TOXICOLOGY AND APPLIED PHARMACOLOGY 62, 282-287 (1982)

In Vitro Effects of 10,10'-Oxybisphenoxarsine on Isolated Rat Liver Mitochondria[1]

JOEL M. KRONENBERG AND MICHAEL J. BRABEC

Toxicology Program, Department of Environmental and Industrial Health, The University of Michigan, Ann Arbor, Michigan 48109

Received July 1, 1981; accepted August 31, 1981

In Vitro Effects of 10,10'-Oxybisphenoxarsine on Isolated Rat Liver Mitochondria. KRONENBERG, J., AND BRABEC, M. J. (1982). *Toxicol. Appl. Pharmacol.* **62,** 282-287. 10,10'-Oxybisphenoxarsine (OBPA), a trivalent, heterocyclic organoarsenical, is a potent, broad spectrum antimicrobial agent often incorporated into plasticized polyvinyl chlorides. Although highly toxic, the mechanism of OBPA toxicity is unknown. Since it is hepatotoxic, we investigated the *in vitro* effects of OBPA on rat liver mitochondria. Isolated mitochondria were incubated with 1 to 8 μM OBPA and oxygen consumption was monitored polarographically. State 3 respiration of α-ketoglutarate, pyruvate, isocitrate, and succinate was rapidly inhibited. OBPA also uncoupled oxidative phosphorylation, although this was masked, at higher concentrations, by progressive respiratory inhibition. The electron transport chain appeared unaffected by OBPA, as measured by NADH oxidation, but several tricarboxcylic acid cycle dehydrogenases were inhibited. This inhibition was prevented and, in some cases, reversed by glutathione. It is likely that OBPA, like other trivalent organoarsenicals, exerts its toxic effects by reacting with vital mitochondrial sulfhydryl groups.

10,10'-Oxybisphenoxarsine (OBPA), a trivalent, heterocyclic organoarsenical (Fig. 1), is a potent, broad spectrum antimicrobial agent often incorporated into plasticized polyvinyl chloride products to prevent bacterial and fungal growth. OBPA is a strong irritant, is hepatotoxic, and has an LD50 of 40 mg/kg in rats and 24 mg/kg in guinea pigs (Ballantyne, 1978). Previous work demonstrated that the liver was a major site for the disposition of radiolabel from orally administered [^{14}C]OBPA (Kronenberg and Hartung, 1981). The mechanism of OBPA toxicity is unknown. However, trivalent organoarsenicals are sulfhydryl reagents capable of reacting with a number of cellular enzymes, including many involved in mitochondrial function (Webb, 1966). Therefore, we decided to investigate the *in vitro* effects of OBPA on the performance of isolated rat liver mitochondria.

METHODS

Preparation of Mitochondria. Mitochondria were prepared from 175- to 250-g, fasted male CD rats[2] essentially as described by Brabec *et al.* (1974). The rats were stunned, decapitated, and exsanguinated. The livers were removed and homogenized in ice-cold isolation medium (250 mM sucrose, 1 mM EDTA, 10 mM KCl, 10 mM Tris, pH 7.4). The homogenate was centrifuged for 10 min at 500*g* and the resulting supernatant fluid for 10 min at 7500*g*. The crude mitochondrial pellet was twice resuspended in 250 mM sucrose and centrifuged for 10 min at 4900*g*. The final pellet was resuspended in the isolation medium to give ca. 30 mg protein/ml,

[1] Parts of this work were presented at Toxicology in Michigan Today, Lansing, Mich. May 1981.

[2] Charles River Breeding Laboratories, Inc., Wilmington, Mass.

0041-008X/82/020282-06$02.00/0

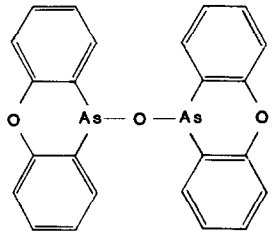

FIG. 1. 10,10'-Oxybisphenoxarsine (OBPA).

as determined by the Biuret procedure (Gornall *et al.*, 1949).

