Ochratoxin A, an Inhibitor of Mitochondrial Transport Systems†

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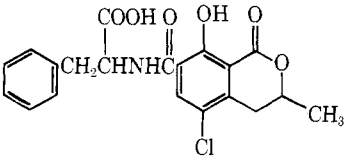
ABSTRACT: The effects of ochratoxin A, a mycotoxin produced by *Aspergillus ochraceus* that is responsible for acute liver damage in animals, have been examined in preparations of rat liver mitochondria. Respiration stimulated by ADP is progressively inhibited up to 0.4 mM ochratoxin A. At 2.5 mM succinate, there is also a marked inhibition of *m*-chlorocarbonylcyanide phenylhydrazone stimulated respiration, which can be overcome by increasing the concentration of succinate to 20 mM. Studies of the effect of ochratoxin A on metabolite anion uptake by mitochondria reveal a competitive type of inhibition with respect to the binding of dicarboxylic acids ([^{14}C]succinate and [^{14}C]malonate), adenine nucleotides (ADP and ATP), and inorganic [^{32}P]phosphate. The apparent 50% inhibition of all the anions examined is reached at 0.02–0.05 mM ochratoxin A, or 10–25 nmol/mg of protein. Respiration in whole mitochondria, using 2.0 mM succinate as substrate, is inhibited 64% by 81 nmol of ochratoxin A/mg of protein. In submitochondrial particles prepared by sonication, there is little or no effect on respiration supported by 1 mM NADH or succinate up to 830 nmol of ochratoxin A/mg of protein. Under these conditions, ochratoxin A therefore acts as a competitive type of inhibitor of mitochrondrial transport carrier proteins. In isotonic 0.16 M monovalent salt solutions, ochratoxin A selectively causes swelling of nonenergized mitochondria in the presence of Na^+ and NH_4^+(Cl), but not with K^+, Li^+, Rb^+, or Cs^+(Cl). Lowering the osmolarity to 0.08 M brings about a swelling with all monovalent cations except Li^+.

The toxic nature of certain metabolic products of the *Aspergillus* genus of fungi toward poultry, fish, animals, and even man, is well documented (Kraybill and Shimkin, 1964; Wogan, 1965; Wright, 1968). The best known toxin is aflatoxin B_1, produced by *Aspergillus flavis*, which, when chronically ingested, produces carcinoma of the liver (Ciegler *et al.*, 1971). Another species of *Aspergillus*, *Aspergillus ochraceus*, produces a mycotoxin designated ochratoxin A (OTA),[1] which is



commonly found in poor quality corn (Shotwell *et al.*, 1969), and if injected into ducks and rats, causes acute liver injury (Theron *et al.*, 1966; Scott, 1965). At the subcellular level, Moore and Truelove (1970) demonstrated a complete inhibition of mitochondrial state III respiration with 0.4 mM OTA. The known effect of atractyloside (Klingenberg and Pfaff, 1966) and oligomycin (Ernster and Lee, 1964) in blocking ADP-stimulated respiration prompted us to examine in more detail the site of action of OTA on mitochondria isolated from rat liver. The results indicate that OTA nonselectively but competitively inhibits several transport processes in mitochondria.

Materials and Methods

Crystallization of Ochratoxin A. Spores of *A. ochraceus* NRRL 3174 were obtained from Dr. C. W. Hesseltine, U.S.D.A., Peoria, Ill., and inoculated into 100 ml of starter medium composed of 4% sucrose, 2% yeast extract, and 10 mM asparagine. Preliminary studies showed that the inclusion of at least 3 mM asparagine led to fourfold increase in ochratoxin A production. Reddy *et al.* (1971) have also shown that the presence of asparagine is essential for high yields of aflatoxin from cultures of *Aspergillus parasiticus*. After 3 days at 25°, the starter culture was shaken well, and 5-ml aliquots were added to 8–10 2.8-l. Fernback flasks containing 300 ml of the sucrose–yeast–asparagine media. After 6–7 days at 25°, the medium was filtered through a double layer of cheesecloth, and the mycelial mat was washed with a small volume of distilled water and combined with the filtrate. The washed mat, which preliminary experiments indicated contained about 5% of the ochratoxin A, was discarded. The medium ws passed slowly through a short column of 100 g of Dowex 1-formate (50 mesh) in a cylindrical 1-l. separatory funnel (Davis *et al.*, 1972). Ochratoxin A, trapped in the top of the column, was eluted with 10 N formic acid in 50% methanol, and detected by thin-layer chromatography on silica gel plates (Nesheim, 1969). The fractions containing ochratoxin A were pooled, extracted four times with equal volumes of chloroform, concentrated, and applied to a chloroform-packed, benzene-equilibrated silica gel column. Elution was carried out with benzene–acetic acid (88:12, v/v), and ochratoxin A was crystallized according to Nesheim (1969) from benzene. Based on thin-layer chromatography, the final preparation contained no more than 1–2% ochratoxin B. The yield from 2 l. of medium was about 100–120 mg.

