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THE COMPETITIVE INHIBITION OF NITROREDUCTASE BY SOME ANALOGUES OF NITROFURANTOIN

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Abstract—The ability of guinea pig liver microsomal and soluble preparations to reduce *P*-nitrobenzoic acid has been investigated. Furadantin and some related nitrofuran analogues have been shown to inhibit competitively the nitroreductase reduction of *P*-nitrobenzoic acid and the enzyme inhibitor characteristics are reported.

FURADANTIN (Nitrofurantoin) is widely used as a urinary antiseptic and its metabolic fate has received attention in both mammalian[1] and bacterial systems.[2] Reduction of the nitro group of nitrofurantoin by nitroreductase to the corresponding amino furantoin would appear to be an obvious metabolic route, but although evidence exists for the formation of amine, it has never been isolated.[1] Indeed, work in these laboratories has shown that aminofurantoin itself is metabolised further by rat liver homogenate.[3] However, nitroreductase readily reduces derivatives of nitrobenzene, e.g. chloramphenicol and *p*-nitrobenzoic acid to the corresponding amines.[4] There is a marked difference between the aromatic character and the chemical reactivity of nitrobenzene and nitrofuran which may explain the inactivity of nitroreductase towards the latter. In view of,

(i) the structural and isosteric similarities of the two substituted systems and,

(ii) the high nitroreductase activity towards *p*-nitrobenzoic acid,

it was thought probable that an enzyme-nitrofuran interaction would occur. The reduction of *p*-nitrobenzoic by nitroreductase was therefore investigated alone and in the presence of Nitrofurantoin and its analogues.

MATERIALS AND METHODS

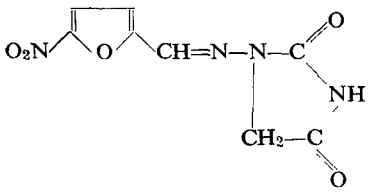
p-Nitrobenzoic acid (BDH) was used as a substrate for studying the inhibition of nitroreductase present in guinea pig liver. The nitrofurans used as inhibitors in these studies were nitrofurantoin* (1.(5 nitro-2-furfylidene amino) hydantoin) Fig. 1, NF62* (5 nitro-2-furaldehyde 4′ methyl semi carbazone) Fig. 2 and NF57* (5 nitro-2-furylmethyl-Ketone semi carbazone).

Tissue preparation

The livers from exsanguinated male guinea pigs (Pilbright strain, 200–250 g) killed by cervical fracture were rapidly removed and immediately chilled to 0° by immersion

* We are grateful to Norwich Pharmacol Co. New York for supplying the compounds.

in isotonic KCl. The tissue (4 vol. of isotonic KCl/g of liver) was homogenised at 0° for 1 min using, an MSE homogeniser and the supernatant was obtained at 10,000 *g* for 30 min in a Sorvall RC2-B Centrifuge. The microsomal fraction was sedimented at 140,000 *g* for 45 min, in an MSE superspeed 40 Centrifuge.



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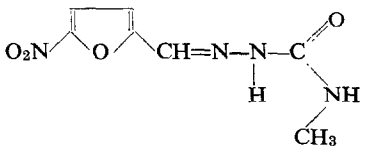

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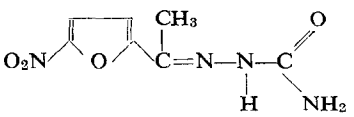

III

The supernatant was reserved and the microsomal pellet was resuspended in phosphate buffer (2 vol./g of liver) followed by a further centrifugation at 140,000 *g* for 30 min. Both the supernatant fraction and the washed microsomal fraction were investigated for nitroreductase activity. All operations were carried out at 0–4° and the supernatant was stored at 0° for 3 days without the loss of reductase activity.

Enzyme activity

The unfortified supernatant fraction was found to reduce *p*-nitrobenzoic acid but a marked increase in enzyme activity was observed in the presence of some added co-factors. The incubation mixture used in these studies had the following composition. NADPH (3·0 μmole), Riboflavin (0·094 μmole), Nicotinamide (4·5 μmole) and G-6-P (17·6 μmole) in Phosphate Buffer pH 7·4 (0·5 ml), liver supernatant (2 ml) and phosphate buffer pH 7·4 (1 ml).

During the inhibitor studies 1 ml of inhibitor in phosphate buffer was added in place of the phosphate buffer. The rate of enzyme reduction of *p*-nitrobenzoic acid was determined at three concentrations (5×10^{-4} to 5×10^{-3} M). Three concentrations of inhibitor were studied (10^{-5} to 10^{-4} M) and the rate of reduction again determined for each concentration of substrate.

