Effects of Glyphosate on Isolated Maize Mitochondria

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ABSTRACT

The effects of the herbicide glyphosate (K^+ salt) on isolated maize mitochondria have been investigated. Protein synthesis, oxygen uptake (state 3 and state 4 respiration) and passive swelling were inhibited at concentrations in the 10^{-6}–10^{-2} M range. No decrease of the respiratory control ratio (*RCR*) or stimulation of ATPase activity by glyphosate (K^+ salt) were observed. It is concluded that the previously reported decrease of the *RCR* and ATPase stimulation by glyphosate (isopropylamine salt) were probably due to the isopropylamine moiety or to impurities of the technical product.

Key words: Glyphosate; Herbicide action; Maize; Mitochondria.

INTRODUCTION

Glyphosate [*N*-(phosphonomethyl) glycine] is a non-specific herbicide which has gained wide use because of its easy translocation within the plant and its ready inactivation and degradation in the soil. Hoagland and Duke (1982) have recently reviewed the biochemical effects of glyphosate on plants and have pointed out that although the interference of this herbicide with aromatic amino acid synthesis and phenolic compound metabolism is probably involved in its primary mechanism of action, other crucial process(es) might be equally affected. Respiration is among the processes which have been implicated in this context, but published evidence is somewhat contradictory. Ali and Fletcher (1978) reported that glyphosate was capable of inducing a dramatic and rapid decline in corn root respiration and concluded that the primary mode of action of the herbicide involves inhibition of respiration in roots resulting in death of the plant. They ascribed the previous report by Sprankle, Meggitt and Penner (1975) of a slower onset of respiratory inhibition to the fact that the latter authors had measured whole plant respiration, instead of the respiration of roots, which was the organ more markedly affected by the herbicide. Cole, Dodge and Casely (1980) also reported a rapid and complete impairment of respiratory competence in the roots, which was concomitant with the induction of phenylalanine ammonia–lyase. In contrast, other reports have indicated either no effect on respiration of roots (Hanson and Rieck, 1975; cited by Hoagland and Duke, 1982) and isolated cells (Brecke and Duke, 1980) or even an enhancement of respiration (Abu-Irmaileh, Jordan and Kumamoto, 1970). The only reported experiments carried out with isolated mitochondria (Olorunsogo, Bababunmi and Bassir, 1979) indicated an uncoupling of respiration in isolated corn-shoot mitochondria by the isopropylamine salt of the herbicide. However, the interpretation of these observations is obscured by the fact that the effect of herbicide dosage on respiratory uncoupling was erratic and by the reported evidence of the uncoupling effect of certain amines (Skulachev, Jasaitis, Navickaite, Yaguzhinsky, Liberman, Topaili and Zofina, 1969; cited by Dawson and Selwyn,

1974). The present study is an investigation of the effects of glyphosate on mitochondria, which stemmed from the initial observation of an inhibition of mitochondrial protein synthesis by pure glyphosate.

MATERIALS AND METHODS

Glyphosate

The acid form (96·7% pure), kindly supplied by Monsanto (Spain), was used throughout this study as a 100 mol m^{-3} stock solution which was brought to pH 7·2 with KOH. When indicated in the text, a concentrated solution of the isopropylamine salt (460 g dm^{-3}), or its commercial formulation, 'Round Up', (360 g dm^{-3} plus surfactant), were also used.

Preparation of mitochondria

Zea mays L., hybrid stock G-4507 (Mahissa, Spain) was germinated in the dark for about 4 d and mitochondria were isolated essentially as described by Forde, Oliver and Leaver (1979). About 16 g of hand-dissected coleoptiles were homogenized in 2 vols of 0·4 mol dm^{-3} mannitol, 8·0 mol m^{-3} cysteine, 0·1% (w/v) bovine serum albumin (BSA), 1·0 mol m^{-3} EGTA, 10 mol m^{-3} MOPS. brought to pH 7·8 with KOH. The mitochondria in the homogenate were pelleted between 1000 × *g* and 10 000 × *g* and further purified by sucrose gradient centrifugation (10%–55%, w/w, sucrose in 1·0 mol m^{-3} EGTA, 0·1% BSA, 10 mol m^{-3} Tricine, pH 7·2) at 40 000 × *g* for 60 min in a SW-41 rotor at 4 °C. Protein was quantitated by the method of Bradford (1976).