Mitochondria. Rat liver mitochondria were prepared as previously described (Meisner *et al.*, 1972). Protein was determined by a modified biuret procedure (Kroger and Klingenberg, 1966).

Submitochondrial particles were prepared by suspending twice-washed rat liver mitochondria at 25 mg/ml in a medium of 10 mM Tris–Mops, 1 mM succinate, 1 mM ATP, and 5 mM $MgCl_2$ (pH 7.4), sonicating with four, 15-sec bursts at maximum setting (Artek Dismembrator), at a temperature of −10°. Unbroken mitochondria were removed by centrifuging for 15 min at 10,000 rpm, followed by a 100,000*g*, 40-min, centrifugation of the supernatant. The pellet was made up to 20

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[1] Abbreviations used are: OTA, ochratoxin A; Mops, morpholinopropanesulfonic acid; MCCP, *m*-chlorocarbonylcyanide phenylhydrazone.

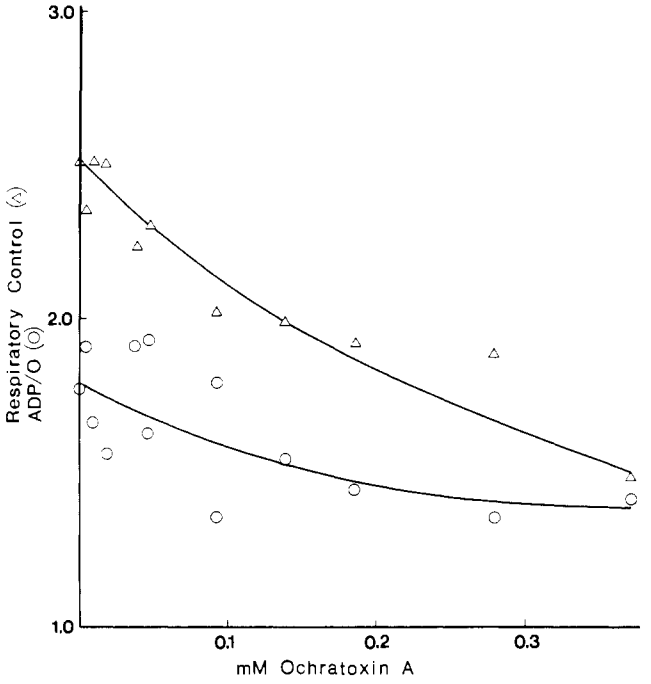

FIGURE 1: Effect of ochratoxin A on the ADP:O and respiratory control ratios in mitochondria. Respiration rates of rat liver mitochondria (1.12 mg) were measured as described in Methods, with 10 mM succinate, 10^{-6}M rotenone, followed by 0.2 mM ADP. Temperature, 30°. Respiratory control and ADP:O ratios are measured according to Chance and Williams (1956). Ochratoxin A was dissolved in absolute alcohol, and added as indicated.

mg/ml with 0.25 M sucrose–10 mM Mops (pH 7.4) and frozen at −80° in 10% dimethyl sulfoxide until needed.

Exchange of Adenine Nucleotides. Mitochondria were labeled with 0.5 μCi of [^{14}C]ADP, and the exchange of unlabeled ADP or ATP was measured using the "inhibitor stop" method (Pfaff *et al.*, 1969; Meisner, 1971). The exchange was carried out at 0 or 10° in a medium of 0.2 M sucrose–10 mM Mops (pH 7.2)–20 mM KCl, and terminated by the addition of 10 μM atractyloside. After centrifugation, radioactivity in the supernatant was determined by scintillation counting. Adenine nucleotides were determined enzymatically (Lamprecht and Trautschold, 1958).

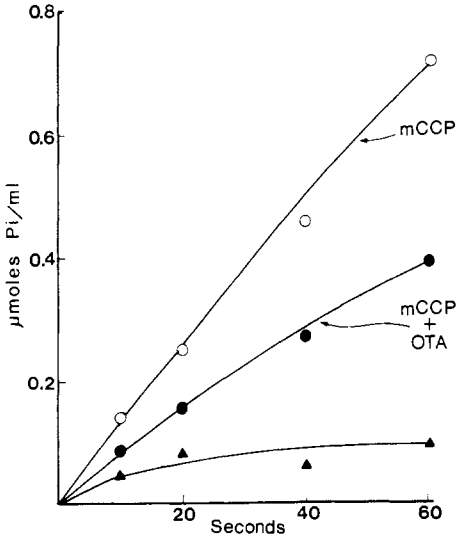

FIGURE 2: Effect of ochratoxin A on *m*-chlorocarbonylcyanide phenylhydrazone stimulated ATPase activity. Mitochondria (2.6 mg/ml) were incubated in 80 mM KCl–5 mM Mops (pH 7.2) at 25° for 1 min. The reaction was started with 1.25 mM Tris–ATP, and terminated at the indicated times by pipetting into perchloric acid. When added, mCCP = 0.5 μM (open circles); ochratoxin A = 0.1 mM (closed circles); oligomycin = 5 μg/ml (triangles).