Respiration rates. Respiration rates were determined polarographically in a YSI Model 53 Oxygen Monitor, as described by Brabec *et al.* (1974). Mitochondria were assayed at 25°C in Oxygen Electrode Buffer (150 mM sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM KH_2PO_4, 20 mM Tris, pH 7.4). Succinate oxidation rates were determined in 7.4 mM succinate and 10 μM rotenone. Isocitrate, α-ketoglutarate, and pyruvate oxidation rates were determined in the presence of 1 mM malate and 3.7 mM of the respective substrate. Unless otherwise specified, 2.3 mg mitochondrial protein was in a total chamber volume of 2.7 ml. All mitochondrial preparations had a respiratory control ratio (with succinate) greater than 5. To determine the effects on state 3 respiration, OBPA[3] (dissolved in 20–40 μl EtOH) was added to the reaction chamber shortly after the addition of excess ADP (4 μmol). State 4 effects were determined by adding OBPA to the reaction chamber during the state 4 respiration following the addition of 0.2 μmol ADP. To measure NADH oxidation, mitochondria (normally impermeable to NADH) were made permeable by swelling in 40 mM KH_2PO_4 (pH 7.4) and 0.3 mM $CaCl_2$ for 1 min before adding 10 μmol NADH followed by OBPA. EtOH controls were run for all experiments.

Enzyme assays. All enzymes were assayed at 25°C by following the reduction of NAD(P) at 340 nm. To

[3] No detectable impurities by HPLC or TLC.

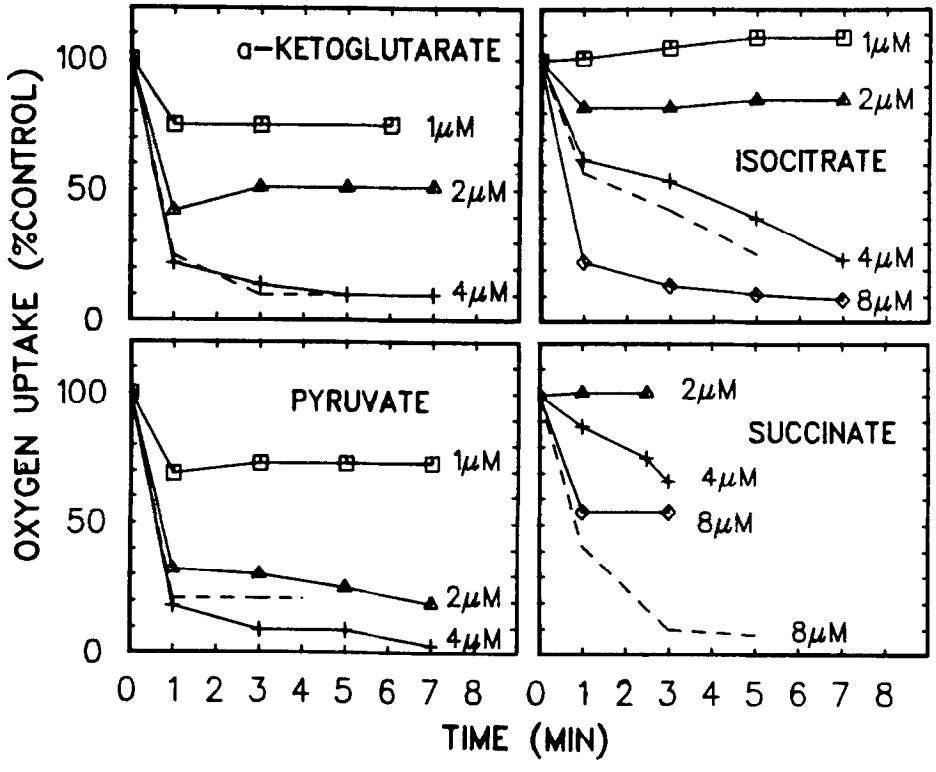

FIG. 2. The effects of OBPA on state 3 oxidation of α-ketoglutarate, pyruvate, isocitrate, and succinate. Mitochondria were incubated with 1 to 8 μM OBPA as described under Methods. Solid lines represent 2.3 mg mitochondrial protein and the indicated OBPA concentration. Dashed lines represent 1.15 mg mitochondrial protein and 2 μM OBPA, unless otherwise marked. Average 100% oxygen uptake rates (nmol O_2/min/mg protein) were α-ketoglutarate, 19.0; pyruvate, 14.9; isocitrate, 22.3; succinate, 65.4.

