Reiner

DATA EVALUATION RECORD

(1) CHEMICAL: Trichlorfon

(2) TYPE OF FORMULATION: Crystalline material, 98.7% pure

(3) CITATION: Reiner, E., Krauthacker, B., Simeon, V., and Skrinjaric-Spoljar, M. 1975. Mechanism of inhibition in vitro of mammalian acetylcholinesterase and cholinesterase in solutions of O,O-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate (trichlorfon). Biochem. Pharmacol. 24:717-722

(4) REVIEWED BY:

Donald L. Hill
Staff Scientist
Southern Research Institute
Birmingham, Alabama 35255
(205) 323-6592

Signature:_____

Date:_____

Janna Strobel-Stevens
Staff Scientist
Southern Research Institute
Birmingham, AL 35255
(205) 323-6592

Signature:_____

Date:_____

(32B-0056)

(5) APPROVED BY:

Signature:_____

Date:_____

(6) TOPIC: The study has information pertinent to discipline toxicology, topic biochemistry. It relates to none of the Proposed Guidelines data requirements.

(7) CONCLUSIONS:

(a) Trichlorfon decomposes at similar rates in phosphate buffer, pH 7.4, and in the same buffer in the presence of enzyme preparations of cholinesterases (from human, horse, and rat plasma) and acetylcholinesterases (from bovine erythrocytes and rat brain).

(b) Although the rate of inhibition of bovine erythrocyte acetylcholinesterase is the same for DDVP, a chemical product of trichlorfon, at pH 6.0 and 7.4, the rate of enzyme inhibition in trichlorfon solutions is 30 times greater at pH 7.4 than at pH 6.0. This information is consistent with the fact that trichlorfon is more stable at acidic pH values and decomposes to DDVP at alkaline pH values.

(c) The rates of spontaneous reactivation of both acetylcholinesterase and cholinesterase, after inhibition in trichlorfon solutions and after inhibition by DDVP, were the same.

The above kinetic results demonstrate that trichlorfon itself is not a direct inhibitor of mammalian cholinesterases and that its product, DDVP, can account for the inhibition observed.

CORE CLASSIFICATION: Not applicable

(8) MATERIALS AND METHODS: Trichlorfon (98.7% pure) and DDVP, the chemical degradation product of trichlorfon (93% pure), were obtained from Bayer Ltd., Leverkusen, W. Germany, and the World Health Organization, Geneva, Switzerland, respectively. Stock solutions (50 mM) were prepared in water (trichlorfon) or dimethylformamide (DDVP) and diluted with water or buffer before use.

The following sources of acetylcholinesterase and cholinesterase were used:

- Purified bovine erythrocytes (Winthrop Ltd., New York)
  Used for assay: 5 µg/ml (spectrophotometric method)
  250 µg/ml (pH-stat method)
- Purified horse serum (Sigma Chemical Co., St. Louis, MO.)
  Used for assay: 6 µg/ml
- Human erythrocytes (washed twice with 0.15 M NaCl and made up to the original volume with 0.15 M NaCl)
  Used for assay: 1.67 µl/ml
- Rat brain (homogenized in 0.15 M NaCl, 160 mg of brain/ml)
  Used for assay: 1.25 mg/ml
- Human plasma (from heparinized blood)
  Used for assay: 6.7 µl/ml
- Rat plasma (from heparinized blood)
  Used for assay: 3.3 µl/ml

The spectrophotometric assays were accomplished at 25°C or 37°C in 0.1 M of phosphate buffer (pH 7.4) by a reported procedure (Ellman et al. 1961. Biochem. Pharmacol. 7:88). Enzyme (2.7 ml) and inhibitor (0.3 ml) were incubated for a given time before substrate (50 µl of 1 mM acetylthiocholine) was added. The reaction mixture contained the thiol reagent, 5,5'-dithiobis-2-nitrobenzoate (0.33 mM). The absorbance at 412 nm was recorded at 15-second intervals for 2.5 minutes.

The pH-stat procedure (Jensen-Holm et al. 1959. Acta Pharmacol. Toxicol. 15:384) was used to assay for enzymatic activity in 1.0 M phosphate buffer (pH 6.0 or 7.4) at 37°C or 25°C. Under a stream of nitrogen, enzyme and inhibitor were incubated for a given time before acetylcholine was added for a final concentration of 10 mM. The liberated acetic acid was titrated with 0.02 M of NaOH in a Syringe Burette Unit attached to a titrator and titrigraph.

