affected by either oligomycin or atractylate. These results are those that would be expected if ADP acted by removing

endogenous phosphate, thereby preventing phosphate from causing a competitive inhibition of the effect of arsenate, as suggested by Bruni *et al.* (1964b). However, this hypothesis appears to be inconsistent with the results shown in Fig. 3. Mitochondria showed an increased rate of oxidation of glutamate together with malate on the addition of arsenate, and ADP enhanced this effect. Significantly, the addition of atractylate caused an immediate decrease in the rate of oxygen consumption to the level observed with arsenate alone, and oligomycin reversed the effect of arsenate. This effect of atractylate in both abolishing and rapidly reversing the effect of ADP on the interaction of arsenate with the respiratory chain is revealed by the experiments shown in Figs. 4 and 5, in which the changes in fluorescence of mitochondrial reduced nicotinamide nucleotides were followed. Oligomycin prevented the oxidation of reduced nicotinamide nucleotides induced by the interaction of arsenate and ADP with the respiratory chain, whereas atractylate prevented only the effect of ADP (Fig. 4). When

Fig. 2. Effects of atractylate and oligomycin on arsenate-stimulated succinate oxidation. In each case the medium contained succinate (5 mM), rotenone (0.17 μ M) (to prevent the oxidation of malate), KCl (80 mM) and tris-HCl buffer, pH 7.2 (20 mM). The temperature was 30° and the total volume 3.0 ml. Additions were: A, 0.1 ml. of rat-liver mitochondria (10 mg. of protein); B, arsenate (1.2 mM); C, ADP (120 μ M); D, dinitrophenol (100 μ M). (a) Control; (b) atractylate (50 μ M) present initially; (c) oligomycin (3 μ g.) present initially.

Fig. 3. Effects of atractylate and oligomycin added after arsenate and ADP on the oxidation of glutamate together with malate. Conditions were as given for Fig. 2, except that succinate was replaced by glutamate (5 mM) plus malate (5 mM). Additions were: A, 0.1 ml. of rat-liver mitochondria (10 mg. of protein); B, arsenate (1.2 mM); C, ADP (120 μ M); D, atractylate (50 μ M); E, oligomycin (3 μ g.); F, dinitrophenol (100 μ M). The concentration of dinitrophenol used caused a non-linear rate of respiration because of the inhibitory effect of high concentrations of this phenol (Chappell, 1964a).

Fig. 4. Oxidation of endogenous reduced nicotinamide nucleotide caused by arsenate and ADP, in the presence of atractylate and oligomycin. Rat-liver mitochondria (8.4 mg. of protein) were suspended in 3.0 ml. of medium containing succinate (4 mM), KCl (80 mM) and tris-HCl buffer, pH 7.2 (20 mM). Additions were: A, arsenate (1.2 mM); B, ADP (170 μ M). (a) Control; (b) atractylate (100 μ M) present initially; (c) oligomycin (4 μ g.) present initially.

Fig. 5. Effect of atractylate and oligomycin on the arsenate-ADP-induced oxidation of mitochondrial nicotinamide nucleotides. Rat-liver mitochondria (7.6 mg. of protein) were first incubated with pentachlorophenol (4 μ M) in medium containing KCl (80 mM) and tris-HCl buffer, pH 7.2 (20 mM), for 2 min. followed by the addition of 50 mg. of bovine plasma albumin (to remove the pentachlorophenol) (Chappell & Crofts, 1965b) and DL- β -hydroxybutyrate (5 mM). Additions were: A, arsenate (1.2 mM); B, ADP (170 μ M); C, atractylate (100 μ M); D, oligomycin (4 μ g.).

atractylate was added after arsenate and ADP, reduction of nicotinamide nucleotide occurred immediately and rapidly, and to a level observed when only arsenate was present. The addition of oligo-

mycin caused further reduction to a level observed in the absence of arsenate or ADP (Fig. 5).