Protein synthesis by isolated mitochondria

The procedure described by Forde, Oliver and Leaver (1978) was followed throughout, except that 2% cycloheximide (Forde *et al.*, 1979) was used in the incubation media. Mitochondria (80–250 μg of protein) were incubated with vigorous shaking in 70 mm^3 of a medium containing 0·26 mol dm^{-3} mannitol, 90 mol m^{-3} KCl, 10 mol m^{-3} $MgCl_2$, 10 mol m^{-3} Tricine (pH 7·2), 5·0 mol m^{-3} sodium phosphate (pH 7·2), 1·0 mol m^{-3} EGTA, 25 μmol of 19 amino acids (excepting methionine), 2·0 mol m^{-3} dithiothreitol, 1·0 mol m^{-3} GTP, and 5·0 μCi of [^{35}S]methionine (800 Ci $mmol^{-1}$). Aliquots of 3·0 mm^3 were counted after precipitation with cold 10% trichloroacetic acid in a liquid scintillation counter (Intertechnique). Incubations were carried out for 60 min at 25 °C. Energy was provided either by the inclusion of 10 mol m^{-3} sodium succinate/6·0 mol m^{-3} ADP or 8·0 mol m^{-3} creatine phosphate/25 μg, creatine phosphokinase/6·0 mol m^{-3} ATP. The initial inhibition experiment was carried out with a commercial formulation of glyphosate (isopropylamine salt, surfactant included). All subsequent experiments were performed with pure glyphosate (K^+ salt). After incubation, the samples were fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 12·5% acrylamide) according to Laemmli (1970). Fluorography of the labelled proteins was carried out according to Laskey and Mills (1975).

Measurements of respiratory activity

Oxygen uptake was measured in a Clarke electrode. Purified mitochondria were resuspended in a medium containing 0·4 mol dm^{-3} mannitol, 10 mol m^{-3} KCl, 5·0 mol m^{-3} $MgCl_2$, 1·0 mol m^{-3} EGTA, 0·1% (w/v) BSA, 0·8 mol m^{-3} ATP, 10 mol m^{-3} K-phosphate, pH 7·2, and 10 mol m^{-3} succinate as substrate. Effects on state 4 respiration were measured by addition of the test chemical following ADP depletion during the period of state 3 respiration initiated by the addition of a limited amount of ADP (0·2 μmol). The effect on state 3 respiration was determined by adding the test chemical after an excess of ADP (1·0 μmol). Respiratory control ratios for O_2 utilization were determined from the oxygen-electrode traces recorded in response to ADP additions.

ATPase activity

The assay was carried out according to McMurray and Begg (1959), as described by Bababunmi and Bassir (1972). The incubation medium was 0·108 mol dm^{-3} sucrose, 75 mol m^{-3} KCl, 50 mol m^{-3} Tricine, at either pH 7·4 or pH 8·5. Mitochondria (~600 μg of protein) were incubated in a volume of 200 mm^3 for 20 min, at 20 °C. The reaction was stopped by adding 2% H_2SO_4, 0·5% SDS. The phosphate liberated was quantified by its colour reaction with 0·5% ammonium molybdate (absorbance at 750 nm).

Swelling and contraction of mitochondria

The mitochondrial suspension was transferred to the swelling medium (0·2 mol dm^{-3} KCl, 20 mol m^{-3} Tricine buffer, pH 7·5, and 0·1% BSA) and passive swelling was measured spectrophotometrically by the changes in light-scattering at 520 nm, as described by Stoner and Hanson (1966). Test chemicals were added as indicated in Fig. 4. Contraction was induced by addition of 1·0 mol m^{-3} ATP and 1·0 mol m^{-3} $MgCl_2$.

