INHIBITION OF GLYCOLLATE OXIDASE FROM PEA LEAVES

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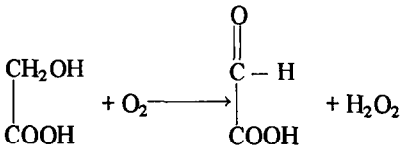
1. Introduction

Glycollate oxidase (glycollate: oxygen oxidoreductase, EC 1.1.3.1) is a flavoprotein catalysing the oxidation of glycollic acid to glyoxylic acid [1].



This enzyme is found in high concentrations in the peroxisomes of photosynthetic tissue and is believed to play an important role in photorespiration [2]. Photorespiration has an incompletely understood function in plant metabolism [3]. In order to investigate further the role of glycollate oxidase in photorespiration it was considered necessary to find an enzyme inhibitor which would be effective in vivo.

Zelitch [4] and Corbett and Wright [5] have investigated aldehyde bisulphite addition compounds as inhibitors. These are powerful competitive inhibitors of glycollate oxidase but are non-specific and ineffective on whole plants.

Recent work has shown the efficacy of acetylenic substrate analogues as irreversible inhibitors of certain flavoproteins [6] and pyridoxalphosphate-containing enzymes [7]. In the case of FMN-containing oxidases the mode of action is thought to involve reaction at position 4a of the isoalloxazine ring of FMN in an analogous manner to the natural substrate. Deprotonation of the α-carbon then leads to formation of an allenic intermediate which can participate in an addition reaction with the nitrogen in position 5 of the isoalloxazine ring. The result is a modified co-factor incapable of participating in further redox reactions [8].

The paper by Walsh et al. [9] concerning inhibition of bacterial L-lactate oxidase by the substrate analogue 2-hydroxy-3-butynoic acid (HBA) seemed relevant to the problem of glycollate oxidase inhibition. Since glycollate oxidase will catalyse the oxidation of straight-chain L-2 hydroxy acids up to L-2-hydroxycaproate in addition to glycollic acid [10], it was considered that HBA might be a candidate inhibitor.

In this paper we show that HBA is an irreversible inhibitor of glycollate oxidase and compare the type of inhibition with that observed by Walsh for L-lactate oxidase. Inhibition of glycollate oxidase in vivo is also reported.

Abbreviations:
HBA, D/L 2-hydroxy-3-butynoic acid;
NaHBA, D/L sodium 2-hydroxy-3-butynoate;
MeHBA, D/L Methyl 2-hydroxy-3-butynoate.

2. Materials

Glycollate oxidase was purified from the leaves of 2–3 week old pea plants (cv. Suttons Phenomenon) using the method of Kerr and Groves [10]. The specific activity of enzyme used in these experiments was in the range 8–10 μmol O_2/min/mg biuret protein.

MeHBA was synthesised as a racemate from methyl glyoxylate using the method of Verny and Vessière [11]. Methyl glyoxylate was prepared by the periodic acid oxidation of dimethyl tartrate in ethereal solution [12]. HBA was prepared from MeHBA by an ester exchange reaction [13] and isolated as the sodium salt (NaHBA). 3-Butynoic acid [14] and D/L 2-hydroxy-3-butenoic acid [15] were also prepared.

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3. Experimental

3.1. *Enzyme inhibition*

Enzyme assays were carried out in an oxygen electrode thermostatted at 30°C. The reaction mixture (3.2 ml) contained: Tris/HCl buffer pH 8.3, 1.5 mmol, FMN 0.2 μmol, NaN_3 (to inhibit any residual catalase activity) 3 μmol, enzyme approx. 0.5 unit. The reaction was started by adding sodium glycollate solution. Light was excluded from the reaction vessel to prevent photo-oxidation of the FMN. The reaction was followed by the uptake of oxygen.

The activity value of Chappell [16], 0.445 μg atoms of oxygen per ml at 30°C for air-saturated buffer was used.

Enzyme and NaHBA solution were incubated in the cell of the oxygen electrode for a timed interval before addition of 30 μl of 1.0 M sodium glycollate. The initial rate of oxygen uptake in μmol min^{-1} (V_t) was measured for t sec incubation of enzyme and inhibitor. Control rates (V_o) were measured after the same pre-incubation times without inhibitor. This was to compensate for a slow fall in enzyme activity when incubated under the conditions of the assay. The high final substrate concentration (1×10^{-2}M) was used to inhibit further reaction of NaHBA with the enzyme.