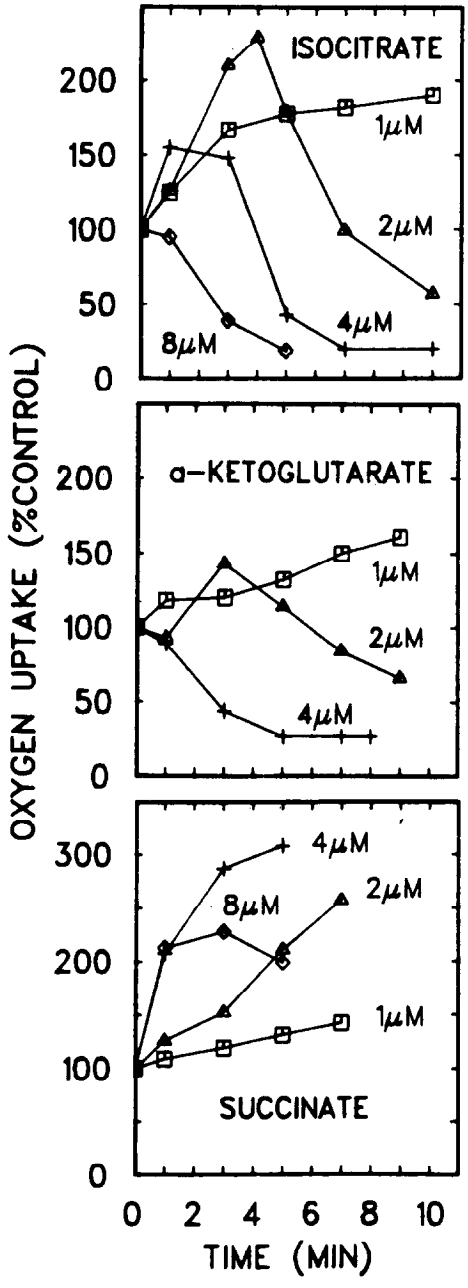

FIG. 3. The effects of OBPA on state 4 oxidation of α-ketoglutarate, isocitrate, and succinate. Mitochondria (2.3 mg protein) were incubated with 1 to 8 μM OBPA as described under Methods. Average 100% oxygen uptake rates (nmol O_2/min/mg protein) were: α-ketoglutarate, 5.5; isocitrate, 5.0; succinate, 10.6.

assay α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, whole mitochondria (2.3 mg protein) were incubated (±OBPA) in Oxygen Electrode Buffer with 1.4 mM NAD, 1 mM ADP, and 7 mM α-ketoglutarate or pyruvate (2.7 ml total volume). After 2 min, the mitochondria were solubilized with 0.1 ml 3% (w/v) Triton X-100 and the reaction was initiated with 0.54 μmol CoASH. Both assays were linear for about 1 min.

NAD-specific isocitrate dehydrogenase (NAD-ICDH) and NADP-specific isocitrate dehydrogenase (NADP-ICDH) assays were done essentially as described by Plaut (1969) and Cleland *et al.* (1969), respectively, using the 26,000*g* supernatant fraction from sonicated mitochondria. The mitochondrial supernatant fraction was incubated for 5 min in 3.0 ml 33.3 mM Tris (pH 7.4), 1.33 mM $MnSO_4$, 0.09 mM NAD(P), and 0.5 mM KCN. Enough supernatant fraction was used to give a ΔA/min of 0.01–0.05 (0.3–2.1 mg protein). The NADP-ICDH assay was initiated with 5 μmol isocitrate while 5 μmol isocitrate and 2 μmol ADP initiated the NAD-ICDH reaction. A blank lacking NAD was needed to determine NAD-ICDH activity because of an interfering reaction involving OBPA. This reaction was insignificant compared to the other enzyme activities assayed.

RESULTS

The effects of OBPA on mitochondrial state 3 respiration are seen in Fig. 2. The oxidation of all substrates was rapidly inhibited by 1 to 8 μM OBPA. The extent of inhibition varied with the substrate as well as the amount of mitochondria present. This inhibition was unaffected by the addition of 2,4-dinitrophenol, a common uncoupling agent. OBPA also uncoupled mitochondrial respiration. When added during state 4 respiration (Fig. 3), OBPA caused an initial increase in oxygen uptake followed, at higher concentrations, by progressive respiratory inhibition which masked the uncoupling. The addition of ADP during this period of increasing inhibition had no effect.

To determine if the inhibition of respiration was caused by electron transport chain inhibition, the effect of OBPA on NADH oxidation was examined. The NADH oxidation rate of mitochondria (rendered permeable to NADH by swelling) incubated for 2 min with 8 μM OBPA was 100.8 (±4.8) nmol/min as compared to 97.6 (±7.4) nmol/min in control mitochondria. Therefore,

TABLE 1

THE EFFECTS OF 10,10'-OXYBISPHENOXARSINE ON α-KETOGLUTARATE DEHYDROGENASE (α-KGDH) AND PYRUVATE DEHYDROGENASE (PDH) ACTIVITIES

	α-KGDH		PDH	
Treatment	Sp Act[a]	% control	Sp Act[a]	% control
EtOH	50.9 ± 1.6	100	17.0 ± 0.5	100
OBPA, 4 μM	27.0 ± 0.7	53	4.9 ± 0.4	29

[a] nmol NADH/min/mg mitochondrial protein, mean ± SE, $n \geq 7$.