RESULTS

Protein synthesis by isolated corn-shoot mitochondria was inhibited by very low concentrations of a commercial glyphosate preparation (isopropylamine salt plus surfactant), as shown in Fig. 1. To ascertain that the observed effect was due to glyphosate, and not to the amine or the surfactant, the acid form of the herbicide was brought up to pH 7·2 with KOH and added to the incubation mixture at different concentrations, the amount of K^+ contributed in this way being negligible in comparison with the K^+ concentration (10^{-1} mol dm^{-3}) of the incubation media. Protein synthesis was inhibited both when supported by an external energy source (creatine phosphokinase/creatine phosphate) and when energy was supplied by succinate respiration (Fig. 1). The inhibition with the K^+ salt was less pronounced than with the commercial formulation. An enhancement of protein synthesis was observed at 10^{-2} mol dm^{-3} glyphosate in the case of the succinate-mediated synthesis, which was consistent over several experiments, although its magnitude was variable. Protein patterns after electrophoresis and fluorography were essentially the same as those reported by Forde *et al.* (1978, 1979) and the inhibition by glyphosate affected all the observed proteins in a similar way (results not shown).

Glyphosate (K^+ salt) was found to inhibit oxygen consumption both in state 4 (ADP absent from the medium) and in state 3 (excess ADP in the medium) of respiration (Fig. 2A). This inhibition increased with concentration, but was not complete even at 10^{-2} mol dm^{-3}

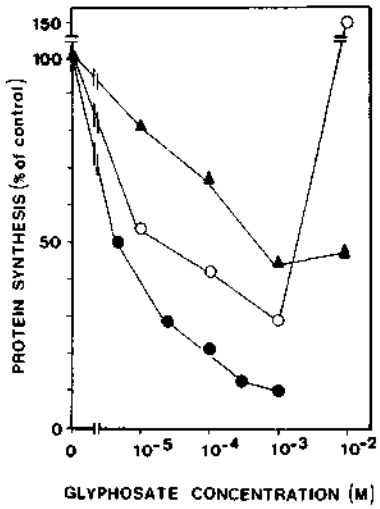

FIG. 1. Inhibition by glyphosate of protein synthesis by isolated maize mitochondria. Mitochondria (80–250 μg of protein) were incubated with 5·0 μCi of [^{35}S]methionine (800 Ci $mmol^{-1}$) in a final volume of 70 mm^3. Two 3·0 mm^3 aliquots were taken for counting and the rest was used for electrophoresis and fluorography. (–▲–▲–) Pure glyphosate (K^+ salt), creatine phosphate/creatine kinase as energy source, 100% = 50 000 ct. min^{-1} (–○–○–) Pure glyphosate (K^+ salt), succinate as energy source, 100% = 9500 ct. min^{-1} (–●–●–) Formulated product (isopropylamine salt plus surfactant), creatine phosphate/creatine kinase as energy source, 100% = 17 500 ct. min^{-1}. Controls with acetate as energy source yielded <10% of the insoluble ct. min^{-1} obtained with succinate.

