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Effects of paraquat and related herbicides on the acetylcholinesterase of rat lung

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Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride) is a diquaternary nitrogen compound which is in common use as an herbicide. It is closely related (Fig. 1) to diquat and morfamquat, which are also herbicides. A general property of diquaternary amines, such as hexamethonium (Fig. 1), and of ring-contained quaternary nitrogen compounds, such as *N*-methylpyridinium, is the inhibition of acetylcholinesterase [1]. Compounds which are congeners of *N*-methylpyridinium are inhibitors of the anionic site of acetylcholinesterase [1]. Anticholinesterase potency is increased by linking two quaternary ammonium nuclei by a series of methylene groups, as in hexamethonium. The distance between the quaternary nitrogens in paraquat is approximately the same as in hexamethonium.

A further analogy between paraquat and anticholinesterase agents is found in the fact that, in animals and in humans, the predominant toxic symptom produced by paraquat is pulmonary edema followed by interstitial pulmonary fibrosis [2]. Inhibition of acetylcholinesterase in the lung causes accumulation of tracheobronchial secretions, and bronchiolar secretion occurs after large doses of pilocarpine, a cholinergic agonist [3].

Paraquat warrants study since it is widely used in agriculture and because it is a good tool for studying cellular proliferation and fibrosis in the lungs. It has the advantage of not readily being metabolized *in vivo* so that tissue concentration and cellular damage can be related to one chemical species rather than to a series of metabolites. We have considered the possibility that inhibition of acetylcholinesterase may contribute to the toxicity of the three bipyridinium herbicides.

The three bipyridylium herbicides tested for their inhibition of acetylcholinesterase were paraquat dichloride, diquat dibromide monohydrate (*N*,*N*′-ethylene,2,2′-bipyridylium dibromide), and morfamquat dichloride ({bis *N* - [(2,6 - dimethylmorpholin - 4 - yl)carbonylmethyl] - 4,4′-bipyridylium}dichloride). All three compounds were supplied by Dr. Michael S. Rose, Imperial Chemical Ind., Ltd., Alderly Park, U.K.

The 5,5′-dithiobis-(2-nitrobenzoic acid), acetylthiocholine iodide and butyrylthiocholine chloride were purchased from the Sigma Chemical Co., St. Louis, MO. Neostigmine bromide was obtained from the K & K Co., Plainview, NY.

Male Sprague–Dawley rats, 200–225 g were obtained from Taconic Farms, Germantown, NY, and were maintained on commercial rat chow with water *ad lib*. While the rats were under halothane anesthesia, the lungs were excised, immersed in cold 0.2 M sodium phosphate buffer (pH 8.2), trimmed of connective tissue, blotted, weighed and placed in fresh buffer. Approximately 1 g of tissue was obtained from a 200 g rat. After the tissue was homogenized in a Servall Omnimixer at 10,000 r.p.m. for 15 sec, homogenization was completed with a Brinkman polytron operated for 10 sec at half speed. The volume of homogenate was adjusted so that the final concentration was 20 mg wet weight of tissue/ml.

Acetylcholinesterase and butyrylcholinesterase were determined by a slight modification of the method of Ellman *et al.* [14]. All reactants were at room temperature, except the substrates, acetylthiocholine and butyrylthiocholine, which were kept at 0°. The reaction mixture consisted of

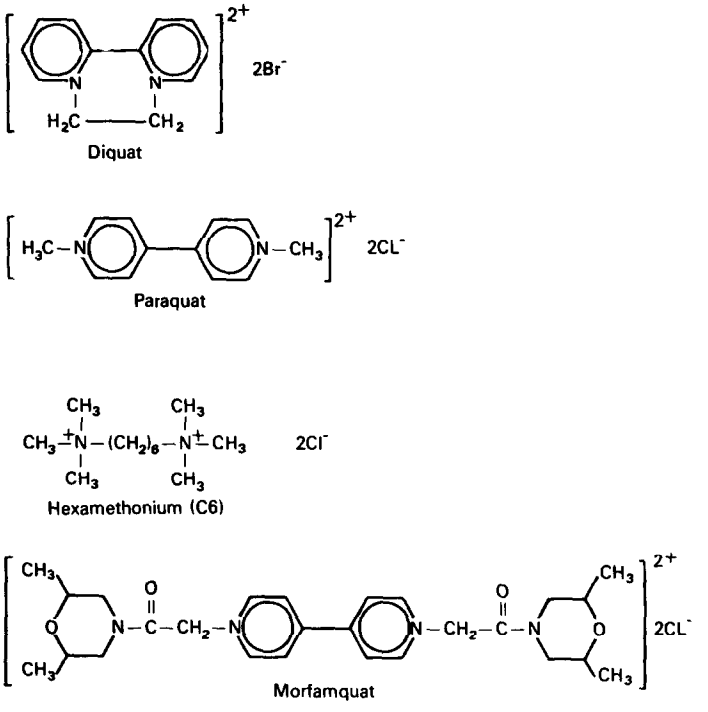

Fig. 1. Structural formulae.

