THE OXIDATIVE METABOLISM OF ALDICARB IN PIGS: IN VIVO - IN VITRO COMPARISON

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SUMMARY

Aldicarb was administered (1 mg/kg b.w.) to four female pigs and the kinetics of its major oxidized metabolites (sulfoxide and sulfone) was followed for 6 hours. The *in vitro* transformations of the carbamate pesticide into these two still active metabolites were also investigated in hepatocytes and in microsomes from pig livers. In all cases, aldicarb was quickly oxidized to the sulfoxide (major metabolite) and only a minor quantity of sulfone was produced. The *in vivo* toxic symptomatology was related to the peak serum concentration of sulfoxide, suggesting that this metabolite is principally responsible for the aldicarb toxicity. Selective *in vitro* inhibition of flavin-containing and cytochrome P-450 monooxygenases confirmed that the former enzymes catalyze mainly sulfoxide production whereas the latter that of sulfone.

KEY WORDS

aldicarb, pigs, *in vitro* metabolism, microsomes, hepatocytes, kinetics

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INTRODUCTION

Aldicarb, 2-methyl-2-(methylthio)-propionaldehyde-O-(methylcarbamoyl) oxime (ALD), a widely used carbamate pesticide for tomato, potato and cucumber crops, is highly toxic to mammals after both oral administration and dermal application. In higher plants and in vertebrates it is rapidly metabolized by oxidation at the sulfur atom into aldicarb sulfoxide (ALDSOX) and aldicarb sulfone (ALDSON) /1,2,3/, compounds which have similar toxic activity to the parent compound, i.e., acetylcholinesterase inhibition. *In vitro* studies show that ALDSOX is 23 times as effective an acetylcholinesterase inhibitor as ALD and 60 times as effective as ALDSON /4/; this fact suggests that the metabolite ALDSOX may be the chemical species primarily responsible for the cholinergic effects of ALD administration.

The first aim of this study was to verify whether ALD administered orally to pigs follows the metabolic pattern already observed in other mammalian species and to relate the formation of toxic metabolites to signs of cholinergic poisoning. The second objective was to confirm the involvement of two important microsomal enzyme systems in the oxidation of ALD, namely NADPH flavin-containing monooxygenases and cytochrome (cyt.) P-450 monooxygenases, which catalyze the transformation of many pesticides into sulfoxide and sulfone metabolites, respectively /5/. The qualitative and quantitative production of oxidized metabolites was studied using microsomes and hepatocytes obtained from pig liver and the data obtained from these *in vitro* systems were compared to the results of *in vivo* experiments.

MATERIALS AND METHODS

Chemicals

ALD and ALDSON were purchased from Dr. Ehrenstorfer (Augsburg, Germany), ALDSOX was synthesized by acidic oxidation of ALD with equimolar *m*-chloroperoxybenzoic-acid dissolved in dichloromethane at 4°C for 15 min. Recovery of ALDSOX was about 60% and its identity confirmed by HPLC-MS. Glucose-6P, Glucose-6P dehydrogenase and NADP were purchased from Boehringer-Biochemia-Robin (Milano, Italy); salts and solvents (analytical grade) were from Baker (Deventer-Holland); Williams' medium E (WME),

fetal calf serum, penicillin, insulin and streptomycin were from Flow Laboratories (Rickmansworth, UK).

Animals

For the *in vivo* experiments, four young healthy sows (five months old, 60 ± 4 kg) were used, housed individually in stalls with free access to standard laboratory diet and tap water.

Livers used for microsome preparation were collected from four sows aged about three years; livers for the hepatocyte cultures were obtained from four one year-old sows. All the animals were chosen from those scheduled at the slaughterhouse and killed by electric stunning followed by exsanguination. A piece of each liver was removed and infused with ice-cold 0.9% NaCl and transported to the laboratory in the same ice-cold solution. In our experience, there are no significant differences between one and three year-old female pig liver microsomes regarding cyt. P-450 content and related enzyme activities.

***In vivo* kinetics**

The animals were administered ALD (1 mg/kg) by oral bolus. Blood samples were collected from the auricular vein before administration and after 5, 15, 30, 45, 60, 120, 240 and 360 min. Serum samples were obtained by centrifugation for 15 min at 2000 *g*; ALD and its metabolites were routinely extracted twice by adding 10 ml of dichloromethane to 1 ml serum sample.