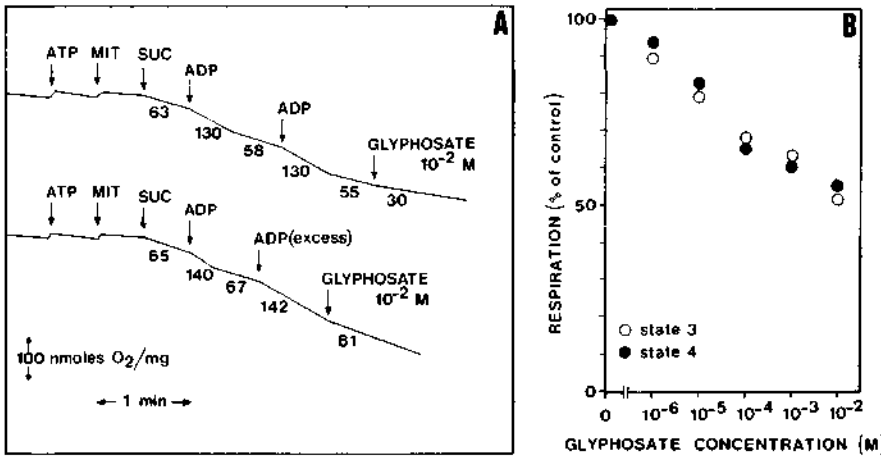

FIG. 2. Effect of glyphosate (K^+ salt) on respiration of isolated mitochondria (A) Oxygen electrode traces corresponding to state 4 (upper trace) and state 3 (lower trace) respiration. The following additions were made to a 2·5 cm^3 electrode chamber: 2·0 μmol ATP, 400 μg (state 4) and 800 μg (state 3) mitochondrial protein (MIT), 23 μmol succinate (SUC), 200 nmol ADP or 1·0 μmol ADP (excess), numbers under the traces indicate nmol of O_2 min^{-1} mg^{-1}. (B) Variation of inhibition with glyphosate concentration.

(Fig. 2B). Respiratory inhibition was similar in both states and the respiratory control ratio (*RCR*) was not significantly affected. In a separate experiment, the K^+ salt and the isopropylamine salt (without surfactant) were compared at a concentration of 10^{-3} mol dm^{-3} and while the K^+ salt did not affect the *RCR* (<5%), the isopropylamine salt markedly decreased the *RCR* (about 38%). No significant enhancement of ATPase activity by glyphosate (K^+ salt) was observed either at pH 7·4 or at pH 8·5 (Table 1).

Corn mitochondria undergo swelling in the presence of KCl. A significantly slower rate of swelling is observed when glyphosate (K^+ salt) is added to the medium (Fig. 3A) and this effect is concentration dependent (Fig. 3B). Glyphosate (K^+ salt)-treated mitochondria are still able to contract when ATP–Mg^{2+} is added (Fig. 3C). Oligomycin also retards swelling, but dinitrophenol (DNP) accelerates the process (Fig. 3D). The isopropylamine salt of glyphosate has the same slowing effect, on a molar basis, as the K^+ salt, but prevents the contraction mediated by ATP–Mg^{2+} (Fig. 3E), a situation which is similar to that of oligomycin (Fig. 3D). The experiment in Fig. 3F was carried out to ascertain that glyphosate retards the swelling of mitochondria but does not induce contraction.

TABLE 1. *Effect of glyphosate on ATPase activity*

Glyphosate (K^+ salt) (mol dm^{-3})	Activity (nmol mg^{-1} min^{-1})[a]	
	pH 7·4	pH 8·5
0	52	59
10^{-4}	54	54
10^{-3}	57	63

[a] Average of 3 determinations. Phosphate liberated in 20 min at 20 °C was measured in an incubation mixture of 200 mm^3 containing about 600 μg of protein.

