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Effect of Glyphosate on Rat Liver Mitochondria *In Vivo*

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The pesticide N-phosphonomethylglycine (glyphosate) was introduced by the Monsanto Company in 1971 as a unique product with broad-spectrum, post-emergence, and non-selective herbicidal properties (EVANS 1972, TERRY and MAGAMBO 1972). Although, MONSANTO (1971) reported that glyphosate is relatively non-toxic when administered orally to rats (LD50 > 4,000mg/kg), it has recently been revealed that the herbicide is probably more toxic (LD50 192-279 mg/kg) when administered intraperitoneally (OLORUNSOGO et al. 1977). It has been shown that the symptoms which accompanied glyphosate intoxication include elevated rectal temperature, asphyxial convulsion and rigor mortis (OLORUNSOGO 1976). In view of the fact that these signs are also caused by a number of chemical compounds which interfere with the process of energy metabolism in the mitochondrion (DIECHMANN et al. 1942, PIESS and FIELD 1948, BUFA et al. 1963, PARKER 1965), attempts were made to investigate the 'in vivo' effects of glyphosate on the process of energy conservation in rat liver mitochondria.

MATERIALS AND METHODS

Treatment of animals. Five sets, each of five female rats (Wistar strain) obtained from the University of Ibadan, Preclinical Animal Breeding House and weighing approximately 250g per rat, were placed in separate cages. The isopropylamine salt of glyphosate (MON-0139, obtained from the Monsanto Company, Brussels, Belgium) was used throughout the studies. Four out of the five sets of animals were intraperitoneally dosed with 15, 30, 60, and 120 mg glyphosate/kg, respectively. The fifth group of animals received a comparable volume of doubly distilled sterile water which was the vehicle used for injecting the pesticide. All other aspects of care and handling were identical in both experimental and control rats.

Preparation of mitochondria. Rats were sacrificed by cervical fracture 5h after dosing and the livers were immediately excised and placed in ice-cold 0.25M sucrose. The animals were killed after 5h because results from experiments in our laboratory revealed that maximal increases in rectal temperatures in glyphosate-poisoned rats were obtained 4.5 - 5.5h after a single intraperitoneal dosing.

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Ten per cent homogenates were prepared and immediately centrifuged at 4°C for 5 min at 600g in an MSE angle 13 refrigerated centrifuge. The mitochondrial pellet, which was sedimented at 5,000g (20 min), was washed twice by resuspending in 0.25M sucrose and sedimented again at 12,400g (10min). The mitochondrial pellet was suspended in a volume of 0.25M sucrose so that a final concentration of 40mg protein per mL of suspension was obtained. Mitochondrial protein was determined by the biuret method.

Measurement of oxygen uptake. Oxygen uptake was measured polarographically with a Clark oxygen electrode supplied by the Yellow Springs Instrument (Ohio). The temperature of the reaction chamber was held at 26°C with a thermostatically controlled bath. The volume of the reaction medium was 3.0mL and it contained 5mM MgCl, 5mM KCL and 20mM phosphate buffer; pH was 7.4. Volumes of the order of 0.01mL were added to the medium for all compounds. The pH of such solution was previously adjusted to 7.4.

Measurement of adenosine triphosphatase (ATPase) activity. ATPase activity was determined by a slight modification of the method of LARDY and WELLMAN (1953). Each reaction vessel contained 65mM Tris-HCl buffer pH 7.4, 0.5mM KCl, 1mM ATP, 25mM sucrose and the test compound (or distilled water) in a total volume of 2.0mL. The reaction, which was started by the addition of the mitochondrial fraction, was allowed to proceed for 5 min with constant shaking at 27°C. The reaction was stopped by the addition of 8mL of 10 per cent solution of trichloroacetic acid to the contents of each tube which were then centrifuged in an MSE centrifuge at 500g. The supernatant was kept for phosphate determination. The zero time tube was prepared by adding an aliquot of the ATP solution to the reaction vessel after the addition of 8mL of a 10 per cent solution of trichloroacetic acid. Inorganic phosphate was estimated in each supernatant fraction according to the method described by BASSIR (1963).

Measurement of the activities of some NAD-specific dehydrogenases. The activities of the NAD-specific dehydrogenases (isocitrate, glutamate, and β-hydroxybutyrate) were determined spectrophotometrically by following the rate of formation of the reduced coenzyme NADH at 370nm (RENDINA 1971). The reaction medium contained the following in a total volume of 3mL, 2.2mL of phosphate buffer, pH 7.4, 0.2mL of 0.01M NAD and 0.2mL of the substrate (0.1M sodium isocitrate, or 0.1M sodium glutamate, or 0.1M sodium β-hydroxybutyrate). 0.3mL of doubly distilled water or the test material was added to make 2.9mL. The reaction was initiated by adding 0.1mL of the mitochondrial suspension which had been preincubated with KCN to block the cytochrome oxidase system of the mitochondrial respiratory chain. The rate of the reaction was follwed by mea-

suring the rate of change of absorbance at 370nm at 30 sec interval for 7min in a Pye Unicam SP600 spectrophotometer.