In both assays, enzyme activity was linear with time for both control and inhibited enzyme samples.

The stability of trichlorfon was measured polarographically at 25°C or 37°C (Giang and Caswell 1957. J. Agr. Food Chem. 5:753) with a dropping Hg cathode and Hg pool anode. The half-wave potential was -0.8 V. Before measurement, gelatin and KCl (final concentrations 0.002% and 0.2-0.4 M,

respectively, were added to samples of trichlorfon solutions stored at 25° and then N_2 was bubbled through samples for 10 min.

To evaluate spontaneous reactivation of the inhibited enzyme, preparations of enzyme were incubated with inhibitor at a concentration giving 65-85% inhibition. The preparations were then diluted up to 300-fold with 0.1 M of phosphate buffer, pH 7.4 and kept at 37°C. Samples were withdrawn at various times and assayed spectrophotometrically as described above and previously (in more detail) (Skrinjaric-Spoljar et al. 1973. Biochim. Biophys. Acta 315:363).

(9) REPORTED RESULTS:

Decomposition of trichlorfon and DDVP. As determined polarographically, trichlorfon decomposed in 0.1 M of phosphate buffer, pH 7.4, in a first-order process. The rate constants are at the bottom of Table 2. Trichlorfon was somewhat more stable at 15 mM than at 0.15 mM. The $t_{1/2}$ at 37°C was 2.6 hours (mean for both concentrations) and that at 25°C was 17.5 hours. The activation energy, calculated from the results at 25°C and 37°C, was 122 kJ/mole.

The stability of DDVP was similar to that for trichlorfon. The $t_{1/2}$ for its decomposition at 37°C (0.01 and 0.5 mM in 0.1 M of phosphate, pH 7.4) was 2.9 hours (unpublished

results) and for the same experiments at 25°C, it was 15.6 hours (Reiner et al. 1973. Meeting of Chemists of Croatia, Zagreb, p. 306).

Kinetics of enzyme inhibition by DDVP. The time course of inhibition of various enzymes by DDVP was measured by varying the concentration of DDVP and the time of inhibition. The rate of inhibition follows the kinetics of a bimolecular reaction, for a plot of log enzyme activity versus time was linear, and first-order rate constants of inhibition were linearly related to DDVP concentration. A summary of second-order rate constants for DDVP, measured in 0.1 M of phosphate buffer, pH 7.4, are in the following table:

Table 1

Second-order Rate Constants for Inhibition of Acetylcholinesterase and Cholinesterase by DDVP

Enzyme	$10^{-4} \times k_a$ (M^{-1} min^{-1}) 25°C	37°C
Acetylcholinesterase		
Bovine erythrocytes	2.62	4.7* (4.2 at pH 6)*
Rat brain	6.85	15.4**
Cholinesterase		
Human plasma	50.1	87.0**
Horse plasma	7.77	8.78
Rat plasma	6.90	18.3**

*Measured in 1 mM phosphate buffer, pH 7.4

**From Skrinjaric-Spoljar et al. (1973. Biochim. Biophys. Acta 315:363).

Kinetics of enzyme inhibition by trichlorfon. For acetylcholinesterase of bovine erythrocytes and rat brain homogenates and for cholinesterase of human, rat, and horse plasma, plots of log activity versus time gave curves with increasing downward slopes, indicating a continuously increasing concentration of inhibitor.

The authors assumed that DDVP was the only inhibitor formed and calculated its rate of formation by dividing

the inhibition curves into segments and calculating the slopes of these segments. The slopes were considered to be proportional to the inhibitor concentration, and these concentrations were calculated from the results (in Table 1) for DDVP as an inhibitor. The concentration of trichlorfon remaining was then calculated. By plotting log [trichlorfon] versus time, the rate constants for its decomposition in the presence of the enzymes were obtained. These values are listed in the following table:

Table 2

Decomposition of Trichlorfon in 0.1 M Buffer

Enzyme	Trichlorfon (mM)	pH	10^4 x k (min^{-1}) 25°C	37°C
Acetylcholinesterase				
Bovine erythrocytes	0.10	6.0		0.73±0.12[a]
	0.10	7.4		22.2±1.7[a]
	0.15	7.4	3.69	
	0.50	7.4	2.80	
Rat brain	0.15	7.4	5.08	28.2
	0.50	7.4	3.48	36.4
Cholinesterase				
Human plasma	0.01	7.4	7.08	41.6
	0.05	7.4	2.39	46.2
	0.10	7.4	4.17	
Horse plasma	0.10	7.4	3.97	39.4
	0.50	7.4	2.68	30.8
	1.00	7.4	1.46	
Rat plasma	0.15	7.4	3.13	20.0
	0.50	7.4	2.47	22.2
None	0.15	7.4	7.27±0.39[b]	53.1±1.2[b]
	15	7.4	6.05±0.52[b]	37.1±1.2[b]

[a]1 mM phosphate buffer, mean ± standard errors for 4 experiments.

[b]Rate constants ± standard errors for 18-40 determinations, determined polarographically.

Although the range of rate constants for experiments at pH 7.4 is fairly wide, there is no trend in the values, either with respect to source of enzyme or to trichlorfon concentration. The rate constants as calculated from the kinetics of enzyme inhibition are equivalent to or even lower than those obtained by the polarographic method.

At pH 6.0, the rate constant for decomposition of trichlorfon is greatly reduced, indicating that the compound is about 30 times more stable at this pH.

Spontaneous reactivation of the inhibited enzyme. The rates of spontaneous reactivation were determined after inhibition of acetylcholinesterase (human and bovine erythrocytes) and cholinesterase (human plasma) in trichlorfon solutions. For human erythrocyte acetylcholinesterase, the rate constant (K_{+3}) for spontaneous reactivation was 0.0138 ± 0.0011 min^{-1} (mean of 5 experiments). The K_{+3} obtained after inhibition by DDVP was 0.0136 min^{-1}. The rate of reactivation of acetylcholinesterase from human erythrocytes was stated to be the same after inactivation solutions of trichlorfon and by DDVP.

(10) DISCUSSION: Many of the data presented in this manuscript are based on the assumption that DDVP is the only active product formed in the chemical degradation of trichlorfon. This is a reasonable assumption, for although other products

might be formed, there is no reason at present to assume that they are involved in the inhibition of esterases.

The kinetic evidence that trichlorfon itself is not an inhibitor is impressive. The rates of trichlorfon decomposition in buffer (determined polarographically) and in the presence of various cholinesterases and acetylcholinesterases (determined enzymatically) are not greatly different. In fact, they are generally slower in the presence of enzymes. If trichlorfon had inhibitory properties, the rate of decomposition would be larger in the presence of the enzymes being inhibited. (Although the enzymatic assay used for calculating decomposition rates for trichlorfon is complicated, it appears to be reasonably reliable).

The conversion of trichlorfon to DDVP is not likely to be enzymatic, for, although crude enzyme preparations were used, its rate of decomposition was not faster in their presence.

Further evidence that trichlorfon is not a direct inhibitor was derived from assays accomplished at different pH values. The rate of inhibition of bovine erythrocyte acetylcholinesterase by DDVP was the same at pH 6.0 and 7.4, but the rate of enzyme inhibition by trichlorfon solutions was 30 times faster at pH 7.4, a pH at which trichlorfon is known to be converted rapidly to DDVP.

These results are said to agree with those of Metcalf et al. (1959. J. Econom. Entomol. 52:44) and Miyamoto (1959. Botyu-Kagaku 24:130).

The similar rates of reactivation of enzymes inactivated either by DDVP or by trichlorfon solutions provide further evidence that trichlorfon itself is not a direct inhibitor. These observations are said to be similar to those of Arthur and Casida (1957. J. Agr. Food Chem. 5:186).

The authors point out that although there are differences in the calculated rate constants for trichlorfon decomposition, these differences are probably related to lack of sensitivity of the assay, for less than 2% of the trichlorfon had to decompose to DDVP to account for the observed enzyme inhibition.

A number of other investigators have not considered the DDVP formed during the time of incubation as responsible for cholinesterase inhibition.

The results reported here agree with those of Miyamoto (1959. Botyu-Kagaku 24:130), who concluded that trichlorfon was not an inhibitor of fly-head acetylcholinesterase.

(11) TECHNICAL REVIEW TIME: 10.0 hours