Preparation of microsomes and hepatocytes

Microsomes were prepared from liver homogenates in phosphate buffer 0.1 M, pH 7.4 and 1.15% KCl solution (1:1, v:v) as reported previously /6/ and stored under liquid nitrogen pending assay.

Pig hepatocytes were isolated as described by Hoogenboom *et al.* /7/. An aliquot of the final cell suspension was used to determine the number and fraction of trypan blue-excluding cells as reported by Jauregui *et al.* /8/. For monolayer cell cultures viable cells (mean viability obtained 90-95%) were diluted up to 1 million/ml; 2.5 million in 2.5 ml of medium were seeded into plastic, 60 mm diam. dishes. WME supplemented with 5% fetal calf serum, insulin 0.5 mg/ml,

glutamine 2 mM, penicillin (50 IU/ml), and steptomycin (50 mg/ml) was routinely used.

After 4 hours incubation the cells were attached and half of the dishes prepared were used immediately for metabolic assay, replacing media with 2.5 ml of WME containing 100 μM aldicarb. The other half were washed with WME only and left to incubate for a further 20 hours before exposure to ALD.

Microsome metabolic assay

Microsome fractions were defrosted at 37°C, immediately suspended in 0.1 M phosphate buffer (pH 7.4) to give a protein concentration of about 2 mg/ml measured with the biuret method according to Gornall and Bardawill /9/, and their cyt. P-450 content determined by the Omura and Sato method /10/. The metabolic assays were carried out aerobically, by incubating tubes at 37°C containing microsomal protein 3 mg, $MgCl_2$ 5 mM, NADP 2 mM, Glucose-6-P 10 mM, 3 units of Glucose-6-P dehydrogenase, ALD 100 μM and phosphate buffer 0.1 M, pH 7.4 to a final volume of 1 ml. The reactions were stopped by adding 10 ml of dichloromethane after 0, 5, 15, 30, 45 and 60 min. Two samples were prepared for each time. Blank samples with ALD but no cofactors were incubated for the same times to check for spontaneous reactions.

Thermal inactivation of flavin-containing monooxygenase was carried out as reported by Ziegler /11/. The tubes were then reequilibrated for two minutes at 37°C and the metabolic assay carried out as reported above. P-450 enzymes were inactivated by saturating 1 ml of the protein solution with carbon monoxide; the inactivated preparations were assayed for ALD transformation as described above.

Metabolic assays with hepatocytes

To relate monooxygenase activity to age of culture, metabolic assays were carried out in cell monolayers four hours after seeding and 24 hours after seeding. To carry out the assay, the supernatant was discarded and replaced with 2.5 ml WME containing ALD 100 μM and incubated at 37°C for 0, 15, 30 min and 1, 2, 3, 20 hours. At each time three dishes were taken, two aliquots (1 ml) of medium were collected from each and extracted with 10 ml of dichloromethane. To check for substrate stability and spontaneous oxidation, ALD (50 μM) and

ALDSOX (20 μM) were incubated in WME only under the same conditions.

HPLC analysis

The dichloromethane combined samples were evaporated to dryness under nitrogen stream and the residues resuspended in 500 μl of HPLC water and filtered before HPLC injection. HPLC analysis was performed as reported previously /12/.

RESULTS

***In vivo* kinetics**

Figure 1 shows the concentration versus time profile of ALD, ALDSOX and ALDSON. After oral administration ALD mean serum concentrations were clearly above the detection limit (30 ng/ml) only at 15 min (65 ± 27 ng/ml) and 30 min (55 ± 19 ng/ml), whereas at 45 min and 60 min they were already close to this value, suggesting fast absorption and presumably extensive metabolism of the molecule. ALDSOX serum levels increased (linearly for the first hour) to reach a peak concentration (186 ± 45 ng/ml) 2 h after administration. At the last samplings it was still present in high concentrations: 135 ± 41 ng/ml at 4 h and about 60 ng/ml after 6 h. Only small quantities of ALDSON were detected at 1 h (53 ± 12 ng/ml) and 2 h (59 ± 18 ng/ml) after administration.

Mild toxic symptoms (salivation) were observed in the treated animals after 15 min. Severe symptoms of acetylcholinesterase inhibition (myosis, fasciculation, diarrhoea and weakness) were observed in all animals 90-120 min after oral administration of ALD. All animals had completely recovered 4-6 h later.

