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EFFECTS OF ANIONIC XENOBIOTICS ON RAT KIDNEY

I—TISSUE AND MITOCHONDRIAL RESPIRATION

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Abstract—The polar 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) metabolite, 2,2-bis (*p*-chlorophenyl)acetic acid (DDA), and the phenoxyacetic acid herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), were previously shown to be accumulated to high levels in liver and kidney via the organic acid transport system, raising the possibility of organ-specific toxicity secondary to transport. In these studies, accumulation of DDA was shown to depress oxygen consumption by renal cortical slices at high doses (0.1 and 1 mM). Isolated renal and hepatic mitochondria were uncoupled by low doses of DDA (5–10 nmoles/mg mitochondrial protein). Maximal uncoupling was seen at 50–70 nmoles/mg. 2,4-D and 2,4,5-T also produced uncoupling, but at doses of 70 nmoles/mg or higher. All agents were more effective with α-ketoglutarate or pyruvate-malate as substrate than with succinate. With succinate as substrate (but not α-ketoglutarate or pyruvate-malate), all three agents also depressed State 3 (ADP-stimulated) respiration. Again, DDA was more effective than 2,4-D or 2,4,5-T. These results suggest that accumulation of these or other anionic xenobiotics may lead to toxicity in those tissues possessing the organic anion transport system.

Many foreign compounds like the phenoxyacetic acid herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D¶) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), are themselves organic anions; still more are converted to organic anions by metabolism [1]. As such, their excretion is primarily determined by secretory transport on the organic anion system [2–4]. On the one hand, this transport greatly speeds their elimination from the body, thus reducing the risk of systemic toxicity. On the other hand, it leads to extensive intracellular accumulation of potentially toxic agents in those tissues possessing the transport system, notably kidney, liver, and choroid plexus. Intracellular pesticide levels are often ten to fifty times those of the extracellular fluid *in vitro* and *in vivo* [2, 3, 5–8]. Thus, it would appear that significant potential exists for selective organ-specific toxicity secondary to organic anion transport.

Perhaps the most likely consequence of such high intracellular concentrations of organic anions would be disruption of respiration and oxidative phosphorylation. Certainly, many of these compounds, like the classical uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNP), have a lipid soluble region, usually aromatic, and a polar or charged region [9, 10]. Therefore, the experiments reported below were undertaken to examine the nature of the interaction of three anionic xenobiotics with tissue and mitochondrial respiration. The three agents chosen were 2,4-D and 2,4,5-T, because of their extremely widespread use, which accounts for over 20 per cent of total pesticide application today [11], and 2,2-bis(*p*-chlorophenyl)acetic acid (DDA), the primary polar metabolite of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) [12, 13]. Each of these compounds has already been shown to undergo extensive organic anion transport *in vitro* in renal and hepatic slices [5, 8, 14], in the isolated perfused kidney [15], and *in vivo* [2–4]. Byczkowski [16] has also presented evidence that DDT metabolites, including DDA, do indeed alter hepatic mitochondrial respiration. DNP was examined to provide a positive control with which to compare the effects of the other anions.

METHODS

Animals

Long–Evans rats were purchased commercially or raised in the departmental animal quarters. They were maintained at 22° and were fed standard rat chow until use. They were then decapitated and the kidneys were rapidly excised and placed in ice-cold Cross–Taggart saline, pH 7.4 [17].