The mitochondria were incubated with pentachlorophenol to cause removal of endogenous substrate and then bovine plasma albumin was added to remove the phenol and prevent its uncoupling action. The addition of DL- β -hydroxybutyrate then caused rapid reduction of endogenous nicotinamide nucleotide. Under these conditions the mitochondria are in the resting state (state 4) and β -hydroxybutyrate is the major substrate (see Chappell & Crofts, 1965b).

If the action of ADP on the rate of arsenate-stimulated respiration were consequent on the removal of phosphate, then it is clear that atractylate should not inhibit when added after ADP; indeed, if anything, it should enhance the rate of respiration by preventing the access of ATP to the mitochondrial adenosine triphosphatase. An alternative explanation of the effect of ADP on arsenate-stimulated respiration is depicted in Scheme 1. The major postulate of this Scheme is that the formation of a phosphorylated or arsenylated intermediate as a result of electron transport is dependent on the presence of ADP. That is, that the enzyme involved in ATP synthesis requires the formation of a ternary complex involving enzyme, ADP and phosphate (or arsenate). The stimulation by arsenate alone could possibly be due to the endogenous adenine nucleotide present. Under these conditions, atractylate would produce no effect, since this compound prevents only the access of external adenine nucleotides. The enhancement of the rate of arsenate-stimulated respiration by externally added ADP would be due to the formation of external ADP-arsenate, i.e. the rate-limiting reaction in the absence of externally added ADP is the spontaneous hydrolysis of internal ADP-arsenate.

Scheme 1, if it is correct, would explain the paradox that the rate of incorporation of ^{18}O from water into ATP exceeds the rate of incorporation into phosphate (Cohn, 1953; Cohn & Drysdale, 1955; Drysdale & Cohn, 1958), even in submitochondrial particles where permeability barriers and 'compartmentation' would be expected to be minimal (Chan, Lehninger & Enns, 1960).

Effect of atractylate on substrate-level phosphorylation. It was shown by Hunter (1951) that dinitrophenol did not affect the substrate-level phosphorylation associated with the oxidative decarboxylation of α -oxoglutarate. Consequently, a phosphate requirement for dinitrophenol-stimulated α -oxoglutarate and glutamate oxidation by mitochondria is apparent (Borst & Slater, 1960, 1961; Chappell, 1961, 1964a; Charles, Tager & Slater, 1963). However, a requirement for ADP is not apparent unless oligomycin is also present (Chappell & Greville, 1961; Charles *et al.* 1963). It has been concluded by

Scheme 1. A possible representation of the requirement for ADP for arsenate-stimulated respiration. It is visualized that the enzyme, E, has a very much decreased affinity for phosphate or arsenate in the absence of ADP.

Chappell & Greville (1961) that in the absence of oligomycin the rate of dinitrophenol-stimulated hydrolysis of internal ATP is adequate for the requirements of the succinyl-CoA synthetase, but that in the presence of oligomycin, which inhibits dinitrophenol-stimulated adenosine triphosphatase (Lardy, Johnson & McMurray, 1958), the provision of ADP becomes the rate-controlling process, thus accounting for the requirement for ADP. It was also concluded that oligomycin does not inhibit the succinyl-CoA-synthetase reaction, and this has been demonstrated directly by Danielson & Ernster (1963), who showed that oligomycin has no effect on the phosphorylation associated with the anaerobic dismutation of α -oxoglutarate in the presence of NH_4^+ .

In essence, with mitochondria suspended in the presence of dinitrophenol and oligomycin the oxidation of glutamate is dependent on added ADP. This system enables the effect of atractylate on substrate-level phosphorylation to be determined (Fig. 6). Atractylate completely abolishes the effect of added

ADP (Fig. 6d). In contrast, in the absence of oligomycin, conditions under which the turnover of internal adenine nucleotide is adequate, atractylate had no effect (Fig. 6b). This latter result indicates that atractylate, like oligomycin, has no effect on the substrate-level phosphorylation and, unlike oligomycin, does not inhibit the dinitrophenol-stimulated hydrolysis of internal ATP. Bruni *et al.* (1964b) have shown that atractylate inhibited the phosphate esterification associated with the anaerobic dismutation of α -oxoglutarate and NH_4^+ when glucose and hexokinase acted as acceptor, i.e. atractylate inhibited the phosphorylation of external ADP.

