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INHIBITION OF ALDEHYDE DEHYDROGENASE BY 2-CHLOROACETOPHENONE AND THE RESULTANT EFFECTS ON THE CATABOLISM OF NOREPINEPHRINE IN BRAIN*†

CHARLES O. RUTLEDGE and RICHARD A. DEITRICH‡
Department of Pharmacology, University of Colorado School of Medicine, Denver, Colo. 80220, U.S.A.

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Abstract—Rabbit brain cortex slices were incubated with ^{14}C-norepinephrine and the amounts of ^{14}C-phenolic acids and ^{14}C-phenolic glycols formed were measured. Pretreatment of rabbits with 2-chloroacetophenone, 300 mg/kg, results in an inhibition of the formation of phenolic acids (especially dihydroxymandelic acid) and a corresponding stimulation of the formation of phenolic glycols. Similar results were observed when the 2-chloroacetophenone was incubated *in vitro* with brain tissue slices. The concentrations of 2-chloroacetophenone required to inhibit an isolated enzyme preparation of aldehyde dehydrogenase and to inhibit the production of dihydroxymandelic acid in brain tissue slices are similar, which suggests that the effect of 2-chloroacetophenone on norepinephrine metabolism is due to inhibition of aldehyde dehydrogenase. The decrease in aldehyde dehydrogenase activity leads to an increase in substrate available for reduction to phenolic glycols and probably accounts for the observed stimulation of phenolic glycol formation. The effect of 2-chloroacetophenone on norepinephrine metabolism or on isolated aldehyde dehydrogenase could be prevented with glutathione. In addition, the effect on aldehyde dehydrogenase could be reversed with sulfhydryl reagents. This suggests that the interaction of 2-chloroacetophenone with sulfhydryl groups may be important in the inhibition of aldehyde dehydrogenase.

DEAMINATION of norepinephrine by monoamine oxidase (EC 1.4.3.4) results in the formation of 3,4-dihydroxyphenylglycolaldehyde[1,2] which, in the presence of aldehyde dehydrogenase (EC 1.2.1.3), can be rapidly oxidized to 3,4-dihydroxymandelic acid (DOMA).[3,4] The aldehyde can also be reduced in intact tissue to 3,4-dihydroxyphenylglycol (DOPEG).[5–7]

The relative importance of these two pathways appears to vary with species, tissue and the substrate. In rabbit brain cortex slices, reduction is the major route of metabolism of 3,4-dihydroxyphenylglycolaldehyde, although oxidation is also quantitatively important.[7] The two products, DOMA and DOPEG, are further metabolized by catechol-*o*-methyl transferase to vanilmandelic acid (VMA) and 3-methoxy-4-hydroxyphenylglycol (MOPEG) respectively.[6–8] Thus, total deamination of norepinephrine can be measured by isolating the amounts of DOMA, DOPEG, VMA, and MOPEG which are formed.

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Aldehyde dehydrogenase is inhibited by a number of substances which react with sulfhydryl groups.[9] The present study was designed to investigate the inhibition of aldehyde dehydrogenase by 2-chloroacetophenone, a potent lacrimator and a common constituent of tear gas preparations. 2-Chloroacetophenone is a potent inhibitor of a variety of sulfhydryl enzymes.[10] The inhibition of aldehyde dehyrogenase was used as a tool to investigate dynamic relationships between oxidation and reduction of 3,4-dihydroxyphenylglycolaldehyde in the metabolism of norepinephrine in brain tissue.

METHODS AND MATERIALS

Measurement of norepinephrine catabolism in brain cortex slices

Adult male rabbits weighing between 2·0 and 2·5 kg were killed by injection of air into a lateral ear vein. The brains were rapidly removed and eight slices of cortex (approximately 150 mg) were incubated in 5·0 ml of Krebs–Henseleit solution for 10 min at 37° in a 95 per cent O_2–5 per cent CO_2 atmosphere. Subsequently [^{14}C]-norepinephrine was added and the flasks were incubated an additional 30 min. In some samples, 2-chloroacetophenone was added prior to the 10-min incubation period. 2-Chloroacetophenone was added in an ethanol solution to assure solubility. The final concentration of ethanol, 0·1 per cent, was used in control samples as well as in all samples to which different concentrations of 2-chloroacetophenone were added. In animals which were pretreated with 2-chloroacetophenone, the substance was dissolved in 3·0 ml of 100 per cent ethanol and injected i.p. at a dose of 300 mg/kg (approximately 1·33 g/kg of ethanol). Control animals received only ethanol. Acid control incubation samples were prepared by the addition of 2 ml of 2 N HCl to the flask before the incubation period. All incubations were terminated by the addition of 2 ml of 2 N HCl.

