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A MOLECULAR MECHANISM FOR THE TOXIC ACTION OF MONILIFORMIN, A MYCOTOXIN PRODUCED BY *FUSARIUM MONILIFORME*

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Abstract—Molecular mechanisms of the toxic action of moniliformin, an extremely toxic fungal metabolite produced by *Fusarium moniliforme*, were investigated. Exceedingly low concentrations of moniliformin ($< 5\ \mu M$) selectively inhibited mitochondrial pyruvate and α-ketoglutarate oxidations by 50 per cent. It is suggested that these inhibitory effects could constitute the major molecular mechanism of toxic action and could largely, if not exclusively, account for the clinical symptoms of moniliformin poisoning.

The mycotoxin, moniliformin, may constitute a health risk to human populations on a staple diet of maize. It is produced by *Fusarium moniliforme* Sheldon, the most prevalent fungus on maize kernels in various maize producing countries [1], as well as by *Fusarium moniliforme* Sheldon var. *subglutinans* Wr. and Reink., which also occurs on maize [2]. Possibly because of lack of suitable analytical methods, moniliformin has not yet been found in natural products, but it is produced in the laboratory in extremely high yields (ca 10 g/kg) by pure cultures of *F. moniliforme* var. *subglutinans* grown on maize [2]. These high yields of moniliformin, together with the compound's extreme toxicity, accentuate the potential danger to human health.

Moniliformin was recently shown to be the sodium or potassium salt of 1-hydroxy cyclobut-1-ene-3.4-dione [3] (Fig. 1). The free acid, also known as semisquaric acid, had previously been synthesized by Hoffman *et al.* [4], but its structure is without precedent for a natural product [3].

In addition to its toxicity to plants [5], moniliformin proved to be extremely toxic to cockerels (LD_{50} = 4 mg/kg, oral) [5], ducklings (LD_{50} = 3.68 mg/kg, oral) [2], rats (LD_{50} = 50.0 and 41.57 mg/kg, oral for males and females respectively) [2], and mice (LD_{50} = 24 mg/kg, i.p.) [6]. Kriek *et al.* [2] found that the clinical signs of moniliformin toxicity in both ducklings and rats were "characterised by progressive muscular weakness, respiratory distress, cyanosis, coma and death" which occurred within 1 hr in the case of ducklings and 3 hr in rats. All animals which did not die within these periods recovered completely within 12 hr.

The mechanisms by which moniliformin exerts its biological effects are largely unexplored. Ueno and Shimada [7] could not detect any inhibitory effect of moniliformin on protein synthesis by rabbit reticulocytes. Moniliformin had no effect on lysosome formation or function in mouse liver and kidneys [6]. Lansden [8] found indications of interaction of moniliformin with DNA.

The extremely rapid biological action of moniliformin as well as its high toxicity motivated studies to localize its site of action and determine its biochemical mode of action. The clinical signs of moniliformin intoxication [2], especially the progressive muscular weakness displayed by both ducklings and rats, seemed to indicate an inhibitory effect of moniliformin on energy yielding processes. Since cyanosis did not appear to be pronounced during the early clinical stages, it appeared that moniliformin may impair reactions directly involved in ATP formation, rather than the transport of oxygen to mitochondria.

This paper describes observations on the inhibitory effect of moniliformin on processes involved in energy metabolism which could possibly account for the primary toxic action of this mycotoxin.

MATERIALS AND METHODS

Isolation of rat liver mitochondria. Livers obtained from healthy adult BD IX black rats were weighed and homogenized in 0.25 M Sucrose–1 mM EDTA (pH 7.4) (10 volumes per *g* tissue) for 1 min at maximum revolutions in a Sorvall Omnimixer. The homogenate was strained through cheesecloth and com-

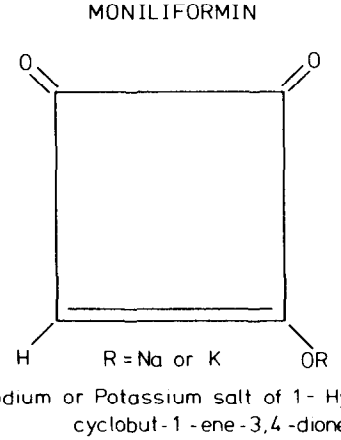

Fig. 1. The chemical structure of moniliformin.

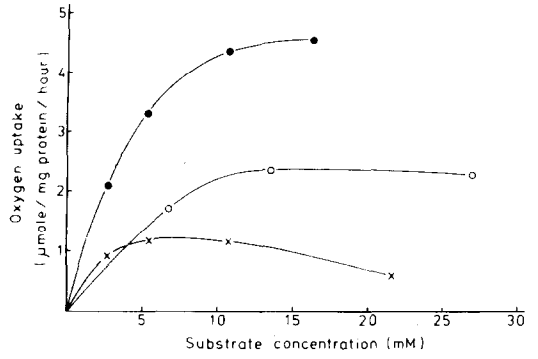

Fig. 2. The effect of substrate concentration on oxygen uptake by isolated rat liver mitochondria oxidizing succinate (●——●), isocitrate (○——○) and fumarate (×——×). Assay mixtures are described in Methods. Each point represents the mean of three determinations.

plete disruption of cells was ensured by treatment in a Dounce homogenizer. The resultant homogenate was centrifuged for 10 min at 700 *g* in a Sorvall RC2B centrifuge and the mitochondria were recovered from the supernatant liquid by centrifugation at 7000 *g* for 10 min. The pellet was resuspended in 0.25 M Sucrose–1 mM EDTA (pH 7.4) (5 volumes per g tissue) and recentrifuged for 10 min at 7000 *g*. The previous procedure was repeated once and the mitochondrial pellet was finally suspended by means of a Dounce homogenizer in 0.25 M Sucrose–1 mM EDTA (pH 7.4) (1 volume per g tissue). This suspension was used in the respiration experiments. All manipulations were carried out at 0–4°C.