Assay of succinate dehydrogenase activity. The method described by SLATER and BONNER (1952) was employed for this determination. The reaction medium contained 1.9mL of a solution of 0.1M sodium succinate pH 7.4, 0.2mL of 0.001M potassium ferric cyanide, 0.3mL of distilled water or the test compound was added to make up 2.9mL of reactants in a 1cm light path cuvette. The reference cell contained water only. After the addition of all reaction components, 0.1mL of the mitochondrial suspension (diluted twice with 0.001M KCN) was added at zero time. The rate of change of absorbance was followed at 400nm for 7min and the enzyme activity computed as described by SLATER and BONNER (1952).

Determination of cytochrome C reductase activity. Cytochrome C reductase activity was measured spectrophotometrically by following the zero-order appearance of the α -peak of reduced cytochrome C at 550nm in the presence of 20mM succinate or 60mM NADH as electron donor(MACKLER and GREEN 1956). Other reactants in final concentration, added to the reaction vessel were 66.6mM potassium phosphate buffer (pH 7.4), 1mM NaCN, 0.027mM cytochrome C, 0.167M sucrose and 0.06mg mitochondrial protein in 3.0mL. The reaction was started by the addition of the ferricytochrome C. Specific activity of the enzyme was expressed as micromoles cytochrome C reduced per mg protein/min.

Measurement of Cytochrome oxidase activity. Cytochrome oxidase activity was estimated as the first-order disappearance of the α -peak of reduced cytochrome C at 550nm (SMITH 1955). Specific activity expressed as micromoles cytochrome C oxidized per mg protein/min was calculated as the product of the first order velocity constant and the initial ferrocytochrome C concentration. Final concentration of reactants were 43.3mM potassium phosphate buffer, pH 7.4, 0.08mM succinate, 0.27mM cytochrome C and 0.020-0.030 mg mitochondrial protein in a final volume of 3.0ml. The reaction was started by the addition of ferrocytochrome.

RESULTS

Fig 1 shows the patterns of the rate of oxygen consumption by mitochondria, immediately after isolation, from the livers of rats 5h after an intraperitoneal dosing with glyphosate. The rates of oxygen uptake (220 and 295 millimicro-atoms O_2 per min), in the absence of added adenosine 5-diphosphate (ADP), by mitochondria from the livers of rats which were given 60 and 120 mg glyphosate/kg body weight, respectively, were higher than the value (132

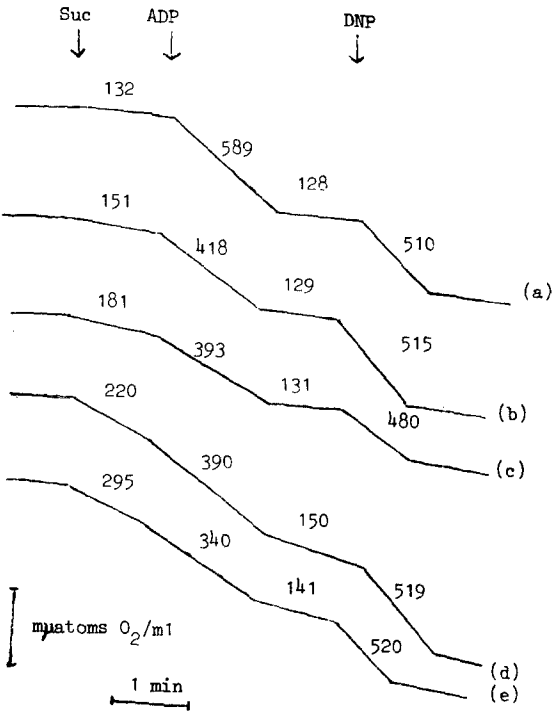

Fig. 1. Oxygen electrode tracings showing the rate of oxygen consumption by mitochondria isolated from the livers of rats 5h after an intraperitoneal dosing with (a) sterile distilled water, (b) 15, (c) 30, (d) 60, and (e) 120 mg glyphosate/kg. Arrows indicate points of successive additions of mitochondria (4mg prot), succinate (3.3mM), ADP (0.3mM) and dinitrophenol DNP, (0.03mM) in a total volume of 3mL. The numbers along the tracings are the rates of respiration expressed as millimicroatoms O_2 per min. Temp. 26°C.

TABLE 1: Respiratory Control Ratio (RCR) of Mitochondria Isolated from the Livers of Rats 5h after an Intraperitoneal Dosing with Glyphosate

Dose of glyphosate mg/kg	Respiratory Control Ratio	%Effect
0	4.45 ± 0.49	
15	3.25 ± 0.56	27.0
30	3.00 ± 0.67	32.6
60	2.50 ± 0.36	43.8
120	2.45 ± 0.24	46.0

Each value is a mean of five different determinations ± standard deviation

millimicro-atoms O_2 per min) obtained with the mitochondria from the control animal. The degree of the stimulation of respiration by the addition of ADP was significantly reduced in mitochondria from the glyphosate-poisoned rats when compared with control (Fig 1). A measure of the ratio of the rate of the ADP-stimulated respiration to that of the ADP-less respiration (state 3/state 4, respiratory control ratio, RCR) reveals reductions in the RCR of the mitochondria isolated from the intoxicated animals (Fig. 1). The relationship between the intraperitoneal dose of glyphosate and the respiratory control ratio of isolated mitochondria is shown in Table 1. At the sublethal dose of 60mg glyphosate/kg, the respiratory control ratio was reduced by 43.8 per cent. A further reduction (46 per cent) in respiratory control ratio was observed when the dose was increased to 120mg glyphosate/kg.