Upon termination of the incubation, 300 μg of each of the following four catabolites were added as carrier substances: DOMA, DOPEG, VMA, and MOPEG. The acidified samples were centrifuged and the supernatants were extracted with ethyl acetate. The ethyl acetate was distilled off in a rotary evaporator and the residue was dissolved in ethyl acetate and applied to silica gel paper for chromatography. After the development of the chromatograms, each substance was eluted with 0·2 N acetic acid and a portion of the eluate was assayed for the metabolites by the phenol reaction.[11] These values were used for the determination of the recoveries. Mean recoveries $\pm$ S.E.M., based upon 98–114 values were as follows: DOMA 28·6 $\pm$ 0·6 per cent; DOPEG, 12·2 $\pm$ 0·3 per cent; VMA, 35·0 $\pm$ 0·7 per cent; and MOPEG, 23·1 $\pm$ 0·5 per cent. Most of the eluate was reduced to dryness; liquid scintillation counting fluid was added and the radioactivity was determined in a Mark I (Nuclear-Chicago) liquid scintillation counter. A complete description of these techniques has been previously presented.[7]

Isolation of aldehyde dehydrogenase

Rabbit brain mitochondria were prepared and sonicated to release aldehyde dehydrogenase as previously described[4] for rat brain, except that no mercaptoethanol was added.

Determination of aldehyde dehydrogenase activity

Activity of aldehyde dehydrogenase was determined by following the rate of formation of NADH fluorometrically. The incubation mixture consisted of 0·015 M pyrophosphate buffer, pH 9·6, 5×10^{-4} M propionaldehyde, 10^{-3} M NAD, 2-chloroacetophenone in 1 per cent ethanol with or without 5×10^{-4} M glutathione or 5×10^{-4} M dithiothreitol, and enzyme. The rate of increase in NADH was linear with protein concentration and with time for several minutes. For the assay, the rate was determined during the first minute of the reaction.

The four deaminated metabolites of norepinephrine—DL-3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylglycol, DL-4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid) and bis-(4-hydroxy-3-methoxyphenylglycol) piperazine salt—were obtained from Regis Chemical Company. Propionaldehyde was obtained from Distillation Products and was redistilled before use. DL-Norepinephrine (carbinol-^{14}C; 31·5 to 45·7 mc/m-mole) was obtained from Amersham-Searle and New England Nuclear Corp.

RESULTS

Effects of 2-chloroacetophenone in vitro *on norepinephrine catabolism in rabbit brain cortex slices*

The metabolism of norepinephrine by rabbit brain cortex slices is not markedly altered by the addition of 0·1 per cent ethanol, which served as the solvent for 2-chloroacetophenone (Table 1). The slight increase in DOMA and DOPEG formation

TABLE 1. EFFECT OF 2-CHLOROACETOPHENONE *IN VITRO* ON NOREPINEPHRINE CATABOLISM IN RABBIT BRAIN*

	DOMA	DOPEG	VMA	MOPEG
Normal	122·1 ± 12·6	119·8 ± 14·2	17·6 ± 1·7	32·1 ± 3·4
Ethanol (0·1 %) control	150·7 ± 10·3	134·6 ± 14·3	19·7 ± 0·8	33·0 ± 3·6
2-Chloroacetophenone (10^{-6} M)	152·6 ± 13·9	168·3 ± 23·4	20·9 ± 0·8	34·2 ± 2·0
2-Chloroacetophenone (3×10^{-6} M)	101·6 ± 7·7†	158·1 ± 17·5	20·1 ± 1·6	33·4 ± 4·7
2-Chloroacetophenone (10^{-5} M)	71·0 ± 9·4‡	162·5 ± 34·5	14·7 ± 1·2†	32·0 ± 2·4
2-Chloroacetophenone (3×10^{-5} M)	68·4 ± 8·1‡	158·0 ± 12·5	14·9 ± 1·2†	29·0 ± 2·6
2-Chloroacetophenone (10^{-4} M)	35·0 ± 7·2‡	152·1 ± 5·4§	13·0 ± 2·3‖	24·1 ± 5·4
2-Chloroacetophenone (3×10^{-4} M)	17·5 ± 2·8‡	122·1 ± 24·1	8·2 ± 1·0‡	19·9 ± 5·1
2-Chloroacetophenone (10^{-3} M)	5·6 ± 2·1‡	24·7 ± 11·4‡	2·3 ± 0·7‡	2·8 ± 0·6‡
Acid control	3·0 ± 0·7‡	3·4 ± 0·9	0·5 ± 0·1‡	0·9 ± 0·3‡