Tissue slice respiration

Thin slices of kidney (~0.5 mm thick) were pre-

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¶ Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDA, 2,2-bis(*p*-chlorophenyl)acetic acid; DDOH, 2,2-bis(*p*-chlorophenyl)ethanol; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; DNP, 2,4-dinitrophenol; α-KG, α-ketoglutarate; P-M, pyruvate-malate; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*′-tetraacetic acid; and Tris, tris(hydroxymethyl)aminomethane.

pared using a Stadie–Riggs microtome. Respiration was measured manometrically on a Gilson respirometer equipped with single valve manometers. Two kidney slices were placed in each 7.5 ml Warburg flask containing 3 ml of incubation medium: Cross–Taggart saline (pH 7.4) alone for the control or saline with DDA in concentrations of 10 μM (2.81 ppm), 100 μM (28.1 ppm) or 1 mM (281 ppm). The center well of each flask contained folded filter paper soaked in 20% KOH for CO_2 absorption. Tissues were distributed randomly among the media throughout the course of the experiment. A run consisted of tissue from one rat exposed to each of the four media; twelve animals were tested.

After a 30-min equilibration period, respiration was monitored at half-hour intervals for 1.5 hr. The experimental temperature was 25°, as this temperature is more suitable for lengthy metabolic studies [5, 17]. Flasks were shaken at 110 oscillations per min. Respiration was calculated as nl oxygen consumed · (mg wet weight)$^{-1}$ · hr^{-1}. Data were analyzed by a two-tailed *t*-test.

Mitochondrial respiration

Rat liver and kidney mitochondria were isolated by the method of Johnson and Lardy [18] in a homogenizing medium of 250 mM sucrose (Schwartz–Mann enzyme grade), 0.1 mM EGTA, and 3 mM Tris–HCl at pH 7.4. EGTA was omitted from the final suspension medium. All reagents used were analytical grade.

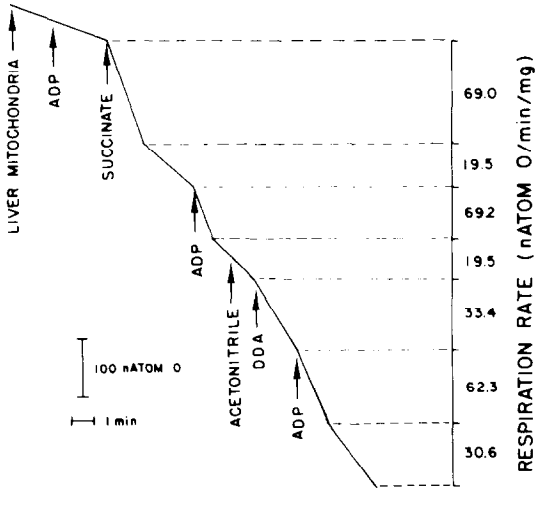

Fig. 1. A typical experimental run showing both a continuous trace of oxygen consumption and the respiratory rate calculated from the slope of the trace. At the left arrow 1.8 mg of hepatic mitochondria was added to the experimental chamber (2.0 ml volume) which had been equilibrated previously with air at 25°. Sequential additions of ADP (0.4 μmole) and succinate (5 mM, chamber concentration) gave the State 3 rate (69.0). Upon utilization of the ADP, the rate returned to State 4 (19.5). A second addition of ADP (0.2 μmole) gave one-half the earlier duration of State 3 rate. Acetonitrile (0.5%) did not alter State 4 respiration, whereas 10 μM (chamber concentration) DDA nearly doubled O_2 utilization. ADP (0.2 μmole) still stimulated oxygen consumption but to a lesser extent than that seen prior to DDA addition.

Respiration was measured polarographically using an oxygen electrode (Yellow Springs Instruments) at 25°. The control incubation medium contained 200 mM sucrose, 3 mM $MgSO_4$, 4 mM K_2HPO_4, and 3 mM Tris–HCl at pH 7.4. In most experiments, the substrate for respiration was 5 mM succinate. Other substrates used were 5 mM α-ketoglutarate and 2.5 mM pyruvate plus 2.5 mM malate. The anions were added to give final concentrations of 1–1000 μM. Since in most experiments the concentration of mitochondria was 1 mg mitochondrial protein/ml, these doses were equivalent to 1–1000 nmoles/mg mitochondrial protein.