Uptake of Substrates. The uptake of [^{14}C]malonate and [^{14}C]succinate was determined as described (Meisner *et al.*, 1972), at 2 or 5° in 0.2 M sucrose, 10 mM KCl, and 5 mM Tris–Mops (pH 6.3), plus 2.5 μg of oligomycin per ml, and 10^{-6} M rotenone (malonate) or 2.5 μg of antimycin A (succinate). Soluene (0.2 ml) was used to solubilize the pellets, which were then counted by liquid scintillation.

Respiration. Mitochondria were introduced into a medium consisting of 0.1 M sucrose, 20 mM KCl, 5 mM $MgCl_2$, 10 mM phosphate, 10^{-6} M rotenone, and 10 mM Tris–Mops (pH 7.2). Respiration rates were determined by polarography, using succinate as substrate, in a total volume of 0.5 ml.

Other experimental methods are described in the appropriate Results section.

Results

Figure 1 shows the effect of OTA on mitochondrial respiration in the presence of rotenone and 10 mM succinate. Up to 0.4 mM OTA, there is a progressive decrease in the respiratory control and ADP:O ratio. In this example, the respiratory control ratio appears to decrease more than the ADP:O ratio, but changing the concentration of succinate or ADP modifies this relative sensitivity. Although not shown, state IV respiration exhibits no noticeable increase in the presence of OTA. Furthermore, when the effects of ochratoxin B were examined on respiration, there was no change in the respiratory control or ADP:O ratio.

Moore and Truelove (1970) have observed that 0.4 mM OTA stimulates state IV respiration, suggestive of a weak uncoupling effect. The polarographic results reported in Figure 1 also indicate a partial uncoupling, as shown by the slight decrease in ADP:O ratio. When ATPase activity was measured, however, there was no activation up to 0.33 mM, or 166 nmol of OTA/mg of protein, even in the presence of 10 mM magnesium. Furthermore, measurements of mitochondrial ATP and ADP reveal no effect of OTA at 0° on the adenine nucleotide level.

Figure 2 indicates that OTA decreases the uncoupler-induced ATPase activity, as measured by P_i release, in much the same manner as oligomycin, a known inhibitor of oxidative phosphorylation. In this case, the addition of 38 nmol of OTA/mg of protein reduces mCCP-stimulated ATPase activity 50%. Unlike inhibitors of oxidative phosphorylation, however, Figure 3A shows that OTA progressively inhibits uncoupler-stimulated respiration. Furthermore, the addition of 20 mM succinate (3B) completely overcomes the inhibition of respiration caused by OTA.

The inhibition of state III respiration reported in this paper, as well as by Moore and Truelove (1970), suggested that OTA may have an atractyloside-like effect. Atractyloside is a toxic glycoside that prevents the binding of ADP or ATP to the adenine nucleotide translocase carrier located on the inner mitochondrial membrane (Bruni, 1966; Klingenberg and Pfaff, 1966). Figure 4 shows a Lineweaver–Burk plot of the effect of OTA on the exchange of external ADP with prelabeled mitochondria. There is an increase in the K_m from 7.1 to 32 μM by 0.14 mM OTA, and no change in the V_{max}, indicating a competition between OTA and the nucleotide. OTA also acts as a competitive inhibitor of the ATP exchange, with a 50% reduction achieved at 0.042 mM OTA.

The inhibition of endogenous phosphorylation has been used by Heldt *et al.* (1965) to emphasize the distinctness of the translocase carrier and the ATP-synthetase system. Atractylos-

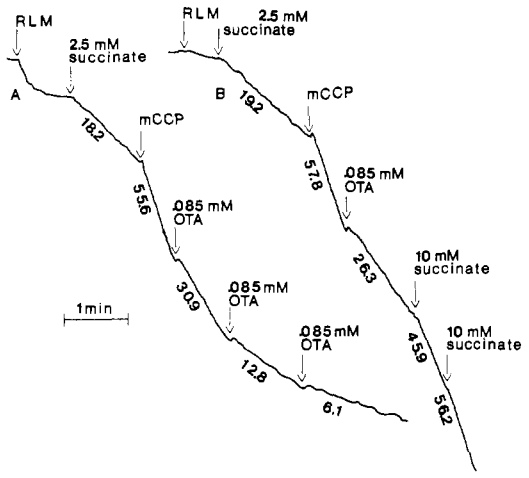

FIGURE 3: Inhibition of mCCP stimulated respiration by ochratoxin A. Mitochondria (0.8 mg) were incubated at 25° in a medium described in Methods, plus 1 μM mCCP, and succinate or ochratoxin A as indicated. Rates are expressed as nmol/mg per min.