* Rabbit brain cortex slices were incubated for 30 min with 10^{-6} M ($1·8 \times 10^{-7}$ to $2·3 \times 10^{-7}$ c) [^{14}C]-norepinephrine. Acid control corresponds to samples in which 2 ml of 2 N HCl were added to the incubation fluid before the incubation. Values are presented as means ± standard error of the mean of 3–11 experiments, in moles $\times 10^{-12}$ (formed from 5000×10^{-12} moles of [^{14}C]-norepinephrine) and represent catabolite in the medium plus that in the tissue. All values are corrected for recovery.

† Significantly different from ethanol control, $P < 0·01$.

‡ Significantly different from ethanol control, $P < 0·001$.

§ Significantly different from normal, $P < 0·05$.

‖ Significantly different from ethanol control, $P < 0·05$.

is not statistically significant. The addition of 2-chloroacetophenone leads to a dose-related decrease in the amounts of DOMA formed with 50 per cent inhibition at $1{\cdot}6 \times 10^{-5}$ M. The formation of VMA is also inhibited, but appears to be less sensitive with 50 per cent inhibition at 2×10^{-4} M. The deaminated phenolic glycol, DOPEG, tends to increase, but this is not significantly different from the ethanol control. 2-Chloroacetophenone, 10^{-4} M, does increase the amount of DOPEG formed when compared to normal, but not when compared to the ethanol control. MOPEG is not significantly altered by 2-chloroacetophenone in concentrations of 10^{-6} to 3×10^{-4} M.

Antagonism of the effect of 2-chloroacetophenone on norepinephrine catabolism by glutathione

Glutathione, 3×10^{-4} M, does not markedly alter norepinephrine catabolism in rabbit brain cortex slices (Table 2). When 2-chloroacetophenone is mixed with glutathione prior to the incubation, the effect of 2-chloroacetophenone on DOMA and VMA formation is completely antagonized.

TABLE 2. ANTAGONISM OF THE EFFECT OF 2-CHLOROACETOPHENONE ON NOREPINEPHRINE CATABOLISM BY GLUTATHIONE*

	DOMA	DOPEG	VMA	MOPEG
Ethanol (0·1%) control	150·7 ± 10·3	134·6 ± 14·3	19·7 ± 0·8	33·0 ± 3·6
2-Chloroacetophenone (3×10^{-5} M)	68·4 ± 8·1†	158·0 ± 12·5	14·9 ± 1·2‡	29·0 ± 2·6
Glutathione (3×10^{-4} M)	162·8 ± 22·5	122·4 ± 8·1	16·9 ± 1·7	35·0 ± 3·3
2-Chloroacetophenone (3×10^{-5} M) + glutathione (3×10^{-4} M)	176·9 ± 26·7	153·4 ± 10·6	20·5 ± 2·2	33·7 ± 3·1
Acid control	3·0 ± 0·7†	3·4 ± 0·9†	0·5 ± 0·9†	0·9 ± 0·3†

* Rabbit brain cortex slices were incubated for 30 min with 10^{-6} M ($1{\cdot}8 \times 10^{-7}$ to $2{\cdot}3 \times 10^{-7}$ c) [^{14}C]-norepinephrine. Acid control corresponds to samples in which 2 ml of 2 N HCl were added to the incubation fluid before the incubation. Values are presented as means in moles $\times 10^{-12}$ (formed from 5000×10^{-12} moles of [^{14}C]-norepinephrine) ± standard error of the mean of 7–11 experiments. All values represent the catabolite in the medium plus that in the tissue. All values are corrected for recovery.

† Significantly different from ethanol control, $P < 0{\cdot}001$.

‡ Significantly different from ethanol control, $P < 0{\cdot}01$.

There are no significant changes in the amounts of phenolic glycols formed during incubation with either 2-chloroacetophenone or glutathione or with the mixture of the two substances.

