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INHIBITION OF PHOTOSYNTHETIC CO_2 FIXATION IN SPINACH CHLOROPLASTS BY α-HYDROXY 2-PYRIDINEMETHANESULPHONATE

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Abstract—Photosynthetic CO_2 fixation in spinach chloroplasts is strongly inhibited by α-hydroxy 2-pyridinemethanesulphonate (α-HPMS). Several chloroplast enzymes, namely carbonic anhydrase, ribulose-diphosphate carboxylase and ribosephosphate isomerase were unaffected by a concentration of α-HPMS sufficient to cause 50% inhibition of chloroplast CO_2 fixation. Phosphopyruvate carboxylase and glycolate oxidase which are both competitively inhibited by α-HPMS, were essentially absent from the chloroplast preparations. Permeability considerations suggest that a site in the chloroplast membrane may be responsible for the inhibition.

INTRODUCTION

THE α-hydroxymethanesulphonates have been widely used in studies on the photosynthetic origin and metabolism of glycolate since their introduction as competitive inhibitors of glycolate oxidase by Zelitch.[1] Accumulation of glycolate following the administration of α-hydroxymethanesulphonates has been observed during photosynthesis in leaves of higher plants[2,3] and in some unicellular algae.[4,5] However, there have also been indications that α-hydroxymethanesulphonates are not highly specific for the inhibition of glycolate oxidase (E.C. 1.1.3.1) in some living cells.[6]

Recently three more enzymic sites of inhibition have been reported for α-HPMS. The inhibition of acetate photoassimilation in *Chlorella pyrenoidosa* by α-HPMS has been attributed to the inhibition of acetyl-CoA synthetase (E.C. 6.2.1.1.).[5] α-HPMS also inhibited phosphopyruvate carboxylase (E.C. 4.1.1.31) and NAD malate dehydrogenase (E.C. 1.1.1.37) from the leaves of several plants,[7] namely spinach, maize and two species of *Atriplex*, one of which (*A. spongiosa*) possesses the β-carboxylation pathway of CO_2 fixation in which phosphopyruvate carboxylase has a key role. The inhibition of this carboxylation reaction by α-HPMS *in vivo* was confirmed by examination of the products of $^{14}CO_2$ fixation.[7]

Although it was reported that 0·4 mM α-HPMS did not inhibit photosynthetic CO_2 fixation in isolated spinach chloroplasts,[8] other α-hydroxysulphonates were found to inhibit photosynthetic CO_2 fixation both in leaf segments[9] and in isolated chloroplasts.[10]

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Recently α-HPMS was shown to inhibit the photosynthetic CO_2-fixation of tobacco leaves by a combination of its effect in reducing the width of stomatal apertures with an inhibition of photosynthesis at the cellular level.[11] The present report demonstrates the inhibitory effect of α-HPMS on photosynthetic CO_2 fixation in isolated spinach chloroplasts and shows further that this inhibition is not attributable to any of the known enzymic sites of inhibition.

RESULTS

Chloroplasts isolated from spinach leaves in the manner described showed rates of CO_2 fixation between 5 and 22 μmoles of CO_2 fixed/mg chlorophyll/hr and maintained these rates for about 30 min. Figure 1 shows the results for two experiments in which inhibition of CO_2 fixation by α-HPMS was studied. The degree of inhibition by 0·7 mM α-HPMS varied within the range 50 ± 10% in all experiments.

As the structure of α-HPMS is analogous to that of glycolate (α-hydroxyacetate), the effects of glycolate on chloroplast CO_2 fixation were determined (Table 1). Glycolate was much less effective as an inhibitor, but the effects of glycolate and α-HPMS applied together were to some extent additive.

TABLE 1. $^{14}CO_2$ FIXATION BY ISOLATED SPINACH CHLOROPLASTS IN THE PRESENCE OF α-HPMS AND ADDED GLYCOLATE

Additions			
α-HPMS 0·74 mM	glycolate (Na) 7·4 mM	Rate of $^{14}CO_2$ fixation (μmoles CO_2/mg Chl.hr)	% inhibition
−	−	11 6	0
+	−	6·7	42
−	+	8 5	27
+	+	5 4	53

Over the range of concentrations tested, α-HPMS had no effect on the proportion of label entering glycolate from fixed $^{14}CO_2$ (Table 2). A reduction in the proportion of label in glycolate always occurred in response to the addition of fructose diphosphate, a finding which supports the derivation of glycolate from sugar phosphate intermediates of the Calvin cycle.