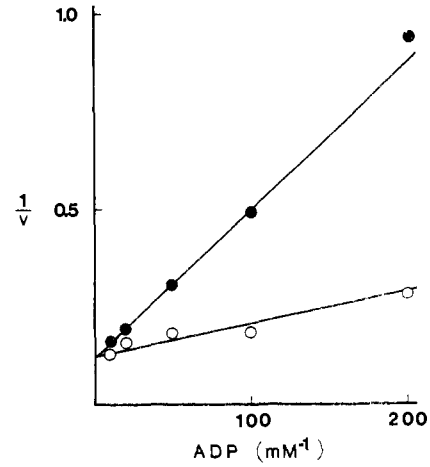

FIGURE 4: Double-reciprocal plot of the rate of ADP exchange in the presence of ochratoxin A. Prelabeled mitochondria (0.98 mg) were incubated at 10° for 1 min with 0.14 mM ochratoxin A, the reaction initiated with ADP as indicated, and stopped at 10 sec with atractyloside. v, nmol/mg per min; (●) plus ochratoxin A.

ide effectively inhibits the phosphorylation of exogenous ADP, but has no effect on intramitochondrial ADP phosphorylation, while inhibitors of oxidative phosphorylation such as oligomycin block only the endogenous synthesis of ADP. Figure 5A shows the effect of 0.24 mM OTA on the rate of endogenous ADP phosphorylation by external 1 mM [^{32}P]P_i at a temperature of 5°. The reaction was started by bubbling 10 cm^3 of air into the anaerobic mitochondrial suspension containing [^{32}P]P_i and the inhibitor in question. It is evident that OTA and oligomycin both completely inhibit phosphorylation of internal ADP, whereas atractyloside has little or no effect. Under these experimental conditions, OTA has no effect on succinate uptake (see Figure 7.). From Figure 5B, the concentration of OTA required for 50% inhibition is 11.4 nmol/mg of protein, or 0.05 mM.

The results thus far indicate that OTA can inhibit the exchange of adenine nucleotides, as well as the phosphorylation of endogenous ADP *via* the ATP-synthetase system. We have previously found that the permeant anion tetraphenylboron can block several mitochondrial transport reactions (Meisner, 1973), and it was important to establish whether OTA had effects on these systems as well. For example, the oligomycin-like effect could be explained just as easily by inhibition of phosphate uptake *via* the phosphate transporter described by Fonyo (1968) and Tyler (1968). When the uptake of [^{32}P]P_i was measured, using 2 mM benzylmalonate to inhibit P_i transport *via* the dicarboxylate carrier, and stopping the reaction with 0.6 mM mersalyl, the results presented in Figure 6 were obtained. The V_{max} of the P_i exchange (59 nmol/mg per min) is not affected by 0.027 or 0.054 mM OTA, but there is a steady decrease in the binding affinity, indicative of a competitive inhibitor. Therefore, the inhibition of [^{32}P]P_i incorporation into intramitochondrially generated ATP is more likely due to a prevention of P_i uptake, rather than a direct effect on the ATP-synthetase reaction.

Polarographic data presented in Figure 7 shows that respiration, when measured in the presence of 10 mM phosphate, is inhibited by OTA at low succinate concentrations. The fact that more than ten times the amount of OTA is required to obtain an inhibition at 2 mM than at 0.2 mM succinate suggests that there is a competition between OTA and succinate for binding to the membrane. This view is supported by measurements of the uptake of [^{14}C]succinate or [^{14}C]malonate to mitochondria in the presence of OTA. The kinetics of malonate uptake (Fig-