Effect of 2-chloroacetophenone pretreatment on norepinephrine catabolism

Pretreatment of rabbits with 2-chloroacetophenone, 300 mg/kg, results in a marked decrease in DOMA formation and an increase in DOPEG and MOPEG formation in brain slices (Table 3). There is no change in the amounts of VMA formed. The total increase in phenolic glycols (DOPEG and MOPEG) is 62·4 $\mu\mu$moles, which closely corresponds to the reduction in the total amounts of phenolic acids formed (61·8 $\mu\mu$moles). Pretreatment with the ethanol solvent for 2-chloroacetophenone does not

TABLE 3. EFFECT OF 2-CHLOROACETOPHENONE PRETREATMENT ON NOREPINEPHRINE CATABOLISM BY RABBIT CORTEX SLICES*

	DOMA	DOPEG	VMA	MOPEG
Normal	122·1 ± 12·6	119·8 ± 14·2	17·6 ± 1·7	32·1 ± 3·4†
Ethanol control (3·0 ml i.p.)	131·0 ± 9·9	111·4 ± 5·0	17·0 ± 0·9	24·0 ± 1·1
2-Chloroacetophenone (300 mg/kg i.p.) in 3·0 ml ethanol	69·2 ± 7·1‡	165·7 ± 6·7‡	17·0 ± 1·0	32·1 ± 2·1§
Glutathione (3×10^{-4} M) *in vitro*	162·8 ± 22·5	122·4 ± 8·1	16·9 ± 1·7	35·0 ± 3·3§
2-Chloroacetophenone (300 mg/kg i.p.) glutathione (3×10^{-4} M) *in vitro*	86·5 ± 12·0§	128·3 ± 9·7	13·8 ± 1·3	28·7 ± 2·3
Acid control	3·0 ± 0·7‡	3·4 ± 0·9‡	0·5 ± 0·1‡	0·9 ± 0·3‡

* Rabbit brain cortex slices were incubated for 30 min with 10^{-6} M ($1·8 \times 10^{-7}$ to $2·3 \times 10^{-7}$ c) [^{14}C]-norepinephrine. Acid control corresponds to samples in which 2 ml of 2 N HCl was added to the incubation fluid before the incubation. 2-Chloroacetophenone was injected 30 min prior to killing the animal. Values are presented as means in moles $\times 10^{-12}$ (formed from 5000×10^{-12} moles of [^{14}C]-norepinephrine) ± standard error of the mean of 7–11 experiments. All values represent the catabolite in the medium plus that in the tissue. All values are corrected for recovery.

† Significantly different from ethanol control, $P < 0·05$.

‡ Significantly different from ethanol control, $P < 0·001$.

§ Significantly different from ethanol control, $P < 0·01$.

alter the amounts of DOMA, DOPEG, and VMA formed from norepinephrine, but does result in a decrease in MOPEG formation.

Glutathione added *in vitro* does not markedly antagonize the inhibition of DOMA formation by 2-chloroacetophenone. The effect of 2-chloroacetophenone on DOPEG formation does appear to be reduced by glutathione. There also appears to be a decrease in VMA formation with the combination of 2-chloroacetophenone and glutathione.

Effect of 2-chloroacetophenone pretreatment on the kinetics of the metabolism of norepinephrine in brain cortex slices

Figures 1 and 2 illustrate the effect of 2-chloroacetophenone pretreatment on norepinephrine metabolism in brain slices. Since we found no evidence that monoamine oxidase is affected by this treatment, it is assumed that the rate of formation of 3,4-dihydroxyphenylglycolaldehyde in both the control and treated animals is the same. Figure 1 shows that the rate of formation of the acid product (DOMA) is inhibited in a classically noncompetitive manner by 2-chloroacetophenone pretreatment.

The influence of 2-chloroacetophenone pretreatment on the rate of formation of the glycol (DOPEG) is seen to be one of stimulation in Fig. 2. The apparent "competitive" nature of this effect indicates that there is no effect of the inhibitor directly on the total catalytic activity of the enzyme responsible for this reduction, but rather that the effect is one of availability of substrate (see Discussion).