NADH oxidation was not inhibited by OBPA and the inhibition of respiration observed earlier must have occurred at sites preceding NAD-CoQ reductase (when using the NAD-linked substrates) and CoQ-cytochrome *b* reductase (when using succinate, a FAD-linked substrate).

Since electron transport did not appear to be the site of respiratory inhibition caused by OBPA, we directly examined the effects of OBPA on several TCA cycle dehydrogenases. Table 1 shows that OBPA was a potent inhibitor of both pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. There are two enzymes involved in the oxidation of isocitrate by rat liver mitochondria, NAD-specific and NADP-specific isocitrate dehydrogenase (Smith and Plaut, 1979). Both were inhibited by OBPA (Table 2).

TABLE 2

THE EFFECTS OF 10,10'-OXYBISPHENOXARSINE ON NAD-SPECIFIC AND NADP-SPECIFIC ISOCITRATE DEHYDROGENASE ACTIVITIES[a]

Treatment	NAD-ICDH	NADP-ICDH
EtOH	100	100.0
OBPA, 2 μM	88.4 (±2.3)	98.0 (±2.0)
OBPA, 4 μM	80.1 (±2.0)	69.0 (±1.0)
OBPA, 8 μM	52.1 (±2.6)	52.5 (±4.5)

[a] Because the specific activity of the sonicated mitochondrial supernatant fraction varied between experiments, all activities are expressed as percentage control, mean ± SE of at least two experiments.

The effect of OBPA on succinate dehydrogenase was not examined.

We also tested the effects of GSH on the inhibition of two of these enzymes by OBPA. GSH (1 mM) protected α-ketoglutarate dehydrogenase from inhibition by 4 μM OBPA and restored most of the activity of previously inhibited enzyme (Table 3). Similar experiments (data not shown) were done with purified NADP-ICDH (Sigma Chemical Co., St. Louis, Mo.). Inhibition by 10 μM OBPA was prevented by 100 μM GSH, but was not reversed by 10 mM GSH.

DISCUSSION

The data presented in this report demonstrate that OBPA, a trisubstituted trivalent organoarsenical, was a potent inhibitor of mitochondrial respiration. OBPA also uncoupled oxidative phosphorylation, although this was masked, at higher concentrations, by progressive inhibition of respiration. The inhibition observed in the presence of ADP (state 3, Fig. 2) occurred more rapidly than the inhibition seen in the absence of ADP (state 4, Fig. 3). This may be caused by conformational changes brought about by ADP (Hackenbrock *et al.*, 1971) leading to altered membrane permeability or by the faster turnover of some component(s) of the TCA cycle during state 3 respiration. The respiratory inhibition observed was apparently not caused by inhibition of

TABLE 3

EFFECTS OF GLUTATHIONE (GSH) ON THE INHIBITION OF α-KETOGLUTARATE DEHYDROGENASE BY 10,10'-OXYBISPHENOXARSINE (OBPA)

Treatment	Specific activity[a] (nmol NADH/min/mg protein)
Expt 1[b]	
EtOH	59.6 ± 1.1
EtOH + 0.5 mM GSH	59.8 ± 1.4
EtOH ± 1.0 mM GSH	53.7 ± 1.6
2 μM OBPA	46.2 ± 1.2
4 μM OBPA	31.2 ± 0.7
4 μM OBPA + 0.5 mM GSH	49.1 ± 0.8
4 μM OBPA + 1.0 mM GSH	54.1 ± 0.9
Expt 2[c]	
EtOH + 1.0 mM GSH	58.8 ± 2.0
4 μM OBPA + H_2O	29.4 ± 0.6
4 μM OBPA + 1.0 mM GSH	48.2 ± 0.7

[a] Mean ± SE, $n \geq 2$.

[b] GSH, if indicated, was added to the mitochondria before OBPA or EtOH. Assay as described under Methods.

[c] GSH, if indicated, was added 2 min after OBPA or EtOH. Mitochondria were then incubated for 1 min and assayed as before.

the electron transport chain since NADH oxidation was unaffected. But, several TCA cycle dehydrogenases were inhibited by OBPA. These enzymes, however, were less sensitive to OBPA inhibition than was mitochondrial respiration. Dissimilar assay conditions may have been responsible for this difference. However, we did not examine the effects of OBPA on substrate transport systems, which may have also been inhibited. The mechanism of uncoupling by OBPA remains unclear It appeared that OBPA caused changes in membrane permeability.