***In vitro* study**

The results obtained after incubating ALD with sow liver microsomes are shown in Table 1. The major metabolite in this system was ALDSOX and its production increased linearly for the first 15 minutes until ALDSON formation began. Neither oxidation nor degradation compounds were observed in blanks prepared to check for spontaneous reactions.

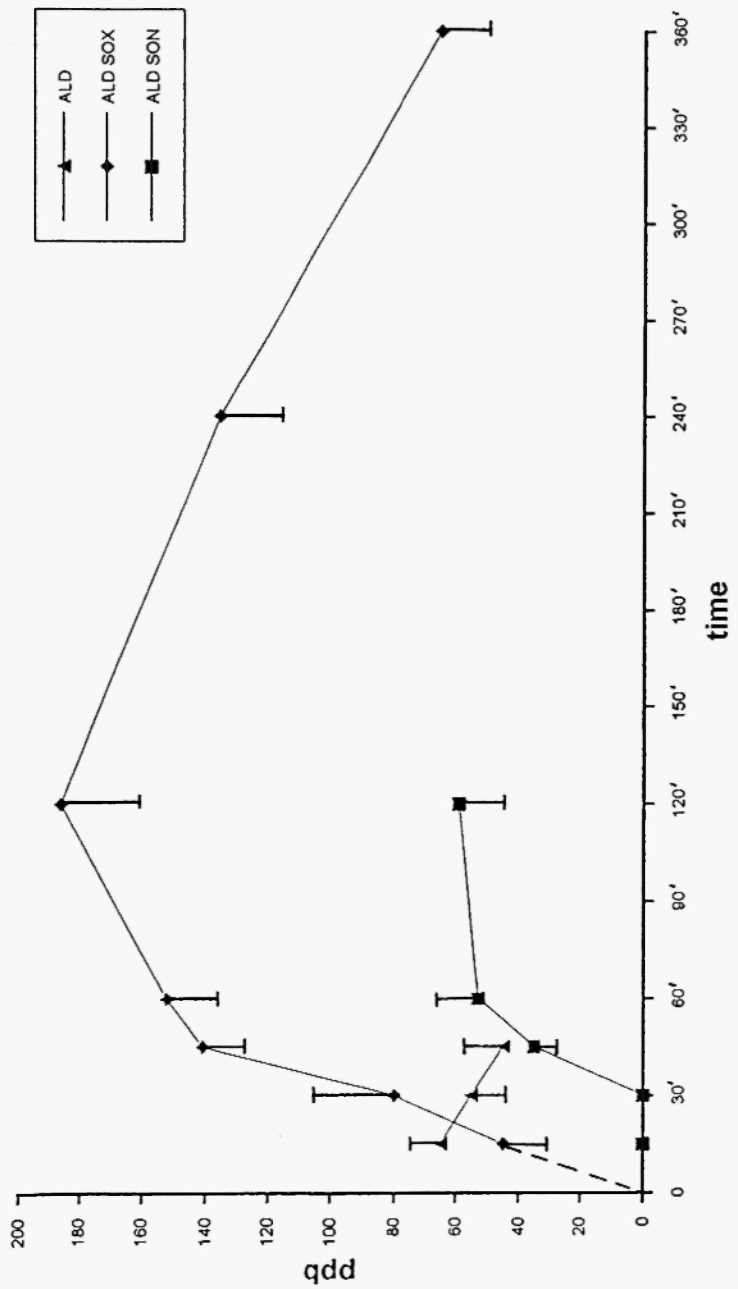

Fig. 1: Kinetic profile of ALD, ALDSOX and ALDSON after the administration of a single oral dose of ALD (1 mg/kg b.w.) to 4 young sows. Data are reported as mean values ± S.D.

TABLE 1

In vitro oxidation of ALD in liver microsome preparations from 4 sows; data are reported as mean ± S.D.

time (min)	ALD (nmol/mg protein)	ALD SOX (nmol/mg protein)	ALD SON (nmol/mg protein)
0	9.31+0.64	0.25±0.02	0.06±0.02
5	4.53±1.22	4.62+0.84	0.11+0.02
15	1.85+0.76	7.19+1.16	0.16±0.05
30	0.85+0.38	8.52+1.39	0.28+0.11
60	0.61±0.55	8.75+1.35	0.31±0.17

TABLE 2

In vitro oxidation of ALD after flavin-containing monooxygenase inactivation by Ziegler's method /11/. Mean inhibition was 80%. Data are reported as mean ± S.D.