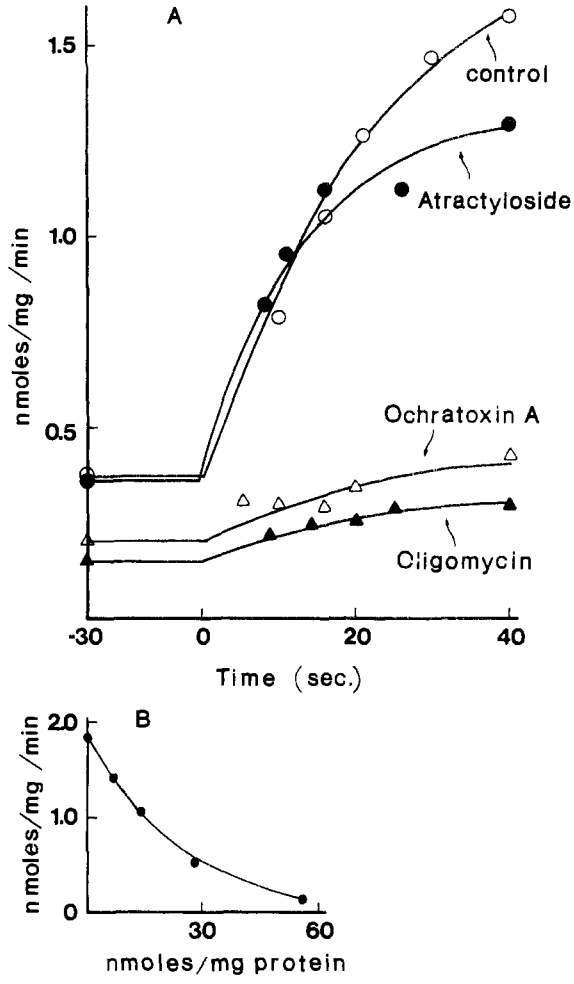

FIGURE 5: (A) Effect of ochratoxin A on phosphorylation of intramitochondrial ADP. Mitochondria (10 mg/ml) were suspended at 5° in 0.25 M sucrose-20 mM triethanolamine-1 mM EDTA-4 mM succinate (pH 7.2). Nitrogen gas was bubbled through the mixture for 10 min to make the suspension anaerobic. Inorganic [^{32}P]phosphate (1 mM, 2 × 10^6 cpm) was added, and the reaction was started 1 min later by injecting 10 cm^3 of air (zero time). When added, ochratoxin A, 180 nmol/mg; oligomycin, 2 μg/mg; atractyloside, 8 μM. The reaction was stopped at the indicated times by adding 100-μl aliquots to 200 μl of 20% perchloric acid, and assaying for organic phosphate (Lindberg and Ernster, 1956). Ordinate, nmol of [^{32}P]P_i incorporated into ATP/min per mg of protein. (B) Incorporation of [^{32}P]P_i into intramitochondrial ADP as a function of ochratoxin A concentration.

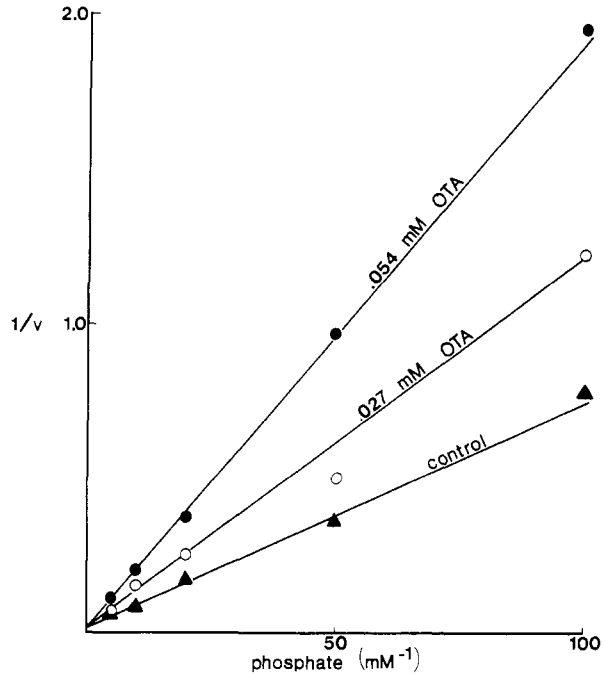

FIGURE 6: Double-reciprocal plot of the effect of ochratoxin A on the P_i exchange. Mitochondria (2.0 mg) were incubated at 2° for 1 min in 0.2 M sucrose, 2 mM benzylmalonate, 10 mM Mops (pH 6.8), 20 mM KCl, plus oligomycin (2.5 μg), and rotenone (10^{-6}M). The reaction was started with [^{32}P]P_i and terminated with 0.6 mM mersalyl. Rates are expressed as nmol/mg per min^{-1}.