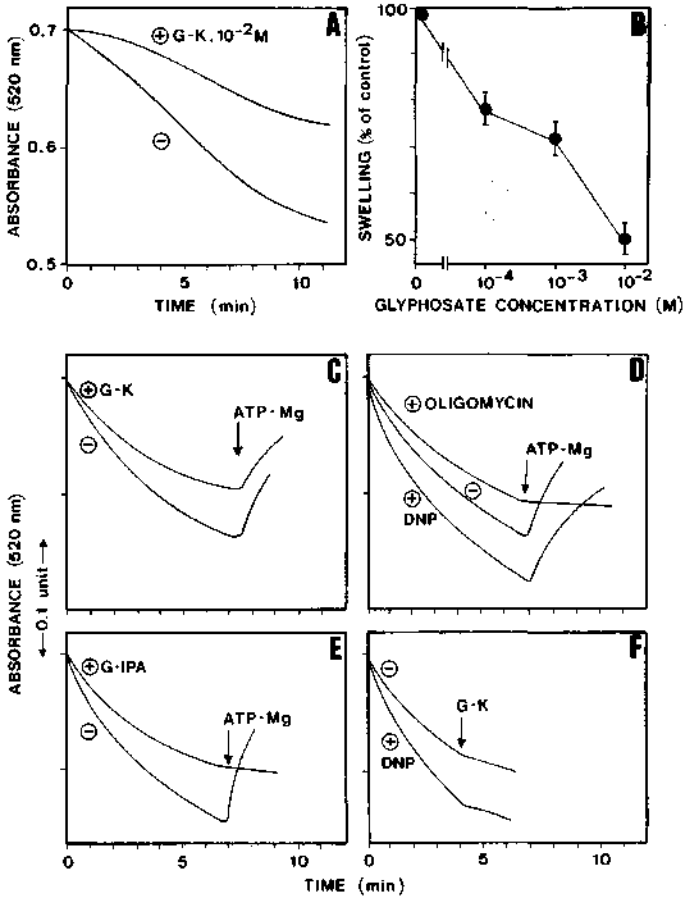

FIG. 3. Effect of glyphosate on passive swelling of mitochondria (90–120 μg cm^{-3}). (A) Effect of initial addition of glyphosate (K^+ salt; G–K). (B) Dosage dependence of swelling inhibition by G–K; 100% swelling = 0·16 Abs/10 min. (C) Contraction mediated by ATP–Mg^{2+} in the presence (5×10^{-3} mol dm^{-3}) and in the absence of G–K. (D) Effects of oligomycin (1·0 μg cm^{-3}) and dinitrophenol (10^{-4} mol dm^{-3}, DNP). (E) Effect of glyphosate (isopropylamine salt. G–IPA). (F) Effect of late addition of G–K.

DISCUSSION

The present evidence confirms that glyphosate markedly affects plant mitochondria, but indicates that the uncoupling effects reported by Olorunsogo *et al.* (1979) for the isopropylamine salt of glyphosate were probably due either to the isopropylamine moiety or to impurities. Indeed, we did not observe either a decrease of the *RCR* or a stimulation of ATPase when the K^+ salt of glyphosate was used in the presence of excess KCl in the medium. In our experiments, the isopropylamine salt did decrease the *RCR* but its effect on mitochondrial swelling was opposite to that of dinitrophenol, which is a typical uncoupler. The effects of the K^+ and the isopropylamine salts on mitochondrial swelling were identical, except that the latter prevented ATP-mediated mitochondrial contraction.

The three observed effects, inhibition of respiration, inhibition of protein synthesis, and retardation of mitochondrial swelling, could be explained in terms of a single mechanism because the alteration of membrane permeability could result in a limitation of the access of the amino acid precursors and the respiratory substrate into the mitochondria or in an alteration of the internal concentrations of ions. However, the present evidence does not exclude the possible independence of the different effects. The creatine phosphokinase/

creatine phosphate energy-generating system is not affected by glyphosate as evidenced by the lack of effect of glyphosate on *in vitro* protein synthesis by the wheat-germ system supported by the same energy source (unpublished results). We have no obvious explanation for the observed enhancement of protein synthesis at 10^{-2} mol dm^{-3} glyphosate.

Glyphosate effects on isolated mitochondria are detected at concentrations as low as 10^{-6} to 10^{-4} mol dm^{-3}, which indicates that these organelles are at least as sensitive to the herbicide as other well studied targets. Thus, the induction of phenylalanine ammonia–lyase in maize seedlings occurs at 10^{-4} to 10^{-3} mol dm^{-3} glyphosate (Hoagland and Duke, 1982); depression of anthocyanin synthesis in excised hypocotyls of buckwheat is detected at 10^{-6} mol dm^{-3} while inhibition of chlorophyll synthesis takes place only at concentrations above 10^{-5} mol dm^{-3} (Holländer and Amrhein, 1980); the conversion of shikimate to anthraquinones is blocked in *G. mollugo* cell cultures at concentrations above 10^{-4} mol dm^{-3} (Amrhein, Brigette, Gehrke and Steinrücken, 1980). However, it should be pointed out that direct comparison of results among different reports is difficult because of two reasons: (i) different glyphosate preparations have been used (pure acid or isopropylamine salt, with or without surfactant) and (ii) there is lack of knowledge of the intracellular glyphosate levels attained in each case. Brecke and Duke (1980) showed that glyphosate was quickly absorbed by whole bean plants but not by individual leaf cells. It would be of interest to investigate whether different types of plant cells have different permeabilities to the herbicide.

ACKNOWLEDGEMENTS

We wish to acknowledge the gift of the glyphosate samples by Dr J. Costa (Monsanto, Spain) and advice on the ATPase assay by Dr R. Serrano.

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