After incubating for 1 hr at 37° under nitrogen the protein was precipitated by the addition of 1 ml of trichloracetic acid solution (5%) and the precipitate removed by centrifugation. Aliquots (3 ml) of the supernatant were removed for analysis. The amount of *p*-aminobenzoic acid formed in 1 hr was determined by the method of Bratton and Marshall[5] and the absorbtion determined at 550 mμ.

Under these conditions no colour is produced with aminofurantoin should any have been formed during the incubation period. The nitroreductase activity of the washed

microsomal preparation was investigated under the above conditions in which the supernatant fluid was replaced by the microsomal preparation.

RESULTS

(1) *Microsomal preparation*

There appeared to be no reducing activity associated with the washed microsomal preparation. It was impossible to detect any *p*-aminobenzoic acid formed during these studies even though the method of assay is sufficiently sensitive to detect concentrations as low as 1–2 μg.

(2) *Supernatant fluid*

The data obtained from experiments using supernatant fluid is presented graphically in Figs. 1, 2 and 3. The inhibitor constants (K_i) obtained by the method of Dixon (1953)[6] were found to be for nitrofurantoin $7{\cdot}5 \times 10^{-5}$, for NF62, $7{\cdot}5 \times 10^{-5}$ and for NF57, 5×10^{-5} M.

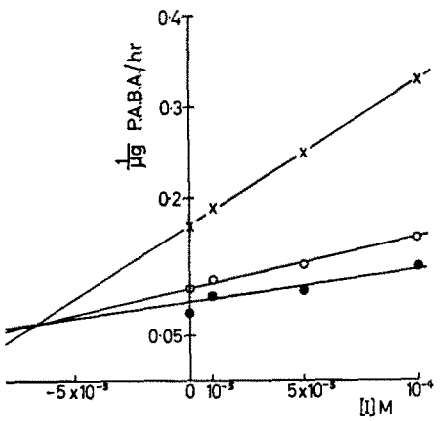

FIG. 1. Inhibition of the enzymic reduction of p-nitrobenzoic acid ([S] = 5×10^{-4}, ×——×; 10^{-3}, ○——○; and 5×10^{-3} M, ●——●) by nitroreductase in the presence of nitrofurantoin.

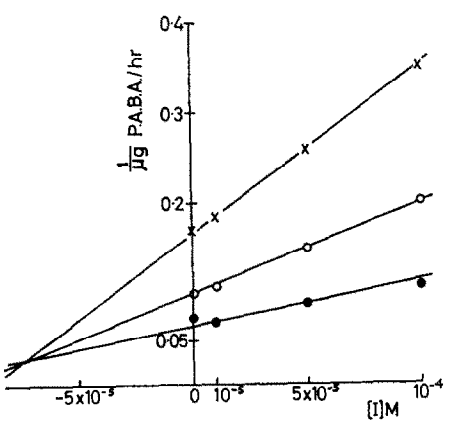

FIG. 2. Inhibition of the enzymic reduction of p-nitrobenzoic acid ([S] = 5×10^{-4}, ×——×; 10^{-3}, ○——○; and 5×10^{-3} M, ●——● by nitroreductase in the presence of NF 62.

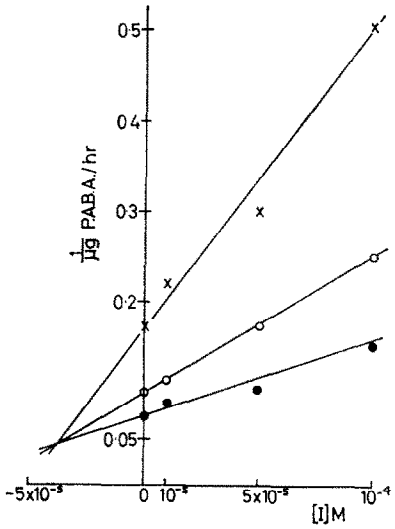

FIG. 3. Inhibition of the enzymic reduction of p-nitrobenzoic acid ([S] = 5 × 10^{-1}, ×——×; 10^{-3}, ○——○; and 5 × 10^{-3} M, ●——● by nitroreductase in the presence of NF 57.

DISCUSSION

In agreement with some early findings[4, 7] but not others,[8] the ability to reduce *p*-nitrobenzoic acid was associated only with the supernatant fraction. Nitroreductase would appear not to be a microsomal but a soluble cytoplasmic enzyme. The results presented in Figs. 1, 2 and 3 establish that the three nitrofurans studied competitively inhibit the reduction of *p*-nitrobenzoic acid and to have an affinity for the enzyme surface. A methyl group α to the furan ring potentiates this affinity; NF57 having the lowest K_i value. The other two nitrofuran derivatives, nitrofurantoin and NF57 in which this methyl group is absent have very similar inhibitor characteristics but are less potent inhibitors of this system than NF57. These and earlier studies show that the nitrofurans have a pronounced affinity for the active surface of nitroreductase but the enzyme has no measurable reducing activity towards these compounds.

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