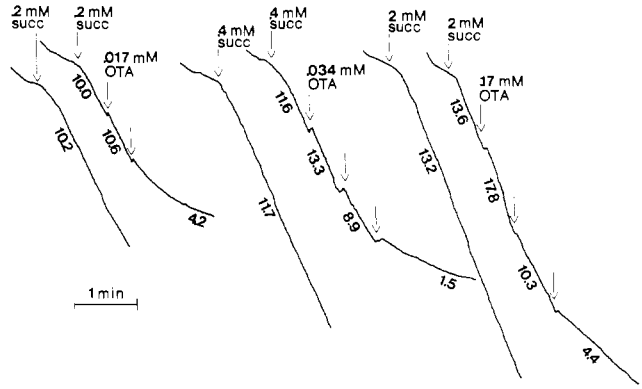

FIGURE 7: Effect of ochratoxin A on respiration in mitochondria at low concentrations of succinate. For details, see Methods. Protein, 3.0 mg. Controls containing up to 4% ethanol showed no effect on the respiratory rate. Rates are expressed as nmol/mg per min.

ure 8) in the presence of 0.033 mM OTA show a common V_{max}, as determined by the least-squares method, indicative of a competitive-type inhibition. Other experiments, not reported here, show that a 50% inhibition of binding of 0.065 mM [^{14}C]malonate is reached at 0.023 mM OTA.

If the inhibitory effect of OTA on respiration is due to interaction with the metabolite carriers, there should be little or no effect on sonicated submitochondrial particles, which are thought to be "inside out" (Lee and Ernster, 1966; Mitchell, 1966), and therefore have carrier sites which are facing inward. In Figure 9, it can be seen that respiration supported by 1 mM NADH or 1 mM succinate (plus rotenone) is only inhibited by 18 and 4%, respectively, compared to control samples. More importantly, the negative results were obtained at OTA concentrations of 830 nmol/mg of protein (NADH) and 625 nmol/mg of protein (succinate). In contrast, respiration rates of whole mitochondria incubated with 2 mM succinate (Figure 7) were inhibited by 64% at 83 nmol/mg of protein. It is therefore apparent that submitochondrial particles are very refractory to the effects of OTA.

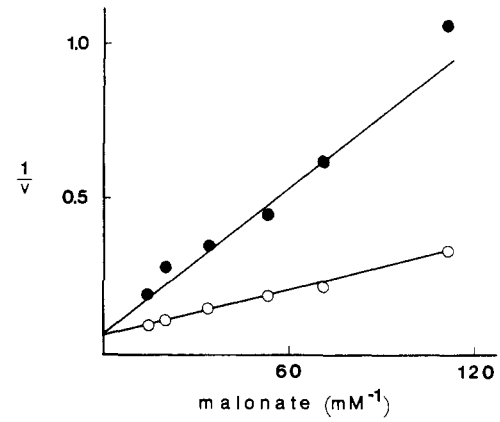

FIGURE 8: Effect of ochratoxin on the uptake of [^{14}C]malonate. Mitochondria (2.0 mg) were incubated at 2° as described in Methods. The uptake was started with [^{14}C]malonate, and stopped at 6 sec by 5 mM benzyl malonate. (●) Plus 0.033 mM ochratoxin A. Rates are expressed as nmol/mg per min^{-1}.

Swelling of mitochondria has been used by Chappell and Crofts (1966) as an indicator of changes in passive ion permeability toward various inhibitors. In Figure 10, mitochondria were added to cuvets containing 0.16 M salt plus 5 mM Tris-Mops (pH 7.2) and optical density changes were measured at 546 nm in the presence of antimycin A and rotenone to block oxidation of endogenous substrates. Of the monovalent cations tested, only Na^+ and NH_4^+ exhibited a pronounced increase in permeability, as reflected in the decreased optical density upon addition of OTA. The initial rates of mitochondrial swelling in Li^+, Cs^+, Rh^+, and K^+ varied between 0.02 and 0.05 ODU per min, compared to 0.12 and 0.20 ODU per min for Na^+ and NH_4^+, respectively. In the absence of energy inhibitors (now shown), there is a pronounced swelling induced by OTA in all salts except LiCl.

The selectivity toward Na^+ and NH_4^+ can also be modified by reducing the osmolarity, as shown in Figure 11. In an 0.08 M KCl solution containing antimycin A and rotenone, addition of 0.083 mM OTA causes and immediate and rapid swelling, which is nearly completely prevented by 5 mM Mg^{2+}. The decrease in optical density was observed with all monovalent cations tested. Lastly, it should be mentioned that there was no discernible effect of OTA on energy-linked, valinomycin-stimulated transport reactions, using a system described by Meisner and Wenner (1970).

The low solubility of OTA in polar solutes suggested that there may be a binding to hydrophobic sites on the mitochondrial membrane. To examine this, mitochondria were incubated for 2 min at 0° with 1.16 mM OTA, and washed once to remove any unbound inhibitor. State IV respiration supported by malonate and pyruvate increased from 5.8 to 13.7 nmol per mg per min, and state III is reduced from 20.2 to 7.8 nmol per mg per min, supporting the view that OTA is bound tightly to the membrane. Recent experiments with ^{14}C-labeled OTA have confirmed and amplified this view.