Examination of the ATPase activities of the mitochondria isolated from the experimental animals reveal an enhancement of the activity of the enzyme in the mitochondria obtained from glyphosate-poisoned rats (Table 2). There is a direct correlation between the intraperitoneal dose of glyphosate and the enchancement of ATPase activity (Table 2).

Table 2 also shows the effect of glyphosate on the activities of some mitochondrial dehydrogenases of rat liver, 5h after a single intraperitoneal dose. The activities of all the dehydrogenases, were slightly enhanced at 60 and 120 mg glyphosate/kg body weight dosage levels.

The activities of some electron transfer complexes of the mitrochondria isolated from the livers of rats 5h after intraperitoneal dosing with glyphosate are shown in Table 3. There is no significant difference in the activities of the cytochrome C reductases and the cytochrome oxidase systems in the mitochondria isolated from glyphosate-poisoned rats when compared with controls.

D I S C U S S I O N

The results presented in Fig. 1 reveal that glyphosate enhanced the rate of oxygen consumption by the mitochondria isolated from the livers of rats 5h after a single intraperitoneal dosing with glyphosate when the reaction medium was deficient in phosphate acceptor. The respiratory control ratios of these mitochondria were also reduced. At 60mg/kg this parameter was reduced by at least 40 percent (Table 1).

The partial reactions of oxidative phosphorylation are presumed to be reflections of the reversibility and multistep nature of the coupling mechanisms. An important partial reaction of oxidative

TABLE 2

Activities of Some Dehydrogenases and ATPase of Mitochondria Isolated from the Livers of Rats 5H after an Intraperitoneal Dosing with Glyphosate

Dose of glyphosate mg/kg	ATPase activity mμmoles Pi/mg prot/min	ICDH	BOHBH	GDH	SDH
		μmole NAD^+ red/mg protein/min			μmole Succ/mg prot/min
0	3.39 ± 0.20	3.75 ± 0.26	5.14 ± 0.31	4.00 ± 0.28	6.24 ± 0.17
15	10.98 ± 0.18	3.79 ± 0.25	5.20 ± 0.34	4.17 ± 0.30	6.89 ± 0.20
30	14.33 ± 0.20	3.90 ± 0.21	5.81 ± 0.27	4.57 ± 0.35	7.10 ± 0.19
60	17.54 ± 0.24	4.71 ± 0.11	5.94 ± 0.29	4.91 ± 0.21	7.68 ± 0.19
120	19.64 ± 0.27	4.17 ± 0.10	6.16 ± 0.28	5.59 ± 0.26	7.94 ± 0.22

Each value is a mean of five different determinations ± standard deviation

ICDH = isocitrate dehydrogenase, GDH = glutamate dehydrogenase

BOHBH = β-hydroxybutyrate dehydrogenase, SDH = succinate dehydrogenase

TABLE 3

The Activities of Some Electron Transfer Complexes of Mitochondria Isolated from the Livers of Rats 5 H After an Intraperitoneal Dosing with Glyphosate

Dose of Glyphosate mg/kg	Cytochrome Oxidase	Succinate Cytochrome C reductase	NADH Cytochrome C reductase
	µmole CytC oxid per mg protein per min	µmoles Cytochrome C reduced per mg protein per minute	
0	3.00 ± 0.21	0.67 ± 0.09	0.40 ± 0.04
15	3.23 ± 0.26	0.62 ± 0.10	0.41 ± 0.06
30	3.08 ± 0.22	0.65 ± 0.06	0.43 ± 0.03
60	3.44 ± 0.27	0.58 ± 0.08	0.44 ± 0.06
120	3.00 ± 0.25	0.60 ± 0.09	0.43 ± 0.05

Each value is a mean of five different determinations ± standard deviation.

phosphorylation is adenosine triphosphatase (ATPase) activity which is characteristically stimulated by dinitrophenol and other uncoupling agents (LARDY and WELLMAN 1953, LARDY et al. 1958, LEHINGER et al. 1958, HEMKER 1961, 1964, PARKER 1965). Because of the enhancement effect of glyphosate on ADP-less respiration and its inhibitory effects on ADP-stimulated respiration in the mitochondria obtained from the livers of glyphosate-poisoned rats, the effect of the herbicide on ATPase activity was investigated. The results presented in Table 2 reveal that ATPase activity was enhanced in the livers of the animals treated with the herbicide. The activities of the dehydrogenases of the common two- or three-site substrates (Table 2) were also slightly enhanced in the hepatic mitochondria of the glyphosate-poisoned animals.

These findings suggest that uncoupling of mitochondrial oxidative phosphorylation may be a major lesion in glyphosate intoxication.

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