Effect of atractylate and oligomycin on the phosphorylation and dephosphorylation of internal ATP. Oligomycin abolishes completely or almost completely the phosphorylation of externally added ADP (Lardy *et al.* 1958) and the incorporation of ^{32}P -labelled phosphate into internal ATP (Slater, Kemp & Tager, 1964) during respiratory-chain phosphorylation. Atractylate 'competitively' in-

Fig. 6. Recordings of the rate of dinitrophenol-stimulated glutamate oxidation in the presence of atractylate and oligomycin. The current from the oxygen electrode was differentiated and recorded after amplification (Chappell & Crofts, 1965a). The reaction medium contained glutamate (5 mM), KCl (80 mM) and tris-HCl buffer, pH 7.2 (20 mM). The temperature was 30°, and the total volume 6.0 ml. Additions were: A, rat-liver mitochondria (11.4 mg. of protein); B, dinitrophenol (100 μ M); C, phosphate (0.84 mM); D, ADP (84 μ M). (a) Control; (b) atractylate (50 μ M) present initially; (c) oligomycin (4 μ g.) present initially; (d) atractylate (50 μ M) and oligomycin (4 μ g.) present initially. On the addition of mitochondria there was an apparent temporary burst in the rate of oxygen consumption due to the fact that the suspension was anaerobic and cold.

Fig. 7. Rate of intramitochondrial ATP synthesis in the presence of atractylate and oligomycin. A 0.1 ml. portion of rat-liver mitochondria (7.2 mg. of protein) was added to 3.0 ml. of a medium containing pentachlorophenol (4 μ M), ethylene glycol bis(aminoethyl)tetra-acetate (0.33 mM), KCl (80 mM), tris-HCl buffer, pH 7.2 (20 mM), phosphate (2.0 mM) and rotenone (0.17 μ M). The temperature was 30°. Bovine plasma albumin (10 mg.) was added to remove the pentachlorophenol (Garbus & Weinbach, 1963; Chappell & Crofts, 1965b). In the control experiment (Δ) succinate (6.7 mM) was added 30 sec. after the albumin. This point is indicated as zero time in the Figure. $\circ$, Atractylate (130 μ M) added 10 sec. before the albumin; $\bullet$, oligomycin (4 μ g.) added 10 sec. before the albumin. The reaction was stopped by addition of HClO_4 at the times indicated.

hibits the phosphorylation of added ADP by intact mitochondria (Bruni *et al.* 1962; Vignais *et al.* 1962), and it has been claimed by Moret, Pinna, Sperti, Lorini & Siliprandi (1963) that it also inhibits the incorporation of ^{32}P -labelled phosphate into internal ATP. It appears therefore that in these respects atractylate and oligomycin are very similar in their action.

However, the experiment shown in Fig. 7 shows that atractylate, at concentrations that inhibited completely the phosphorylation of 300 μ M-ADP, had no detectable effect on the rate or extent of internal ATP synthesis when succinate served as substrate. Similar results have been obtained by M. Klingenberg (personal communication). We have often found that, when DL- β -hydroxybutyrate was used in place of succinate (Moret *et al.* 1963), there was, after a rapid phosphorylation, a slow but extensive dephosphorylation of internal ATP. No ready explanation of this phenomenon can be given at present. This finding does, however, indicate why Moret *et al.* (1963) reported that atractylate inhibited the phosphorylation of internal ADP.

It was also possible to show that atractylate did not affect significantly the dephosphorylation of internal ATP that occurred on the addition of anti-

Fig. 8. Dephosphorylation of intramitochondrial ATP on the addition of antimycin. Rat-liver mitochondria (8.2 mg. of protein) were incubated with medium containing succinate (5 mM), phosphate (2 mM), rotenone (0.17 μ M), KCl (80 mM) and tris-HCl buffer, pH 7.2 (20 mM). The temperature was 30°, and the total volume 3.0 ml. After 2 min. (zero time in the Figure) antimycin (0.6 μ g.) was added. The reaction was stopped at the time indicated. $\circ$, Control; $\bullet$, oligomycin (4 μ g.) added 5 sec. before the antimycin; Δ , atractylate (130 μ M) added 5 sec. before the antimycin.

mycin to mitochondria suspended in the presence of phosphate and succinate, whereas oligomycin markedly inhibited (Fig. 8).