Discussion

The results suggest that OTA acts to produce a competitive type of inhibitory pattern with respect to the mitochondrial transport carriers located in the inner membrane. The fact that the respiration rate in submitochondrial particles is not affected by OTA strengthens the concept that the site of OTA is the surface of the intact mitochondrion. Under the conditions of sonication, the particles have probably lost the properties of

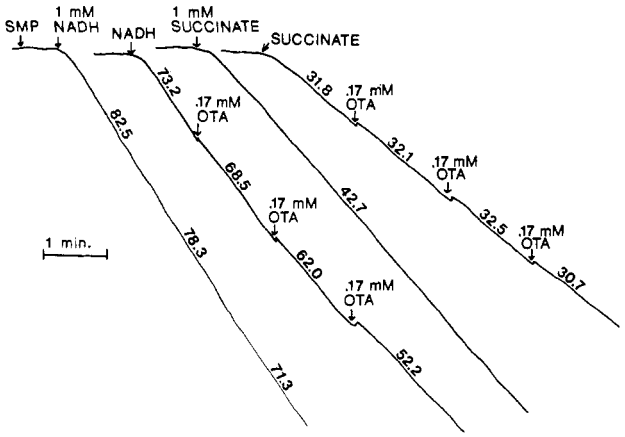

FIGURE 9: Effect of ochratoxin A on respiration in submitochondrial particles. Respiration rates of sonicated particles were measured in 0.25 M sucrose, 10 mM Mops, 5 mM $MgCl_2$, 1 mM P_i (pH 7.4), and either 1 mM NADH or 1 mM succinate (plus rotenone). Protein, 0.3 mg (NADH); 0.4 mg (succinate). Final volume, 0.5 ml, temperature 25°. Rates are expressed as nmol/mg per min.

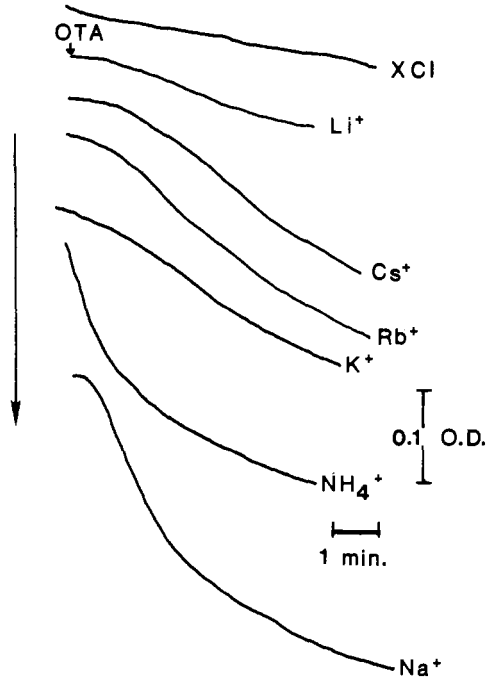

FIGURE 10: Permeability of energy-inhibited mitochondria toward monovalent cations induced by ochratoxin A. Mitochondria (0.43 mg/ml) were incubated with 0.16 M XCl, 5 mM Mops (pH 7.2), 22 μg of antimycin A, and 2.4 μM rotenone. Ochratoxin A, 0.1 mM, was added to induce swelling, measured at 546 nm on an Eppendorf photometer.

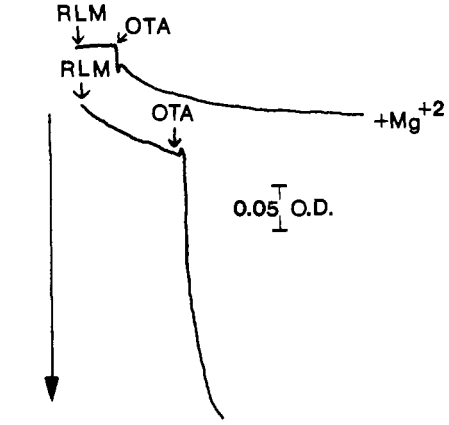

FIGURE 11: Effect of osmolarity and Mg^{2+} on ochratoxin A stimulated swelling in inhibited mitochondria. Mitochondria (0.5 mg/ml) were incubated with 0.08 M KCl and 5 mM Mops (pH 7.2), 1 μg of antimycin A, 10^{-7} M rotenone, and, when indicated, 0.083 mM ochratoxin A or 10 mM $MgCl_2$.

the transport system (Astle and Cooper, 1974), rather than being turned "inside out," as proposed by Lee and Ernster (1966).