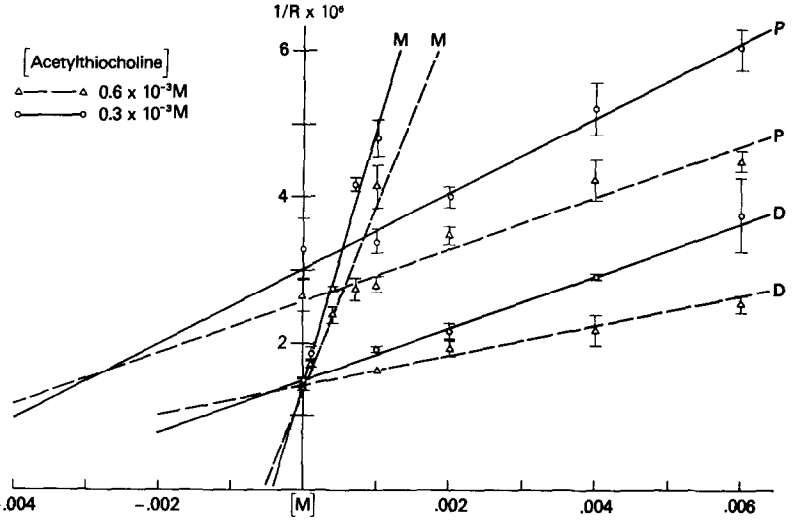

Fig. 2. Dixon plot of $1/R \times 10^6$ where R = moles of substrate hydrolyzed/min/g of tissue and $[M]$ = molar concentration of inhibitor. Key: M = morfamquat; P = paraquat and D = diquat. These lines indicate that the bipyridinium herbicides are competitive inhibitors of acetylcholinesterase and the point where the curves cross = $-K_i$.

5 ml of 0.1 M phosphate buffer (pH 8.0) and drug, 0.75 ml of the homogenate, 0.2 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (0.01 M in 0.1 M phosphate buffer, pH 7.0, containing 15 mg sodium bicarbonate in 10 ml) and 0.04 ml of the substrate (0.075 M acetylthiocholine or butyrylthiocholine).

The reaction mixture was mixed with a vortex mixer and centrifuged (1500 g/min). The optical denisty was read at 420 nm after 7–10 min and was repeated at 10 min intervals until a greater than 0.100 change in optical density was obtained. Protein content was determined by the method of Lowry *et al.* [5] using human serum albumin as a standard. The average amount of protein in the homogenate was 0.125 g/g wet wt of tissue.

Butyrylcholinesterase activity was negligible in our rat lung homogenates in contrast to the results of Ellman *et al.* [4] who reported levels of butyryl ester hydrolysis which were one-third those of the acetyl ester. Neostigmine bromide (1×10^{-5}M) inhibited the hydrolysis of acetylthiocholine iodine by more than 90 per cent.

Inhibition studies were performed using two levels of acetylthiocholine substrate, 0.3 and 0.6 mM. The results were plotted (Fig. 2) according to the method of Dixon which gives K_i directly without calculation [6]. We also determined the slopes of the curves and the intercepts using a standard analysis of variance. The apparent K_i was 2.9×10^{-3} M for paraquat, 2.4×10^{-4}M for diquat and 1.4×10^{-4} M for morfamquat. For comparison, the inhibitory constant, K_i, for hexamethonium on the electric eel enzyme was 4×10^{-4} M, as reported by Changeux *et al.* [7].

Tissue levels for paraquat have been reported by Maling *et al.* [8] to be 15 nmoles/g of lung tissue 6 hr after 15 mg/kg of the base, i.p.; assuming all water in the tissue, the concentration of paraquat was $1.5–2.9 \times 10^{-5}$ M. Sharp *et al.* [9] have shown that the concentration of paraquat in lung is almost constant in the period of 1–7 hr after i.v. administration of 20 mg/kg of the dichloride. The concentration in rat lungs, which they reported, was about 8 μg/g of tissue (31 nmoles/g = 3.1×10^{-5} M) which is in the same range as our *in vivo* data [8].

If paraquat were to be toxic in the lung mainly through the mechanism of inhibition of acetylcholinesterase, it must be assumed that the compound is concentrated 100-fold in a compartment containing acetylcholinesterase.

A preliminary report of this work has been presented [10].

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