Effect of atractylate on ATP-activated succinate oxidation. Azzone & Ernster (1960) showed that liver mitochondria that had been incubated with arsenate and an uncoupling agent showed a diminished ability to oxidize succinate. However, ATP caused a marked activation of respiration. Chappell (1961) showed that the effect of ATP was catalytic and was not inhibited by oligomycin. Experimental evidence was presented that ATP caused a reversal of an oxaloacetate-induced inhibition of succinate dehydrogenase by an unknown mechanism. In view of the implication (because of the lack of oligomycin inhibition) that ATP was not acting in this system through the respiratory-chain phosphorylation system, it was decided to determine the effect of atractylate. Fig. 9 shows the results of a series of experiments in which it is demonstrated that oligomycin enhances and atractylate inhibits the ATP-induced activation of succinate oxidation. In each

case the mitochondria were incubated with pentachlorophenol for 1½–2 min., and then succinate and rotenone (to prevent further production of oxaloacetate) were added. ATP caused a marked activation of succinate oxidation (Fig. 9a), and oligomycin added before the nucleotide caused a marked increase in the effect of ATP (Fig. 9b). In contrast, atractylate caused a complete inhibition of the ATP effect (Fig. 9c). It was shown in other experiments that the effects of ATP and atractylate were 'competitive'.

Effect of atractylate on the reduction of nicotinamide nucleotide by palmitate, palmitoyl-CoA and palmitoyl-carnitine. Isolated liver mitochondria that had been incubated with pentachlorophenol followed by bovine plasma albumin (Chappell & Crofts, 1965b) showed no or little reduction of nicotinamide nucleotide on the addition of palmitoyl-CoA. The further addition of DL-carnitine caused a marked reduction of nicotinamide nucleotide (Fig. 10b). Carnitine caused no reduction in the absence of palmitoyl-CoA. Palmitoylcarnitine caused a very rapid and extensive reduction of nicotinamide nucleotide (Fig. 10a) (Chappell, 1964b). In both these systems neither oligomycin nor atractylate had any detectable effect. In contrast, when free palmitate was used, conditions under which the addition of both ATP and carnitine was necessary to observe nicotinamide nucleotide reduction, oligomycin enhanced and atractylate inhibited the reduction of nicotinamide nucleotide (Figs. 11a and 11b). Fig. 11(b)

Fig. 9. Effect of atractylate and oligomycin on the ATP-induced activation of succinate oxidation. The medium contained KCl (80 mM) and tris-HCl buffer, pH 7.0 (20 mM). The total volume was 6.0 ml. Additions were: A, rat-liver mitochondria (11.4 mg. of protein); B, pentachlorophenol (3 μ M); C, succinate (5 mM); D, rotenone (0.17 μ M) (to prevent further oxidation of malate and consequent inhibition by oxaloacetate produced); E, ATP (0.4 mM); F, oligomycin (4 μ g.); G, atractylate (67 μ M). (a) Control; (b) oligomycin (4 μ g.) added as indicated; (c) atractylate (67 μ M) added as indicated. Where the recordings are interrupted thus —||—, a downward deflexion occurred. This artifact was due to the addition of an ethanolic solution, which, because of the high solubility of O₂ in ethanol, caused an apparent evolution of O₂.