Glycollate oxidase inhibition in the presence of substrate was measured by using 2,6-dichlorophenolindo-phenol as electron acceptor. The buffer system and volumes used in this assay were identical with those in the oxygen electrode assay except that solutions were purged with nitrogen. Enzyme, inhibitor and glycollate were placed in 10 mm cells in the 30°C thermostatted ceil block of a Unican SP8000 spectrophotometer. At measured times after adding inhibitor to enzyme plus glycollate 2,6-dichlorophenolindophenol (0.10 μmol) was added. The reaction was followed by decrease in the absorbance at 602 nm due to the reduction of 2,6-dichlorphenolindo-phenol.

3.2. *Leaf disc experiments*

1 cm diam discs cut from pea leaves were agitated in flasks containing 3 ml of aqueous inhibitor solution buffered at pH 8.0 with sodium phosphate. Controls were run using buffer without inhibitor. Experiments were performed at 30°C either in the dark or under standard conditions of illumination. At timed intervals after adding inhibitor solution groups of five discs were removed, washed thoroughly in distilled water, homogenised in Tris/HCl buffer 0.1 M pH 8.3, centrifuged and the supernatant assayed for glycollate oxidase activity as above.

Leaf discs incubated with inhibitor were also assayed for glycollate content using the method of Calkins [17]. It was found necessary to analyse the incubation medium as well as the disc homogenate for glycollate, since glycollate apparently diffuses out of the discs.

3.3. *Inhibition of glycollate oxidase in whole plants*

Various species of plants including pea, sugar beet, kale and maize were grown under standard lighting, humidity and temperature conditions in Hoagland nutrient medium. Either NaHBA or MeHBA at concentrations varying from 5×10^{-5}M to 5×10^{-4}M was incorporated in the nutrient medium. Inhibitors were applied to the plants for times varying between 2 hr and 24 hr. The nutrient medium containing inhibitor was then replaced by fresh nutrient medium and the plants grown for a further 24 hr before the leaves were harvested. Glycollate oxidase was assayed in the homogenate supernatants as in the leaf disc experiments and compared with controls grown without inhibitor on the basis of units of enzyme per gramme fresh weight and as units per mg biuret protein. These two methods gave comparable results.

4. Results and discussion

4.1. *In vitro inhibition of glycollate oxidase*

A plot of fractional inhibition of glycollate oxidase activity (V_t/V_o) versus time for four concentrations of NaHBA is shown in fig.1. In contrast to bacterial L-lactate oxidase, glycollate oxidase does not oxidise NaHBA prior to inhibition of the enzyme. There was no oxygen uptake when glycollate oxidase was incubated with NaHBA up to concentrations of 5×10^{-2}M. From fig.1 it can be seen that time-dependent inhibition cannot be represented by a single exponential function. The curvature of the plots in fig.1 could be due to (i) the racemic nature of the inhibitor (ii) the possibility that the enzyme exists in several aggregation states [10] which are inhibited at different rates (iii) dissociation of the FMN-inhibitor adduct from the enzyme. Measurements of the initial rates from fig.1 (first order in inhibitor concentration)