Trivalent organoarsenicals inhibit and uncouple mitochondrial respiration (Fluharty and Sanadi, 1963; Webb, 1966). Monosubstituted trivalent organoarsenicals (e.g., lewisite and phenylarsenoxides) exert their effects by forming a stable dithioarsinite ring structure with susceptible dithiols (e.g., dihydrolipoic acid). A few disubstituted trivalent organoarsenicals have been studied (diphenylchloroarsine and phenarsazines). These can inhibit monothiol enzymes (e.g., NADP-ICDH), probably by forming a stable monothioarsinite (Lotspeich and Peters, 1951; Peters, 1955). OBPA, however, is a *tri*substituted trivalent organoarsenical. Several TCA cycle dehydrogenases, known to be susceptible to sulfhydryl inhibition (Webb, 1966; Plaut, 1970) were inhibited by OBPA. Excess GSH, a monothiol, was able to prevent and reverse inhibition of α-ketoglutarate dehydrogenase, a dithiol enzyme. GSH, however, would only prevent, not reverse, inhibition of isocitrate dehydrogenase, a monothiol enzyme. This suggested that OBPA forms a monothioarsinite complex with monothiols that is more stable than that formed with dithiols, in a manner similar to that of *di*substituted trivalent organoarsenicals.

ACKNOWLEDGMENTS

This work was supported, in part, by NIEHS Training Grant T32 ES06072. OBPA was donated by Ventron Corporation, Beverly, Massachusetts.

REFERENCES

BALLANTYNE, B. (1978). The comparative short-term toxicology of phenarsazine oxide and phenoxarsine oxide. *Toxicology* **10,** 341–361.

BRABEC, M. J., GRAY, R., AND BERNSTEIN, I. A. (1974). The restoration of hepatic mitochondria during recovery from CCl_4 intoxication. *Biochem. Pharmacol.* **23,** 3227–3238.

CLELAND, W. W., THOMPSON, V. W., AND BARDEN, R. E. (1969). Isocitrate dehydrogenase (TPN-specific) from pig heart. In *Methods in Enzymology* (J. M. Lowenstein, ed.), Vol. XIII, pp. 30–33. Academic Press, New York.

FLUHARTY, A. L., AND SANADI, D. R. (1963). On the mechanism of oxidative phosphorylation. VI. Localization of the dithiol in oxidative phosphorylation with respect to the oligomycin inhibition site. *Biochemistry* **2,** 519–522.

GORNALL, A. G., BARDAWILL, C. J., AND DAVID, M. M. (1949). Determination of serum proteins by means of the Biuret reaction. *J. Biol. Chem.* **177,** 751–766.

HACKENBROCK, C. R., REHN, T. G., WEINBACH, E. C., AND LEMASTERS, J. J. (1971). Oxidative phosphorylation and ultrastructural transformation in mitochondria in the intact ascites tumor cell. *J. Cell Biol.* **51,** 123–137.

KRONENBERG, J., AND HARTUNG, R. (1981). Disposition of 10-10′ oxybiphenoxarsine (OBPA) in rats, guinea pigs and rabbits. *Toxicologist* **1,** 93.

LOTSPEICH, W. C., AND PETERS, R. A. (1951). The action of sulfhydryl inhibitors upon isocitrate dehydrogenase with especial reference to the behavior of some trivalent arsenicals. *Biochem. J.* **49,** 704–709.

PETERS, R. A. (1955). Biochemistry of some toxic agents. Present state of knowledge of biochemical lesions induced by trivalent arsenical poisoning. *Bull. J. Hopkins Hosp.* **97,** 1–20.

PLAUT, G. W. E. (1969). Isocitrate dehydrogenase (DPN-specific) from bovine heart. In *Methods of Enzymology* (J. M. Lowenstein, ed.), Vol. XIII, pp. 31–42. Academic Press, New York.

PLAUT, G. W. E. (1970). DPN-linked isocitrate dehydrogenase of animal tissues. *Curr. Top. Cell. Regul.* **2,** 1–27.

SMITH, C. M., AND PLAUT, G. W. E. (1979). Activities of NAD-specific and NAD-specific isocitrate dehydrogenases in rat liver mitochondria. *Eur. J. Biochem.* **97,** 283–295.

WEBB, J. L. (1966). Arsenicals. In *Enzyme and Metabolic Inhibitors*, Vol. 3, pp. 595–793. Academic Press, New York.