time (min)	ALD (nmol/mg protein)	ALD SOX (nmol/mg protein)	ALD SON (nmol/mg protein)
0	9.21±0.84	0.05±0.02	0.05±0.01
15	8.12+0.86	1.33±0.07	0.10±0.04
30	8.25+0.58	1.64±0.22	0.18±0.03
60	6.73±1.09	3.15±0.16	0.25±0.07

The data obtained after thermal inactivation of the microsome preparations (to selectively inhibit flavin-containing monooxygenase) are shown in Table 2. The extent of inhibition at the various incubation times was about 80%. The mean cyt. P-450 values before and after thermal inactivation were 0.38 ± 0.02 nmol/mg protein and 0.35 ± 0.03 nmol/mg protein respectively, thus cyt. P-450 was not significantly decreased by the treatment. The results obtained after cyt. P-450

inactivation are not reported because, as expected, the amount of ALDSOX formed did not change with cyt. P-450 inactivation with CO, nor was any ALDSON detected.

In hepatocyte cultures ALD was readily metabolized to ALDSOX while ALDSON was formed to only a minor extent (Fig. 2). A small difference was recorded in cyt. P-450 levels measured in cells preincubated for 4 hours and those preincubated for 24 h (about 25% in those incubated for the longer time).

The extent of ALDSON formation seems to be slightly modified by the preincubation times: ALDSON production became evident after 15 min in cells attached for four hours and after 60 min in those attached for 24 hours. After 1 h of ALD incubation, ALDSON production in 4 h preincubated cells was double that of those preincubated, for monolayer formation, for 24 h; this difference became significant at 3 h ($p<0.05$ with Student's t-test) and maintained significance to the last incubation time (20 h).

At 20 h of incubation the ALDSOX concentration had decreased from its peak at 3 h, presumably due to its conversion to metabolites other than ALDSON. No degradation was detected when ALD and ALDSOX were incubated with WME alone.

DISCUSSION

Our *in vivo* kinetic results demonstrate that, in pigs, ALDSOX is the major metabolite, as reported in other animal species. The levels of ALDSOX observed in pigs are lower than those observed in other species (rat and cows) /1,4/, suggesting that ALDSOX is quickly hydrolyzed to less toxic metabolites and/or that the cyt. P-450 oxidation rate is slower in pigs. This is further supported by the facts that the cyt. P-450 content we found in pigs is low compared to other species, such as rabbit, sheep, etc., and that toxic symptoms were evident for two-three hours in this species after administering the very low dose of 1 mg/kg. During the *in vivo* experiment the severity of symptoms attributable to cholinesterase inhibition coincided with the peak serum concentration of ALDSOX, suggesting that this metabolite is mainly responsible for the toxic effect of ALD, as also inferred from *in vitro* studies by Baron and Merriam /4/.

Turning to the *in vitro* studies, we note that thermal inactivation, which irreversibly denatures FAD-containing monooxygenases and leaves cyt. P-450 monooxygenases unaffected, confirmed the