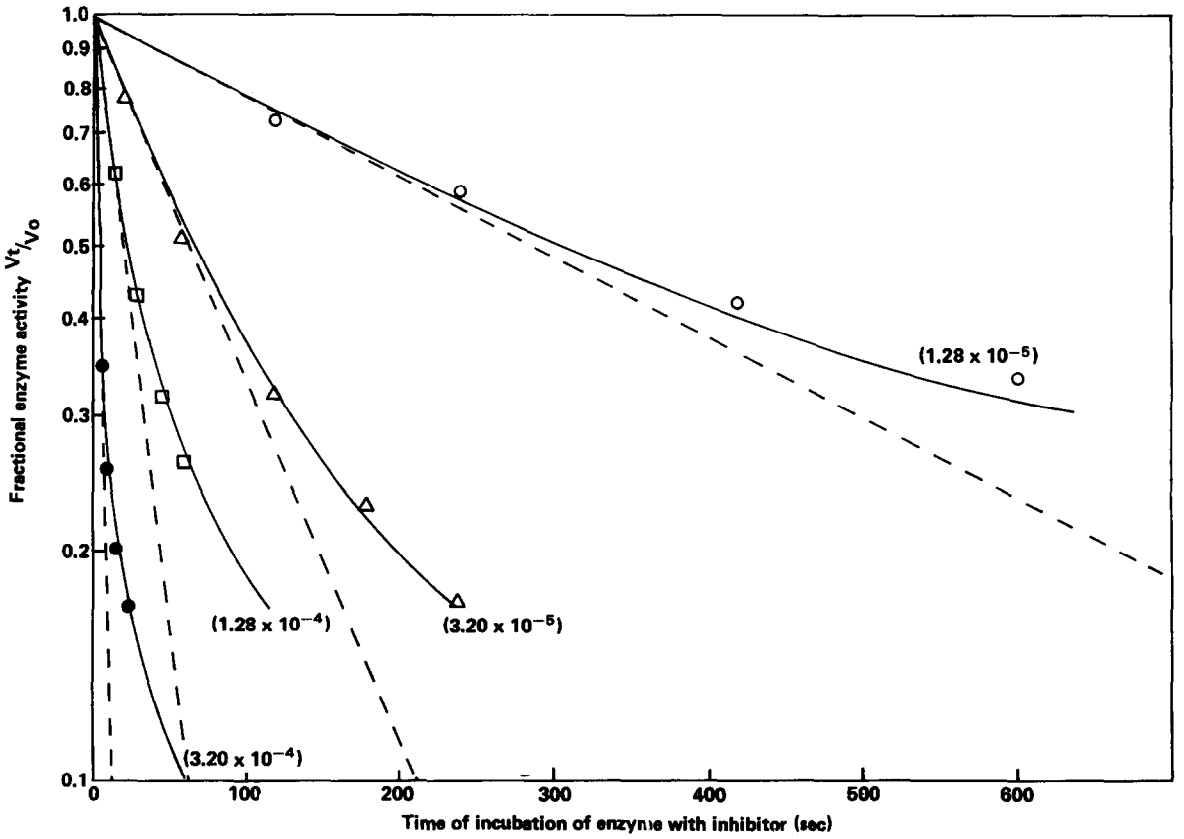

Fig.1. Inhibition of pea leaf glycollate oxidase by NaHBA at 30°C, pH 8.3. Molar concentration of NaHBA are in parentheses. Extrapolated initial rates are indicated (– – –).

gave a value of $3 \pm 0.5 \times 10^2$ 1 mol^{-1} sec^{-1} for the second order rate of enzyme inhibition.

When glycollate oxidase was incubated with 1×10^{-4}M NaHBA in the presence of 1×10^{-3}M sodium glycollate the enzyme was inhibited by 5% after 30 min. This compares with 99% inhibition in the absence of sodium glycollate and indicates that substrate and inhibitor may react with the same site on the enzyme. MeHBA did not inhibit the enzyme at a concentration of 1×10^{-4}M. Inhibition of the enzyme in vivo is presumably due to plant esterase catalysed hydrolysis. When 3-butynoate was incubated with glycollate oxidase no time-dependent enzyme inhibition was observed. However, the compound was found to be a competitive inhibitor with glycollate (K_i; 4×10^{-5}M). 2-Hydroxy-3-butenoate was found to be a substrate of glycollate oxidase (K_m 2×10^{-3}M). These observations suggest that the α-hydroxy group as well as the ethynyl group is necessary to produce an irreversible inhibitor.

4.2. *Effects on glycollate metabolism in pea leaf discs*

Fig.2 shows the time course of inhibition of glycollate oxidase by 3×10^{-4}M MeHBA in illuminated leaf discs and the resultant accumulation of glycollate. Lower concentrations of MeHBA, use of the sodium salt, or treatment of discs in the dark caused a slower rate of enzyme inhibition. Over the 7 hr experimental period 3×10^{-4}M MeHBA caused no detectable effect on respiration of leaf discs under the same conditions.