For each run, control ADP-stimulated (State 3) and ADP-limited (State 4) respiration were measured. Inhibitors were then added. For final inhibitor concentrations of 50 μM or less, inhibitors were added in saline. Higher concentrations required an organic solvent (acetonitrile or ethanol, 0.5%) to carry them into the incubation medium. At this low concentration neither solvent changed control respiration or altered the response to inhibitors (at low [< 50 μM] inhibitor concentrations where this could be tested directly). A typical experiment is shown in Fig. 1. Rotenone was used in some experiments to block NADH formation and was found to have no effect on the respiration rates. Respiration rates were calculated in natoms 0 · (mg mitochondrial protein)$^{-1}$ · min^{-1}. In this calculation, the solubility of oxygen was assumed to be 474 natoms 0/ml at 25° [19]. Protein was determined by the method of Lowry *et al.* [20]. Data were analyzed by the two-tailed *t*-test.

RESULTS

Tissue slice respiration

As shown in Table 1, respiration of rat kidney cortical slices was reduced significantly in the presence of 100 μM DDA. At 1 mM DDA respiration was further reduced to only one-third of control values. Inhibition was already maximal at the time of the first measurements (30–60 min post-exposure). Mean respiration of tissues incubated in 10 μM DDA did not differ significantly from the control. However, several of these slices had oxygen consumption rates which were more than twice that of their paired controls, suggesting that, like DNP, DDA might uncouple oxidative phosphorylation at low concentrations while inhibiting respiration at higher doses.

Mitochondrial respiration

Kidney mitochondria. An initial series of sixteen animals was used to examine the response of isolated

Table 1. Respiration of rat kidney slices exposed to DDA

DDA (μM)	Respiration (nl O_2 · mg^{-1} · hr^{-1})	P value
0	609.6 ± 59.1*	
10	638.2 ± 66.0	NS†
100	475.0 ± 78.6	0.05
1000	200.0 ± 43.9	0.001

* Data are the mean ± S.E. for twelve animals.
† NS indicates a P value of >0.05.

Table 2. Effect of *in vitro* exposure to DDA on the respiration of rat kidney mitochondria*

DDA (μM)	State 4		State 3		Mean RCR†
	O_2 consumption	% Stimulation	O_2 consumption	% Inhibition	
0	99.0 ± 9.0		333.0 ± 21.6		3.6
10	158.4 ± 13.2‡	60	253.2 ± 18.6‡	24	1.6
100	216.6 ± 16.2‡	119	184.8 ± 9.6‡	44	0.8
1000	156.6 ± 12.6‡	58	72.6 ± 13.2‡	78	0.5

* Results are expressed as mean oxygen consumption (natoms $O \cdot min^{-1} \cdot mg^{-1}$) ± S.E. for sixteen animals. The substrate was 4 mM succinate.

† RCR = receptor control ratio, i.e. State 3 oxygen consumption ÷ State 4 oxygen consumption.

‡ Significantly different from the control, $P < 0.001$.

mitochondria to the same concentrations of DDA used with the cortical slices, i.e. 10 μM, 100 μM and 1 mM. As shown in Table 2, resting (State 4) respiration of mitochondria oxidizing succinate was markedly stimulated by all three concentrations of DDA. However, as suggested above, the response was biphasic with maximal stimulation at 100 μM and significantly less stimulation at 1 mM. The response to added ADP was very different from the State 4 results. First, State 3 respiration was inhibited by DDA, not stimulated. The extent of inhibition continued to increase with increasing DDA dosage. Second, only after the lowest dose of DDA (10 μM) did the subsequent addition of ADP stimulate respiration rate. The increased oxygen consumption induced by the higher doses of DDA (100 μM and 1 mM) was inhibited by addition of ADP. The latter phenomenon has been called reverse acceptor control [21, 22] and has been seen only in uncoupled mitochondria after ADP addition. Considered together, these data argue that DDA both uncouples oxidative phosphorylation and inhibits State 3 respiration. It might also be concluded that 10 μM DDA is insufficient to completely uncouple the mitochondria. This conclusion was supported by the observation (four rats) that addition of 10 μM DNP to mitochondria already respiring in the presence of 10 μM DDA further increased State 4 oxygen consumption to the extent produced by 100 μM DDA alone. Addition of DNP to mitochondria exposed to 100 μM or 1 mM DDA produced no further increase in State 4 respiration, indicating that, unlike 10 μM DDA, the higher DDA concentrations produced complete uncoupling or prevented further response to DNP. Thus, the overall pattern of response is much like that of other uncoupling agents where total uncoupling occurs in a narrow concentration range and higher doses inhibit the respiration [23].