Fig. 10. Reduction of mitochondrial nicotinamide nucleotides by palmitoylcarnitine (a) and by palmitoyl-CoA and carnitine (b). A 0.1 ml. portion of rat-liver mitochondria (7.4 mg. of protein) was added to 2.9 ml. of a medium containing KCl (80 mM), tris-HCl buffer, pH 7.2 (20 mM), and malonate (4 mM) (to prevent reduction by endogenous substrate). Additions were: A, pentachlorophenol (4 μ M); B, defatted bovine plasma albumin (50 mg.); C, palmitoylcarnitine (83.5 μ M); D, palmitoyl-CoA (6.6 μ M); E, DL-carnitine (75 μ M). For clarity the initial part of the curve (b) has been omitted.

also shows that, although atractylate inhibited the reduction by palmitate, ATP and carnitine, it did not prevent reduction by palmitoylcarnitine. The findings are summarized in Scheme 2. The results reported above are consistent with this Scheme. Also, the findings by Dr P. K. Tubbs (personal communication) that approx. 50% of the total free CoA of isolated liver mitochondria is acylated by palmitate together with ATP in the absence of carnitine, and that on addition of carnitine the remainder of the CoA is acylated, are entirely consistent with this Scheme.

Fig. 11. Effects of oligomycin (a) and atractylate (b) on nicotinamide nucleotide reduction by palmitate. Conditions were as given for Fig. 10. Additions were: A, pentachlorophenol ($4\mu\text{M}$); B, defatted bovine plasma albumin ($50\mu\text{g}$); C, palmitate ($10\mu\text{M}$); D, ATP ($870\mu\text{M}$) plus DL-carnitine ($75\mu\text{M}$); E, palmitoylcarnitine ($40\mu\text{M}$); F, oligomycin ($4\mu\text{g}$); G, atractylate ($100\mu\text{M}$). For clarity the initial part of curve (b) has been omitted.

Mitochondrial adenylate-kinase activity. Of those mitochondrial enzymes or enzymic systems tested, adenylate kinase was the only one utilizing external adenine nucleotide that was not affected by $100\mu\text{M}$ -atractylate. This finding led to an investigation of some aspects of the properties of this enzyme. It appears that atractylate prevents the penetration of the mitochondrial membrane by adenine nucleotide. If this view is correct then it follows that adenylate kinase is located outside the 'atractylate barrier'. It is predicted from this that the adenylate kinase would not be a 'latent' enzyme, i.e. the activity should not be increased on disruption of the mitochondrial membrane (see Chappell & Greville, 1963). It was shown that freezing-and-thawing the mitochondrial suspension six times or inclusion of 0.01% Triton X-100 (which caused lysis of the mitochondria) in the assay medium was without detectable effect on mitochondrial adenylate-kinase activity. Under these conditions the activity of many NAD- and NADP-linked dehydrogenases (see Bendall & de Duve, 1960) and other enzymes, including thiosulphate transulphurase (rhodanese) (Greville & Chappell, 1959), is increased several-fold. It has been postulated (Chappell & Greville, 1963) that the 'latency' of these enzymes is most probably due to feeble penetration of the mitochondrial membrane by the reactants or products of the enzymes involved, e.g. NAD, which is known to penetrate the mitochondrial membrane slowly (Purvis & Lowenstein, 1961). The lack of effect of atractylate on mitochondrial adenylate kinase and the absence of 'latency' indicate that this enzyme is located on the outside of the mitochondrion. Siekevitz & Potter (1955) concluded that at least a part of the adenylate kinase was located on the surface of the mitochondrion, but the present results with

Scheme 2. Relationship between the carnitine-activation of fatty acid oxidation and the action of atractylate. Cn, Carnitine; $\Rightarrow$, site of inhibition by atractylate.

atractylate do not support their contention that this enzyme is responsible for the transfer of adenine nucleotide across the mitochondrial membrane, since the transfer is blocked by atractylate and adenylate-kinase activity is not inhibited.

Respiration-dependent accumulation of bivalent ions. Under the conditions described by Chappell, Cohn & Greville (1963) 100 μM -atractylate was without detectable effect on the rates and extents of oxygen uptake and H^+ production induced by Ca^{2+} , Sr^{2+} or Mn^{2+} .