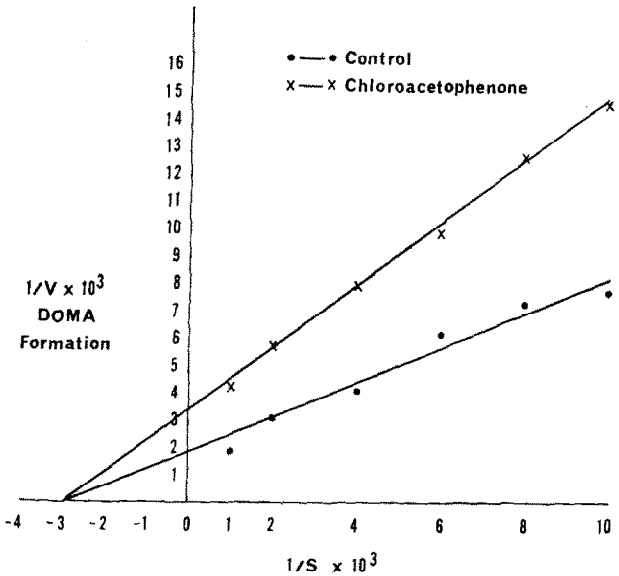

FIG. 1. Lineweaver–Burk plot of the inhibition of DOMA formation from norepinephrine by 2-chloroacetophenone in rabbit brain cortex tissue slices.

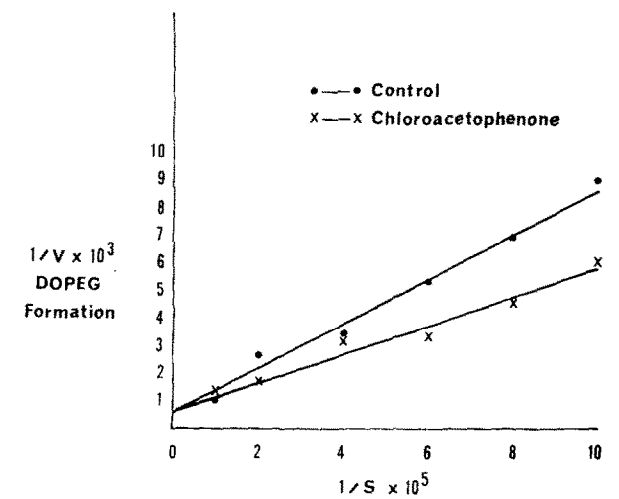

FIG. 2. Lineweaver–Burk plot of the stimulation of DOPEG formation from norepinephrine by 2-chloroacetophenone in rabbit brain cortex tissue slices.

It is important to emphasize that the substrate concentration which is plotted is the norepinephrine concentration in each figure.

Inhibition of rabbit brain mitochondrial aldehyde dehydrogenase by 2-chloroacetophenone in vitro

In a series of four separate experiments, it was found that 2-chloroacetophenone inhibited aldehyde dehydrogenase by 50 per cent at a concentration of $0{\cdot}92 \times 10^{-5}$ M $\pm$ 0·36 M (S.E.M.). Similar inhibition was observed using benzaldehyde as a substrate.

TABLE 4. EFFECT OF SULFHYDRYL COMPOUNDS ON INHIBITION OF RABBIT BRAIN MITOCHONDRIAL ALDEHYDE DEHYDROGENASE BY 2-CHLOROACETOPHENONE

Conditions*	% Inhibition	% Protection or reversal†
2-Chloroacetophenone (5×10^{-5} M) +enzyme	90·3	—
2-Chloroacetophenone (5×10^{-5} M) +dithiothreitol (5×10^{-4} M) +enzyme	19·4	78·5
2-Chloroacetophenone (5×10^{-5} M) +GSH‡ (5×10^{-4} M) +enzyme	15·4	82·9
2-Chloroacetophenone (5×10^{-5} M) +enzyme +dithiothreitol (5×10^{-4} M)	57·6	36·2
2-Chloroacetophenone (5×10^{-5} M) +enzyme +GSH (5×10^{-4} M)	56·0	38·0

* The assay was conducted as described in the text. The fluorometer cell contained buffer and NAD before the inhibitor, enzyme or sulfhydryl reagent additions were made. These additions were made in the order listed. The reaction was initiated by the final addition of the substrate, propionaldehyde. The values are based upon two determinations each, of two enzyme preparations.

† Protection or reversal $= \dfrac{\%\text{ Inhibition control} - \%\text{ Inhibition with protecting agent}}{\%\text{ Inhibition control}}$.

‡ Glutathione.