To establish the dose response and to compare DDA with DNP and the phenoxyacetic acid herbicides, a second series was examined using mitochondria from kidney and liver and xenobiotic concentrations of 1–1000 μM. The effects of these agents

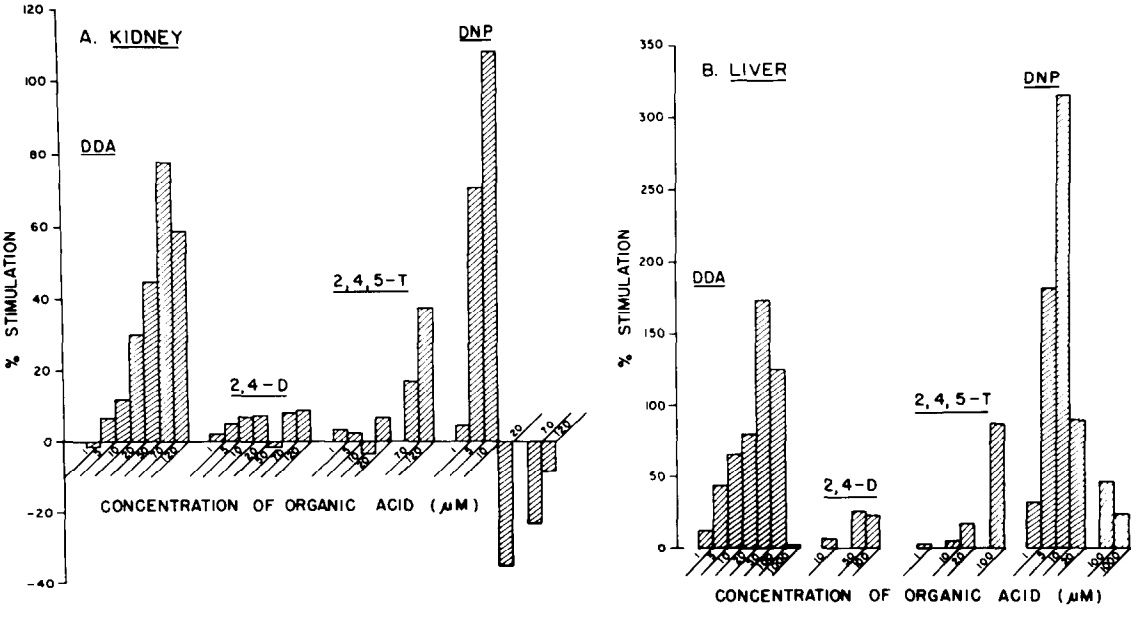

Fig. 2. Stimulation of succinate-based State 4 oxygen consumption by anionic xenobiotics in renal (A) and hepatic (B) mitochondria. The anion concentration (μM) is equivalent to nmoles/mg mitochondrial protein. Blank spaces (unlabeled) indicate that a concentration was not tested (e.g. 50 μM 2,4,5-T in kidney). Negative values indicate inhibition of oxygen consumption.