TABLE 2. EFFECTS OF FRUCTOSE DIPHOSPHATE ON $^{14}CO_2$ FIXATION IN ISOLATED SPINACH CHLOROPLASTS IN THE PRESENCE AND ABSENCE OF α-HPMS

Additions				
α-HPMS 7·1 mM	Fructose diphosphate 1 4 mM	Rate of $^{14}CO_2$ fixation (μmoles CO_2/mg Chl.hr)	% inhibition (−) or stimulation (+)	% of fixed ^{14}C in glycolate
−	−	8·0	0	7·6
+	−	1·37	− 83	7 9
−	+	12 8	+ 60	4 0
+	+	5 2	− 35	3·7

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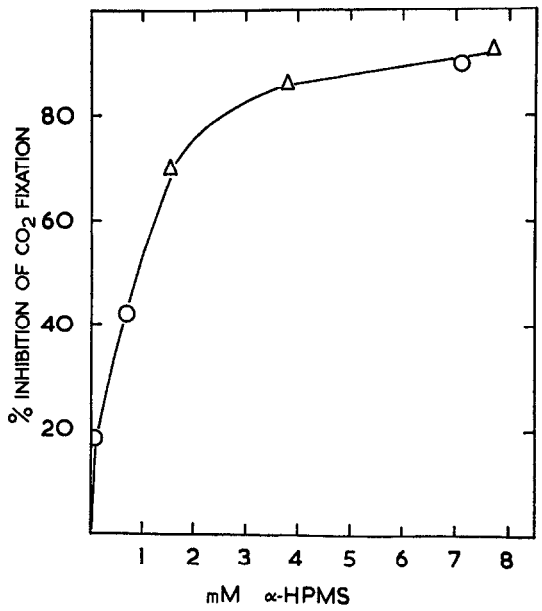

FIG. 1. INHIBITION OF $^{14}CO_2$ FIXATION IN ISOLATED SPINACH CHLOROPLASTS BY α-HPMS. EXPERIMENTAL CONDITIONS WERE AS DESCRIBED IN METHODS.

Ribulosediphosphate carboxylase (E.C. 4.1.1.39) and ribosephosphate isomerase (E.C. 5.3.1.6.) were not inhibited by 1 mM α-HPMS either in chloroplast extracts or in leaf supernatant fractions. Phosphopyruvate carboxylase showed competitive inhibition by 1 mM α-HPMS in supernatant obtained from leaf homogenates prepared with 'Polyclar AT' as described by Osmond and Avadhani.[7] It is apparent that relatively undamaged spinach chloroplasts were obtained in good yield in this work as they contained 40–45% of the extracted ribulosediphosphate carboxylase, which is an enzyme known to be confined to the chloroplasts.[12] As the same chloroplast preparations contained less than 1% of the total extracted phosphopyruvate carboxylase activity, the remainder being in the supernatant, it seems very probable that phosphopyruvate carboxylase is not a chloroplast enzyme in spinach.

Carbonic anhydrase (E.C. 4.2.1.1) was assayed in broken chloroplasts and on the surface of intact chloroplasts prepared from the same leaf homogenate. In neither case was any inhibition by 1 mM α-HPMS detected.

Inhibition of CO_2 fixation by 0·7 mM α-HPMS was almost completely overcome by the addition of 0·1 ml of leaf supernatant, as shown in Table 3. The addition of supernatant alone usually stimulated the observed rate of CO_2 fixation.

DISCUSSION

The inhibition of photosynthetic CO_2 fixation in spinach chloroplasts by α-hydroxysulphonates was previously attributed[10] to the inhibition of glycolate oxidase associated with the chloroplast preparations.[8] However glycolate oxidase has been reported to occur

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TABLE 3. ALLEVIATION OF α-HPMS INHIBITION OF $^{14}CO_2$ FIXATION IN ISOLATED SPINACH CHLOROPLASTS BY LEAF SUPERNATANT

Additions			
α-HPMS 0·71 mM	Supernatant 0 1 ml	Rate of $^{14}CO_2$ fixation (μmoles CO_2/mg Chl. hr)	% inhibition (−) or stimulation (+)
−	−	5·5	0
+	−	2·4	− 56
−	+	9·3	+ 69
+	+	9 0	+ 64

in spinach peroxisomes, but not in the chloroplasts,[13] and in our chloroplast preparations no glycolate oxidase could be detected. Furthermore, accumulation of label from fixed $^{14}CO_2$ into glycolate, a characteristic response to glycolate oxidase inhibition, was not shown by intact chloroplasts (Table 2). Other enzymes known to be inhibited by α-hydroxysulphonates are NADH: malate dehydrogenase which is a mitochondrial and peroxisomal enzyme,[14] and phosphopyruvate carboxylase which does not appear to be a chloroplast enzyme in spinach.

Since chloroplasts are known to have a low permeability to glycolate,[15,16] it is probable that they have a low permeability to α-HPMS and therefore the inhibition of CO_2 fixation may concern the inner or outer chloroplast membranes. In this respect, carbonic anhydrase was a suitable enzyme to examine, since 9–15% of its total activity in the chloroplast is associated with the chloroplast surface.[17] This distribution of the enzyme was confirmed, but neither surface nor total chloroplast carbonic anhydrase activities showed any inhibition by 1 mM α-HPMS. It is suggested that α-HPMS may be affecting other transport processes associated with the chloroplast membrane during photosynthesis.