Table 4 demonstrates the ability of sulfhydryl compounds to prevent inhibition of the enzyme by 2-chloroacetophenone and to partially reverse the inhibition, once established. Dithiothreitol and glutathione are equipotent in preventing and reversing inhibition of the enzyme by 2-chloroacetophenone under the conditions of this study.

Reaction between NAD and 2-chloroacetophenone

During the course of these experiments, it was observed that NAD and relatively high concentrations of 2-chloroacetophenone (10^{-3} to 10^{-4} M) reacted to form a fluorescent product with activation at 350 nm and emission at 495 nm (uncorrected). These wavelengths are similar to those for NADH (340 and 457 nm). The nature of the reaction is currently under investigation. The reaction di dnot interfere with the enzyme assay at the relatively low concentrations of inhibitor and saturating amounts of NAD employed for inhibition studies.

DISCUSSION

The relative importance of the oxidation or reduction of 3,4-dihydroxyphenylglycolaldehyde depends primarily upon the relative K_m values, the V_{max} of each enzyme with the aldehyde as substrate, and the relative accessibility of the aldehyde for each of the enzymes. The concentrations of oxidized and reduced pyridine nucleotides are important when their concentrations fall below saturating values for the two enzymes.

Erwin and Deitrich[4] have demonstrated that aldehyde dehydrogenase isolated from brain tissue is present in both the supernatant and mitochondrial fractions. The K_m

for 3,4-dihydroxyphenylglycolaldehyde with this enzyme was 3×10^{-6} M. Much less is known about aromatic aldehyde reductases. Culp and McMahon[12] isolated an aromatic aldehyde reductase from the supernatant fraction of kidney cortex, but did not determine whether 3,4-dihydroxyphenylglycolaldehyde is a substrate for the enzyme. More recently, Tabakoff and Erwin[13] have isolated a similar enzyme from the supernatant fraction of brain homogenates; this enzyme has a relatively low K_m for phenylglycolaldehydes.

The data from the present study suggest that the two enzymes which metabolize 3,4-dihydroxyphenylglycolaldehyde have access to the substrate. When aldehyde dehydrogenase in brain slices is inhibited by 2-chloroacetophenone, the amounts of newly formed DOMA are decreased and there is a corresponding increase in the amounts of phenolic glycols formed. The most likely explanation for the increase in the amounts of phenolic glycols formed is that there is more substrate available to be reduced by an aldehyde reductase when the oxidation of the aldehyde is inhibited. This is supported by the kinetic data, which show that, as the norepinephrine concentration is increased toward infinity, the V_{max} of the enzyme from the 2-chloroacetophenone animals is not different from that obtained from control animals.

It is a theoretical possibility that 3,4-dihydroxyphenylglycolaldehyde reacts with norepinephrine to form a tetrahydropapaveroline derivative.[14] This does not seem likely since: (1) under our conditions, all of the norepinephrine added can be accounted for as either normetanephrine, phenolic acids or phenolic glycols;[7] and (2) the decrease in phenolic acids after 2-chloroacetophenone pretreatment is accompanied by a corresponding increase in phenolic glycols. This is different from the situation with dopamine, where inhibition of aldehyde dehydrogenase results in an accumulation of the aldehyde[14] which cannot be easily reduced to the alcohol,[7] presumably because of the relatively high K_m and small V_{max} of phenylacetaldehyde with aldehyde reductase.[13]

It is also conceivable that the ethanol used as solvent for 2-chloroacetophenone when the rabbits were pretreated with the inhibitor is converted to acetaldehyde and thus leads to competitive substrate inhibition of aldehyde dehydrogenase.[15] This does not seem likely, since 2-chloroacetophenone *in vitro*, in brain slices and with isolated mitochondrial aldehyde dehydrogenase, produces an effect similar to that observed when the animal is pretreated with the inhibitor.

It is likely that the mechanism of the inhibition of aldehyde dehydrogenase by 2-chloroacetophenone is by attack on the sulfhydryl groups of the enzyme. The effect of the inhibitor can be virtually abolished both *in vivo* and *in vitro* by prior reaction with a sulfhydryl-containing compound. In addition, some reversal of the effect can be obtained *in vitro* by addition of GSH or dithiothreitol to the inhibited enzyme. These results are consistent with previous studies of aldehyde dehydrogenase using sulfhydryl reagents[4,9] as well as with studies on the actions of 2-chloroacetophenone on other sulfhydryl enzymes.[10]

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