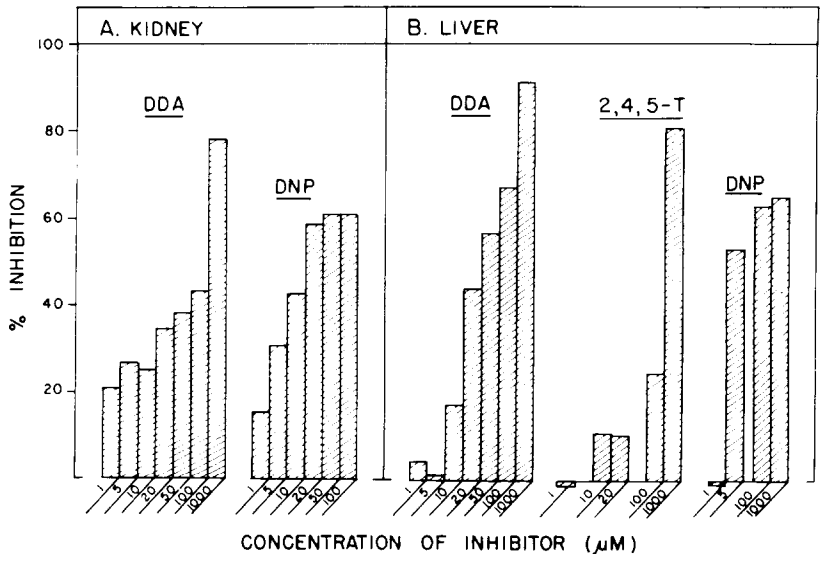

Fig. 3. Inhibition of State 3 oxygen consumption by DDA, 2,4,5-T, and DNP in renal (A) and hepatic (B) mitochondria oxidizing succinate (5 mM). Results for 2,4-D (not shown) were qualitatively similar to 2,4,5-T except even smaller. Other details are as in the legend of Fig. 2.

on State 4 respiration are summarized in Fig. 2. In both tissues DNP and DDA produced a biphasic stimulation of respiration. Maximal stimulation by DDA in renal mitochondria was nearly as great as that produced by DNP (78 vs 109 per cent), but required seven times the dose (70 vs 10 μM). In liver mitochondria, maximal stimulation by DDA was considerably less than that produced by DNP (172 vs 312 per cent), and again it required much more DDA to produce maximal stimulation (50 vs 10 μM). Uncoupling was also produced in both tissues by 2,4-D and 2,4,5-T, but the magnitude was much less and no biphasic response was seen even at 120 μM. Maximal stimulation of oxygen consumption by 2,4-D was only 23 per cent in liver and 8 per cent in kidney. 2,4,5-T was somewhat more effective, producing as much as 88 per cent (liver) and 32 per cent (kidney) stimulation.

The effect of these agents on State 3 respiration was inhibitory rather than stimulatory (Fig. 3). Again DDA and DNP were much more effective than either phenoxyacetic acid herbicide. Only the trichlorinated herbicide (2,4,5-T) produced inhibition greater than 25 per cent at any dose, and even 2,4,5-T required a concentration of 1 mM before inhibition became marked. With DDA, inhibition reached 40 per cent at doses of 100 μM (kidney) and 20 μM (liver), whereas DNP required only 10 μM (kidney) and 5 μM (liver) to achieve the same degree of inhibition. Maximal inhibition at the highest dose tested (1 mM) was slightly greater for DDA than DNP (78 vs 62 per cent for kidney and 90 vs 65 per cent for liver). 2,4,5-T also produced inhibition of this magnitude (80 per cent) after a dose of 1 mM.