The possibility that the inhibition of chloroplast CO_2 fixation by α-HPMS may be brought about by limitation of NADPH and ATP synthesis is rendered unlikely by earlier observations that photoreduction of NADP and photophosphorylation by spinach chloroplast fragments were not appreciably inhibited by sodium sulphite or glyoxal-bisulphite at concentrations of 1 mM or less.[10]

The factors responsible for the alleviation of α-HPMS inhibition (Table 3) have not yet been identified. These factors may well be constituents of the cytoplasm, in which case the direct effect of α-HPMS on the chloroplast may not always be expressed in living cells. Some labelling studies do indicate that cytoplasmic sites are those primarily affected by α-HPMS *in vivo*.[2,3,7] On the other hand, certain effects of α-HPMS on living cells might be explained in terms of a direct interference with chloroplast metabolism. Such effects include the closure of stomates in leaves[11,18] and inhibition of glucose uptake in *Chlorella*.[19] In view of the continued expansion in the range of known inhibitory effects attributable to α-hydroxymethanesulphonates, a degree of caution is recommended in their future application.

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EXPERIMENTAL

Spinach plants (*Spinacia oleracea*, L.) were grown in a glass house or leaves were obtained from local markets. Chemicals used were of the best available purity. Isolation media and α-HPMS solutions were prepared 2 hr before use.

Preparation of Chloroplasts

After preliminary illumination of detached leaves by flotation in a Warburg manometer bath (7000 lx, 20°, 1 hr) chloroplasts were isolated by the procedure of Cockburn *et al.*[20] Laminae were chilled in cold water and blended in an 'Atomix' for 5 sec with a semi-frozen solution containing 330 mM sorbitol, 5 mM $MgCl_2$, 2 mM Na-isoascorbate and 10 mM Na $P_2O_7.10\ H_2O$ at pH 6·5 (100 ml per 20 to 25 g laminae). The macerate was passed successively through 2 then 8 layers of cotton muslin into 50 ml plastic tubes and centrifuged from rest to 4000 *g* to rest within 90 sec. The supernatant was immediately decanted and the chloroplast pellets gently resuspended with several ml of an ice solution containing 330 mM sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na_2EDTA and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) at pH 7·6. Chloroplast extracts were prepared by resuspending pellets in 10 ml of cold water or 1 mM dithiothreitol.

Measurement of Photosynthetic CO_2 Fixation

Within 20 min of isolation, chloroplasts (0·2 ml, containing 40–80 μg chlorophyll) were incubated in 1·0 ml of resuspension medium to which $NaH^{14}CO_3$ (13–14 mM, specific activity 0 50 or 0·98 μc/μmole) had been added. The incubations were performed in manometer vessels in a manometer bath maintained at 20° with continuous illumination of 8000 lx at the level of the vessels. For additions, a 10 mM solution of α-HPMS was prepared in resuspension medium and readjusted to pH 7·6.

After 20 min illumination, vessel contents were boiled for 5 min. Aliquots (0·1 ml) were dried on Al planchettes and counted in a gas flow counter. Control treatments for dark $^{14}CO_2$ fixation plus manipulation time accompanied each experiment, giving rates of 0·04–0·07 μmoles CO_2 fixed/mg chlorophyll/hr. Chlorophyll was measured in 80% acetone extracts.[21]

Separation of Products of $^{14}CO_2$ Fixation

Partial separation of labelled products of $^{14}CO_2$ fixation was achieved using columns of Dowex 1 (acetate) exactly as described by Zelitch.[2] Duplicate 1 ml samples from the 10 ml fraction containing glycolate plus glycerate were counted by scintillation spectrometry in 10 ml of the scintillator described by Bray.[22] Quenching by acetate in the samples was found to be negligible. Over 90% of the radioactivity in this fraction was labile when 1 ml samples were dried on planchettes and could thus be attributed to the glycolate-^{14}C component.

Enzyme Assays

Ribulosediphosphate carboxylase, phosphopyruvate carboxylase and ribosephosphate isomerase were assayed as described by Bradbeer.[23] Carbonic anhydrase was assayed[17] at 0–2° in a 2·5 reaction mixture containing 0·1 mg bromothymol blue indicator in 1 ml of 25 mM veronal buffer, pH 8·2. Saturated CO_2 solution (1 ml) was injected to start the reaction. Sorbitol (330 mM) was incorporated into the veronal-dye solution for comparison of whole with broken chloroplasts.

A 10 mM solution of α-HPMS in water adjusted to the appropriate pH was used for additions in enzyme assays. The protein content of broken chloroplasts and leaf supernatants was determined by the biuret method.[23]

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