The inhibition of respiration in whole mitochondria at low substrate levels is probably due to competitive inhibition by OTA of the dicarboxylate carrier. The modest decrease in ADP-stimulated respiration is due to the partial block in ADP translocation, which allows phosphorylation to proceed, but at a low rate. The inhibition by OTA of the phosphorylation of intramitochondrial ADP can be attributed to a block in the transport of P_i across the membrane, rather than a direct effect on the ATP-synthetase system. The low apparent K_i of 0.05 mM for endogenous ADP phosphorylation is similar to the inhibitory constant found for the P_i transporter. It should be stressed that K_i values reported herein are only apparent, because the low solubility of OTA in aqueous solvents makes it likely that most of the toxin is bound to hydrophobic areas, a view that is supported by recent [^{14}C]OTA binding studies. Thus, in contradistinction to Moore and Truelove (1970), our results do not support a pharmacological effect of OTA on energy-conserving reactions.

Of the alkali metals examined, passive mitochondrial swelling by OTA shows a marked discrimination for Na^+ over K^+, Rb^+, or Cs^+. The lack of swelling with Li^+ is of dubious importance due to the negative results even in the absence of antimycin A and rotenone. It is unclear why OTA is specific for Na^+ among the alkali metals, but a consideration of the structure of OTA suggests that the high charge density of Na^+ (Diamond and Wright, 1969) may be great enough to allow Na^+ to accommodate itself near the electronegative center of OTA. The three cations of weaker charge density would not make such a strong bond. The loss of selectivity toward Na^+ at hypotonic conditions may be due to the greater membrane permeability under these conditions.

No direct evidence has been presented here concerning whether the mitochondrion is the physiological site of action of OTA. However, from the published LD_{50} values of 20–22 mg/kg reported for rats (Purchase and Nel, 1967), and assuming an equal distribution in the body, it may be calculated that this results in a level of OTA of about 0.05 mM in the liver. In preliminary studies we have evidence that forced feeding of an LD_{50} dose of [^{14}C]OTA to rats results, after 3 or 24 hr, in a liver concentration of 0.01 mM OTA. The fact that most of the toxin must be osmotically inactive renders such values open to criticism. Nonetheless, the similarity to the apparent K_i values presented here of 0.02–0.05 mM supports the possibility that the observed pathological symptoms of OTA poisoning are related to its effects on mitochondria, and in particular, on transport carriers found in the membrane.

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Formation of High-Energy Phosphate Bonds Effected by Electron-Deficient Sulfides†

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ABSTRACT: Electron-deficient sulfides are postulated as intermediates in the formation of a phosphorylated sulfonium salt which has previously been suggested as the high energy phosphorylating intermediate of respiratory chain-linked oxidative phosphorylation. To test the ability of electron-deficient sulfides to effect phosphorylation of adenine nun the presence of orthophosphate, an aromatic sulfur cation radical and dication are used as models. Treatment of the tetra-*n*-butylammonium salts of adenosine 5′-monophosphate and orthophosphoric acid in anhydrous acetonitrile with thianthrene perchlorate in the molar ratio of 1:1:2 results in the rapid formation of adenosine 5′-diphosphate and triphosphate in a combined yield of 16% based on the amount of thianthrene perchlorate added or 52% yield, based on the amount of adenosine 5′-monophosphate consumed. The thianthrene perchlorate is converted to thianthrene and thianthrene sulfoxide. Similar reactions with 2,3,7,8-tetramethoxythianthrene diperchlorate in place of thianthrene perchlorate result in the rapid formation of adenosine 5′-diphosphate and triphosphate in a combined yield of 19% based on dication added or 73% yield based on adenosine 5′-monophosphate consumed. Evidence concerning the mechanisms of these reactions is presented and discussed as well as their biological significance. In particular, theoretical consideration of electron-deficient aliphatic sulfides as intermediates in oxidative phosphorylation is presented. A key suggestion is that the oxidation potential of aliphatic sulfides and the stability of aliphatic sulfur cation radicals and/or dications can be affected by neighboring group participation.

The mechanism by which energy is conserved in respiratory chain-linked oxidative phosphorylation remains unknown despite intensive investigation (Lardy and Ferguson, 1969). Three suggestions have been made for the primary energy conserving step: (1) formation of a high-energy chemical intermediate (Lipmann, 1946; Slater, 1953), (2) translocation of ions across a membrane resulting in a potential gradient (Mitchell, 1961, 1966, 1968), and (3) formation of a high-energy conformation of a macromolecule (Boyer, 1965). However, no definitive evidence has established as yet which one of these processes is the primary one.

The hypothesis that a high-energy chemical intermediate is formed in mitochondrial oxidative phosphorylation has inspired several groups to devise model systems in which oxidation is coupled to phosphorylation by such an intermediate. Oxidation

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