To gain additional insight into the nature of these effects, the influence of altered respiratory substrate

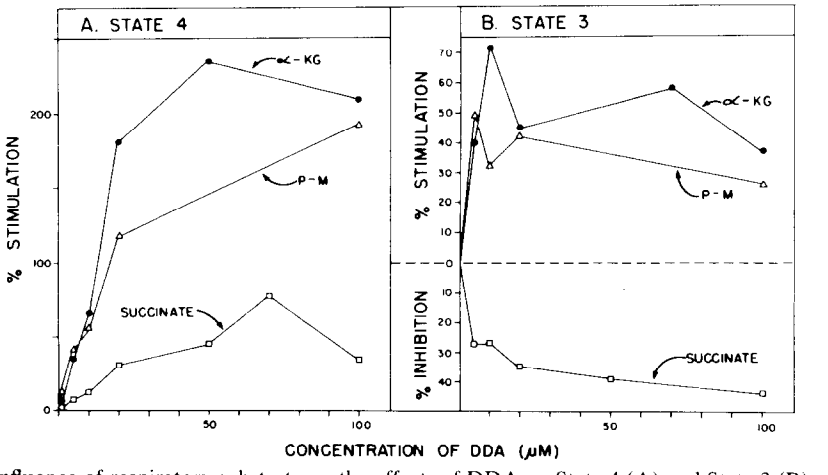

Fig. 4. Influence of respiratory substrate on the effects of DDA on State 4 (A) and State 3 (B) oxygen consumption by renal mitochondria. As in Figs. 2 and 3, DDA concentration (μM) is equivalent to nmoles/mg mitochondrial protein. Substrates tested were succinate (5 mM), α-ketoglutarate (α-KG, 5 mM), and pyruvate (2.5 mM) plus malate (2.5 mM) (P-M). Control values for succinate are shown in Table 2. Control values for α-KG were 36.0 (State 4) and 90.6 (State 3) natoms $O \cdot mg^{-1} \cdot min^{-1}$. Values for P-M were 41.7 (State 4) and 90.6 (State 3).

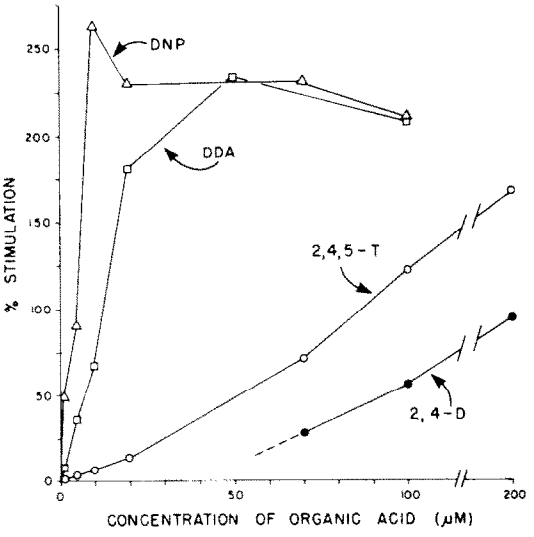

Fig. 5. Comparison of the abilities of DDA, 2,4-D, 2,4,5-T and DNP to uncouple State 4 respiration of renal mitochondria using 5 mM α-ketoglutarate as substrate.

was examined (Fig. 4). State 4 respiration was stimulated much more markedly by DDA with either α-ketoglutarate (α-KG) or pyruvate-malate (P-M) as substrate, but the shape of the curve was not changed and maximal stimulation was observed at 50–100 nmoles/mg. On the other hand, the response of State 3 respiration to DDA was very different for α-KG and P-M. Under these conditions DDA produced stimulation of ADP-stimulated respiration, rather than inhibition as seen with succinate. In addition, each of the other anions also produced a more marked stimulation of State 4 respiration with α-KG (Fig. 5) or P-M (not shown). However, the relative effectiveness of these xenobiotics was similar to that seen for succinate oxidation. DNP and DDA gave maximal response at 10 and 50 μM, respectively, while 2,4,5-T and 2,4-D stimulation continued to increase over the whole range tested (1–200 μM), reaching maximal values of 169 per cent for 2,4,5-T and 96 per cent for 2,4-D. State 3 respiration was always stimulated by the xenobiotics when either α-KG or P-M was the substrate (as shown for DDA in Fig. 4), but the effect never exceeded 30 per cent for either herbicide (not shown).