DISCUSSION

In Table 1 the results of investigations by other authors and those reported in the present paper on the effects of atractylate and oligomycin on a variety of reactions catalysed by mitochondria and involving the utilization of adenine nucleotides are summarized. Atractylate and oligomycin have a similar effect on a number of reactions involving adenine nucleotides, e.g. both compounds inhibit the phosphorylation of external ADP by the respiratory-chain system, and also dinitrophenol-stimulated adenosine triphosphatase. Oligomycin and atractylate differ in their action in the following respects. Atractylate inhibits the substrate-level phosphorylation of externally added ADP, the ATP-dependent activation of palmitate and the stimulatory effect of ATP on the oxidation of succinate by mitochondria that have been incubated with uncoupling agents. Oligomycin either had no inhibitory effect

or often enhanced the action of ATP. On the other hand, oligomycin inhibited the synthesis of internal ATP at the respiratory-chain level and the dephosphorylation of ATP on adding a respiratory inhibitor, and in these cases atractylate had no effect.

These findings are consistent with the hypothesis that atractylate either (1) inhibits the transfer of adenine nucleotide across a membrane or (2) prevents the transfer of 'energy-rich' phosphate across a mitochondrial membrane (Scheme 3). In Scheme 3(a) it is visualized that the adenine nucleotide is the moiety that is transferred, whereas in Scheme 3(b) it is the phosphorylium group which is transferred. In either case the results presented in Table 1 are consistent with inhibition of the transfer process by atractylate.

Some support for Scheme 3(a) is derived from the results of Bruni, Luciani & Contessa (1964a), who showed that atractylate but not oligomycin or antimycin inhibited the binding of ^{32}P -labelled adenine nucleotides to liver mitochondria. M. Klingenberg (personal communication) has shown that ^{14}C -labelled adenine nucleotides exchange with internal adenine nucleotides and that this exchange is inhibited by atractylate. It is visualized that when submitochondrial particles are produced the adenine nucleotide-transferring system postulated in Scheme 3(a) is no longer used, thus accounting for the fact that ox-heart submitochondrial particles catalyse respiratory-chain phosphorylation that is insensitive to atractylate (Löw *et al.* 1963). Some, but not all, of the results obtained by Vignais *et al.* (1962)

Table 1. *Effects of atractylate and oligomycin on reactions utilizing or requiring adenine nucleotides in mitochondria*

References: ¹ Slater *et al.* (1964); ² this paper; ³ but see Moret *et al.* (1963); ⁴ Lardy *et al.* (1958); ⁵ Bruni *et al.* (1962); ⁶ Vignais *et al.* (1962); ⁷ Chappell & Greville (1961); ⁸ Charles *et al.* (1963); ⁹ Danielson & Ernster (1963); ¹⁰ Bruni *et al.* (1964b); ¹¹ Estabrook (1961); ¹² Chappell (1961).

ATP synthesis		Oligomycin	Atractylate
Respiratory-chain level	Internal ADP	Inhibits ^{1,2}	No effect ^{2,3}
	External ADP	Inhibits ⁴	Inhibits ^{3,6}
Substrate level	Internal ADP	No effect ^{2,7,8}	No effect ²
	External ADP	No effect ^{7,8,9}	Inhibits ^{2,10}
Adenosine triphosphatase			
Dinitrophenol-stimulated			
On adding antimycin		External ATP Inhibits ⁴	Inhibits ^{5,6}
Nicotinamide nucleotide reduction by:		Internal ATP Inhibits ²	No effect ²
Palmitate + carnitine + ATP		No effect ²	Inhibits ²
Palmitoylcarnitine		No effect ²	No effect ²
Palmitoyl-CoA		No effect ²	No effect ²
Adenylate kinase		No effect ²	No effect ^{2,6}
Arsenate-stimulated respiration			
Without ADP		Inhibits ¹¹	No effect ²
With ADP		Inhibits ¹¹	Inhibits ADP effect ²
Activation of succinate oxidation by ATP		Enhances ^{2,12}	Inhibits ²

Scheme 3. Alternatives for the possible mechanism of action of atractylate: (a) adenine nucleotide transferred; (b) phosphorylation group transferred. Z, Hypothetical phosphate carrier; $\Rightarrow$, sites of inhibition by atractylate and oligomycin.

with digitonin-treated particles from liver mitochondria may be accounted for in this way.

It appears that the use of atractylate has perhaps revealed the existence of a 'permease' that allows adenine nucleotides to be transferred across the mitochondrial membrane.