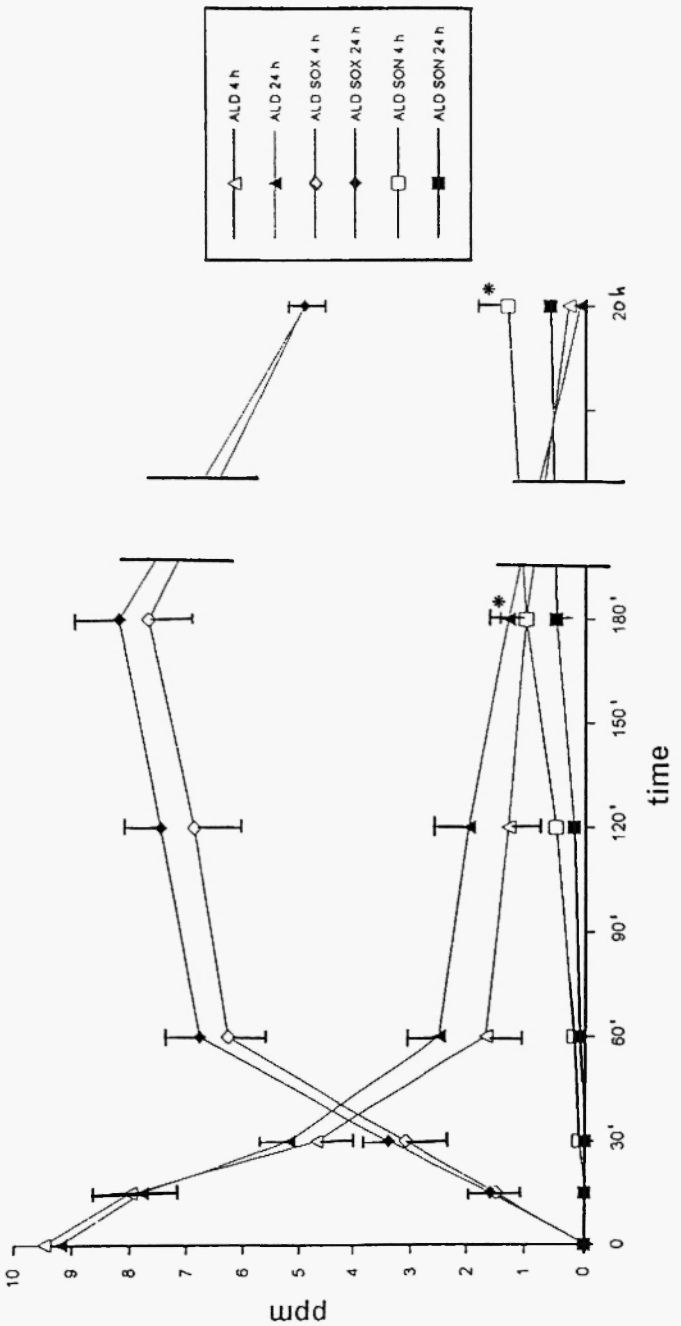

Fig. 2: Comparative oxidation of ALD in pig hepatocytes pre-incubated for 4 h and 24 h. Data are reported as mean values ± S.D. *Significantly different from those preincubated for 24 h ($p<0.05$)

respective roles of the two enzyme systems in the subsequent oxidation of the ALD sulfur, as reported by Smyser *et al.* /13/. However, the thermally inactivated microsomes still produced about 20% of the ALDSOX (and only slightly less ALDSON) originally produced by non-treated microsomes, suggesting either an incomplete inactivation or that active cyt. P-450 can play a small role in oxidation to ALDSOX as already reported for tiobenzamide by Tynes and Hodgson /15/.

The kinetic profiles for the formation of ALDSOX and ALDSON from ALD in cultured hepatocytes and in microsome suspensions are similar, showing that the overall oxidation capacities of these two *in vitro* systems are closely related; in both cases ALDSOX is the major metabolite and ALDSON concentrations increase slowly with time.

It is well known that the age of the monolayer culture can affect the metabolic activity of the hepatocytes by decreasing cyt. P-450 concentrations, as reported by Hoogenboom *et al.* /7/.

Furthermore, it has recently been reported that the decrease of cyt. P-450 concentrations of cultured hepatocytes is more marked during the first hours of incubation /14/. In the present study ALDSON production was significantly lower in hepatocytes preincubated for 24 hours than those preincubated for 4 hours, confirming a decrease in cyt. P-450 activity, whereas the ALDSOX production, not being significantly modified by pre-incubation time, suggests that FAD-containing monooxygenases are less labile.

Both sets of *in vitro* results correlate well with the *in vivo* kinetics: in all cases ALD was quickly oxidized and ALDSOX was the major metabolite. Incubation of ALD with hepatocytes for more than 3 h resulted in a decrease of the ALDSOX concentration, without concomitant increase of ALDSON, presumably due to hydrolytic conversion to more polar, less/non-toxic metabolites /4/. This was not observed in microsomes or when ALD and ALDSOX were incubated in WME alone. Intact cells (hepatocytes) have more extensive metabolic potential and can provide information on further metabolism which might be important for toxicity evaluation.

The results of the present study confirm that flavin-containing monooxygenases play an important role in the bioactivation of sulfur-containing xenobiotic compounds in pigs, as already observed in other mammals by Tynes and Hodgson /15/ and more recently in the trout by Schlenk and Buhler /16/. The toxicity of these compounds is therefore mainly attributable to the transformations brought about by flavin containing monooxygenases. These enzymes, present in high

concentrations in pig liver, have not been investigated in the tissues of other food-producing animals, particularly ruminants. Studies with aldicarb in such species will provide further useful information on this enzyme system, which is probably responsible for the transformation of many drugs routinely used in veterinary practice.

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