DISCUSSION

Beginning in the late 1940's, DDT and its metabolites were shown to alter several steps in mitochondrial respiration leading to inhibition of oxygen consumption, uncoupling, or both depending upon the conditions studied [13, 24–27]. As noted by Nelson [28] and documented above, many of the differences between these studies may be explained in large part by differences in pesticide dose (nmoles/mg mitochondrial protein). For example, at low doses DDA is a potent uncoupler of State 4 respiration, whereas at higher doses this effect is reduced (kidney) or lost (liver) (Table 2, Fig. 2), presumably because inhibition of substrate entry or oxidation occurs at these higher doses, thus obscuring observation of the uncoupling. On the other hand, effects on State 3 respiration continue to increase with dose throughout the concentration range tested (Table 2, Fig. 3). In addition, results may vary both quantitatively and qualitatively with the tissue studied (e.g. Fig. 2) or the substrate utilized (Figs. 4 and 5).

Similarly, a wide variety of respiratory effects has been attributed to other pesticides and foreign organic anions [9, 10, 28–30]. On balance, there appear to be three major mechanisms involved in these effects. First, anionic agents like DNP or pentachlorophenol appear to act as proton carriers, or protonophores, uncoupling by dissipation of the proton gradient which, according to the chemiosmotic theory [31, 32], drives phosphorylation. Second, there are changes in the permeability or fluidity of the mitochondrial membrane, often attributed to "detergent-like" properties of the agent or changes in membrane charge density and distribution [28, 30, 33–36]. Such changes may lead to uncoupling via dissipation of the proton gradient or to inhibition of electron transport, either directly or via altered substrate entry [e.g. Refs. 33 and 36]. Third, they may act by binding to specific membrane components such as the oliogomycin sensitive Mg^{2+}-ATPase [6, 37–39].

Our results with DDA and the phenoxyacetic acid herbicides appear to reflect the influence of several of these mechanisms, each operating over a slightly different concentration range. First, as predicted by their chemical structures (i.e. each contains a charged polar region and a hydrophobic aromatic portion [10], DDA and, to a lesser extent, 2,4-D and 2,4,5-T were able to uncouple oxidative phosphorylation in isolated mitochondria (Table 2, Figs. 2, 4 and 5). Indeed, DDA was a very effective uncoupler and the pattern of its effects was much like DNP. For example, like DNP and other uncouplers, DDA produced a biphasic stimulation of State 4 respiration [23, 40, 41]. Maximal stimulation of either succinate or α-ketoglutarate (α-KG) oxidation occurred at 50–70 μM DDA (i.e. 50–70 nmoles/mg mitochondrial protein). While this dose was five to seven times the dose required for maximal stimulation of State 4 respiration by DNP, the extent of response was quite similar. For example, 50 μM DDA produced 235 per cent stimulation of renal α-KG oxidation (Fig. 5) or 80 per cent stimulation of renal succinate oxidation (Fig. 2), whereas the maximally effective dose of DNP (10 μM) gave only slightly larger responses (264 and 110 per cent respectively). The structure of DDA, the pattern of response, and the observations that DDA may free hepatic mitochondria from oligomycin inhibition [16] without an increase in mitochondrial permeability to large molecules [34] suggest that DDA is a classical type 1 uncoupler (i.e. a protonophore, like DNP or pentachlorophenol, which acts by facilitating the movement of H^+ through the inner membrane, thus collapsing the proton gradient necessary for ATP synthesis [31, 32]). The phenoxyacetic acid herbicides may also act in this way. However, the high concentrations, 100 nmoles/mg or greater (Ref. 42: Figs. 2 and 5), necessary to cause significant uncoupling suggest that other less specific mechanisms (see below) might also be involved.