We thank Miss Jenny Flemans for expert technical assistance, the Royal Society for a Parliamentary grant-in-aid for the construction of the fluorimeter and the Medical Research Council for a grant for expenses.

REFERENCES

- Azzone, G. F. & Ernster, L. C. (1960). *Nature, Lond.*, **187**, 65.
- Bendall, D. S. & de Duve, C. (1960). *Biochem. J.* **74**, 444.
- Borst, P. & Slater, E. C. (1960). *Biochim. biophys. Acta*, **41**, 170.
- Borst, P. & Slater, E. C. (1961). *Biochim. biophys. Acta*, **48**, 362.
- Bruni, A., Contessa, A. R. & Luciani, S. (1962). *Biochim. biophys. Acta*, **60**, 301.
- Bruni, A., Luciani, S. & Contessa, A. R. (1964a). *Nature, Lond.*, **201**, 1219.
- Bruni, A., Luciani, S., Contessa, A. R. & Azzone, G. F. (1964b). *Biochim. biophys. Acta*, **82**, 630.
- Chan, P. C., Lehninger, A. L. & Enns, T. (1960). *J. biol. Chem.* **235**, 1790.
- Chance, B. & Baltscheffsky, H. (1958). *J. biol. Chem.* **233**, 736.
- Chappell, J. B. (1961). In *Biological Structure and Function*, vol. 2, p. 71. Ed. by Goodwin, T. W. & Lindberg, O. London: Academic Press (Inc.) Ltd.
- Chappell, J. B. (1964a). *Biochem. J.* **90**, 237.
- Chappell, J. B. (1964b). *Abstr. 6th int. Congr. Biochem.*, New York, p. 625.
- Chappell, J. B., Cohn, M. & Greville, G. D. (1963). In *Energy-Linked Functions of Mitochondria*, p. 219. Ed. by Chance, B. New York: Academic Press Inc.
- Chappell, J. B. & Crofts, A. R. (1965a). *Biochem. J.* **95**, 378.
- Chappell, J. B. & Crofts, A. R. (1965b). *Biochem. J.* **95**, 393.
- Chappell, J. B. & Greville, G. D. (1961). *Nature, Lond.*, **190**, 502.
- Chappell, J. B. & Greville, G. D. (1963). *Symp. biochem. Soc.* **23**, 39.
- Charles, R., Tager, J. M. & Slater, E. C. (1963). *Biochim. biophys. Acta*, **74**, 33.
- Cohn, M. (1953). *J. biol. Chem.* **201**, 735.
- Cohn, M. & Drysdale, G. R. (1955). *J. biol. Chem.* **216**, 831.
- Danielson, L. & Ernster, L. (1963). *Biochem. biophys. Res. Commun.* **10**, 85.
- Drysdale, G. R. & Cohn, M. (1958). *J. biol. Chem.* **233**, 1574.
- Estabrook, R. W. (1961). *Biochem. biophys. Res. Commun.* **4**, 89.
- Garbus, J. & Weinbach, E. C. (1963). *Fed. Proc.* **22**, 405.
- Greville, G. D. & Chappell, J. B. (1959). *Biochim. biophys. Acta*, **33**, 267.
- Hunter, F. E. (1951). In *Phosphorus Metabolism*, vol. 1, p. 297. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
- Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
- Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Arch. Biochem. Biophys.* **78**, 587.
- Löw, H., Vallin, I. & Alm, B. (1963). In *Energy-Linked Functions of Mitochondria*, p. 5. Ed. by Chance, B. New York: Academic Press Inc.
- Moret, V., Pinna, L. A., Sperti, S., Lorini, N. & Siliprandi, N. (1963). *Biochim. biophys. Acta*, **78**, 547.
- Purvis, L. J. & Lowenstein, J. M. (1961). *J. biol. Chem.* **236**, 2794.
- Siekevitz, P. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 237.
- Slater, E. C., Kemp, A. & Tager, J. M. (1964). *Nature, Lond.*, **201**, 781.
- Vignais, P. V., Vignais, P. M. & Stanislas, E. (1962). *Biochim. biophys. Acta*, **51**, 394.