4.3. *Effects on whole plants*

Table 1 shows that MeHBA applied to the roots of

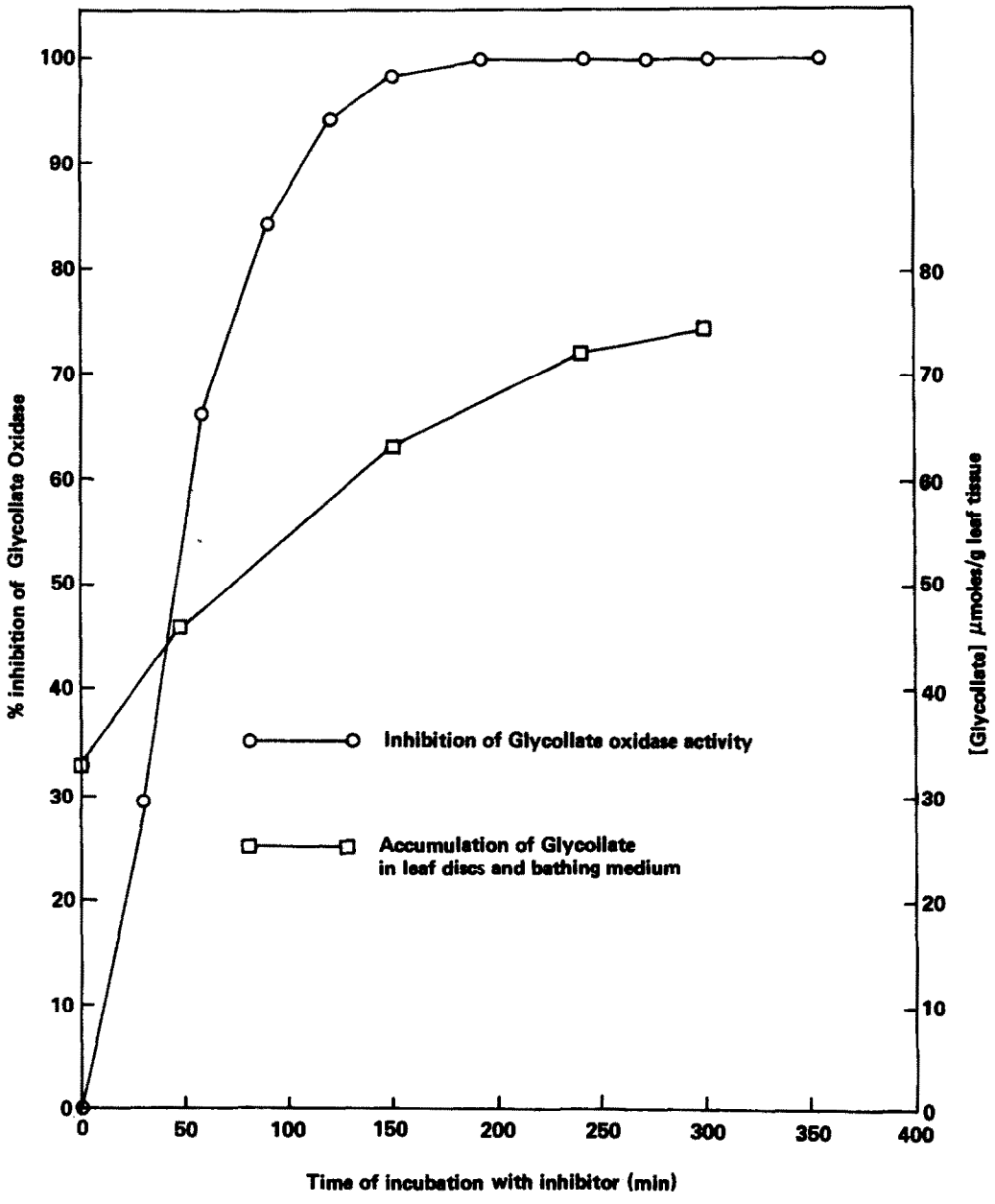

Fig.2. Inhibition of glycollate oxidase and accumulation of glycollate in pea leaf discs incubated in aqueous MeHBA (3×10^{-4}M) at 30°C with illumination.

Table 1
Inhibition of glycollate oxidase in the leaves of whole plants by acetylenic inhibitors applied via the roots

Species	Compound		Time of treatment	% Inhibition
Pea	NaHBA	10^{-4}M	24 hr	16
Sugar beet	NaHBA	2×10^{-4}M	72 hr	75
Kale	MeHBA	5×10^{-5}M	22 hr	42
Kale	MeHBA	10^{-4}M	20 hr	62
Kale	MeHBA	2×10^{-4}M	2 hr	58
Kale	MeHBA	5×10^{-4}M	2 hr	63
Pea	MeHBA	10^{-4}M	6 hr	45
Pea	MeHBA	10^{-4}M	23 hr	76
Pea	MeHBA	5×10^{-4}M	5 hr	78
Maize	MeHBA	10^{-4}M	4 hr	72
Maize	MeHBA	10^{-4}M	6 hr	80
Maize	MeHBA	5×10^{-4}M	5 hr	62

a variety of plant species (pea, maize, kale) causes irreversible inhibition of glycollate oxidase in the leaves. The sodium salt appears to be less active. The degree of inhibition never exceeded 80% although the residual enzyme activity could be inhibited by addition of HBA in vitro. Maize, a 'non-photorespiring' species appeared to be more susceptible although its leaves contained only about 15% of the glycollate oxidase activity per gramme compared with pea and kale.

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