As shown above (Table 2 and Fig. 3), uncoupling

was not the only effect of these agents. Succinate-based State 3 respiration was markedly reduced, as was oxygen consumption in renal slices over a similar range of xenobiotic concentrations (Table 1). These effects are quite similar to those reported for the parent pesticide DDT [24, 25, 27, 43] and more recently for DDA and DDOH by Byczkowski using hepatic mitochondria [16, 36, 44]. A precise understanding of these effects has not yet been achieved, but the most plausible mechanisms appear to involve changes in membrane properties, particularly membrane charge and fluidity [28, 30, 45]. These changes may then lead to changes in conductance (leakiness), electron transport, or depression of substrate exchange reactions [9, 29, 30, 33, 36]. Certainly, the lack of inhibition of State 3 respiration when α-KG or P-M were substrates (Fig. 4) suggests that at least part of this effect arises prior to coenzyme Q, either in succinate oxidation or in its penetration to the interior of the mitochondria.

Irrespective of the exact mechanisms involved, it is clear that at sufficient concentrations DDA and, to a lesser degree, 2,4-D and 2,4,5-T may disrupt respiration. It must then be asked whether these concentrations arise under conditions of environmental exposure and, if so, in which tissues. The results presented above support the basic hypothesis that the extensive intracellular accumulation of foreign organic anions like DDA, 2,4-D and 2,4,5-T by kidney, liver, choroid plexus, or other tissue possessing the organic anion transport system may lead to toxic effects. Indeed, while not expressed in these terms at the time, evidence supporting precisely this idea has been in the literature for many years. In 1965, Haung and Lin [46] demonstrated that DNP was a substrate for the organic acid transport system, and its effectiveness as an uncoupler in liver and kidney was shown to derive from its rapid entry and extensive accumulation in these tissues as well as its potency as an uncoupler [46, 47].

It appears that DDA and the two herbicides may act in the same manner as DNP. They are known to be actively accumulated by kidney [3–5, 8, 14], liver [5], and choroid plexus [7]. They are all capable of uncoupling State 4 respiration (Table 2, Figs. 2, 4, and 5). At somewhat higher doses they inhibit succinate-based State 3 oxygen consumption (Table 2, Fig. 3) and renal slice respiration (Table 1). It is not yet clear whether these effects would occur at environmentally relevant concentrations. However, several pieces of data suggest that altered mitochondrial function might well arise. First, acute and chronic toxicity studies [11], though often done with very high doses, have indicated cellular injury in organs possessing the organic anion system. Furthermore, renal transport studies have shown that acute 2,4,5-T exposure (90 mg/kg) caused inhibition of several independent transport systems [48, 49], an effect consistent with altered respiration. Indeed, reversible inhibition of renal slice oxygen consumption by 2,4,5-T has been shown [50]. Thus, there is suggestive evidence that toxic effects may be produced in tissues capable of organic acid transport. Second, as documented above, the concentrations required for altered mitochondrial function are quite low. Only 5 μM DDA (1.4 ppm) was required to produce nearly 50 per cent stimulation of State 4 respiration by hepatic mitochondria (Fig. 2) and 30 per cent stimulation in renal mitochondria (Fig. 5). Similarly, 2,4,5-T, though less potent than DDA, required only 100 μM to yield 40–100 per cent stimulation (Figs. 2 and 5). Since active organic anion transport in kidney, liver or choroid plexus produces tissue levels ten to fifty times that of the bathing medium or plasma, circulating xenobiotic concentrations of only 0.1 to 5 μM (0.03 to 1.4 ppm) would be required to achieve intracellular concentrations of this magnitude. Concentrations in this range have been reported after acute intoxication by the phenoxyacetic acid herbicides [11]. The impact of chronic low level exposure is less clear. Certainly, the present observations indicate that organ-specific toxicity secondary to active accumulation of these or other anionic xenobiotics warrants further examination.

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