Protein concentration of the mitochondrial suspension was determined by the method of Lowry *et al.* [9].

Assay of oxygen uptake by mitochondria. The effect of moniliformin on oxygen uptake by isolated rat liver mitochondria was determined manometrically on a Warburg apparatus employing various substrates and combinations of substrates in the presence and absence of moniliformin.

The reaction mixtures contained the following constituents in a total volume of 3 ml: 8 mM $MgCl_2$, 50 mM KCl, 0.3 mM ADP, 0.015 mM cytochrome *c*, 0.5 mM NAD, 12 mM NaF, 20 mM KH_2PO_4, final pH 7.4 [10]. Mitochondrial suspensions (0.5 ml) containing 3.5–5 mg protein were added to each flask. The amounts of substrate and inhibitor (moniliformin) are specified in conjunction with the appropriate results.

In all experiments the constituents of the reaction mixture, including moniliformin (dissolved in water), were transferred to the main compartments of the Warburg flasks while the substrate solutions only were placed in the sidearms. Concentrated sodium hydroxide solution (0.2 ml) was placed in the centre well. After an initial equilibration period of 10 min at 37° the substrate was added and the oxygen consumption registered at 10 min intervals over a period of at least 40 min. The rate of oxygen consumption was determined in triplicate over a period when it proceeded linearly with time which usually occurred from 10 to 40 min after addition of the substrates and was expressed as μmoles/mg protein/hr.

Inhibition of oxygen uptake by moniliformin. The effect of moniliformin on metabolic processes was investigated according to the principles described by Webb [11] for the localization of the site of inhibition of a metabolic inhibitor. The basic approach was to test the effect of a wide range of moniliformin concentrations on the rate of oxidation of different substrates added individually to isolated rat liver mitochondria.

The rate of oxidation of a specific substrate was in all cases corrected for endogenous oxygen consumption determined for every moniliformin concentration used.

The concentration of each substrate used in the inhibition experiments was selected from the concentration range exhibiting zero-order kinetics.

Chemicals. All chemicals were of analytical reagent grade. ADP, NAD and cytochrome *c* were obtained from Boehringer Mannheim. Moniliformin was isolated from pure cultures of *F. Moniliforme* var. *subglutinans* grown on maize.*

RESULTS

The effect of substrate concentration on oxygen uptake. Oxygen consumption by isolated rat liver mitochondria at varying substrate concentrations is illustrated in Fig. 2 for the oxidation of succinate, isocitrate and fumarate and in Fig. 3 for the oxidation of citrate, α-ketoglutarate, pyruvate and malate. The substrate concentrations used in moniliformin inhibition experiments (Table 1) were based on these observations.

The effect of moniliformin on oxygen uptake. The inhibitory effect of moniliformin on the rate of oxidation of the individual substrates is illustrated in Fig. 4 for the lower concentration ranges of moniliformin. From these data the moniliformin concentrations inhibiting oxidations by 50 per cent were determined for each individual substrate (Table 1). Basically three different types of inhibitory effect of moniliformin were observed. Firstly, the oxidation of pyruvate,

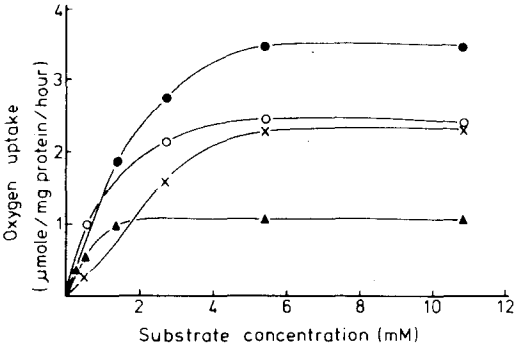

Fig. 3. The effect of substrate concentration on oxygen uptake by isolated rat liver mitochondria oxidizing citrate (●——●), α-ketoglutarate (○——○), pyruvate ×——×) and malate (▲——▲). Assay mixtures are described in Methods. Each point represents the mean of three determinations.

* M. Steyn, P. G. Thiel and G. C. van Schalkwyk, manuscript in preparation.

Table 1. Comparison of moniliformin concentrations causing 50 per cent inhibition of oxygen uptake during the oxidation of different substrates

Substrate	Concn of substrate employed (mM)	Moniliformin concn at 50 per cent inhibition (μM)*
Pyruvate	8.0	5
α-Ketoglutarate	5.4	4
Citrate	8.0	8
Iso-citrate	16.0	9
Fumarate	8.0	140
Malate	5.4	70
Succinate	21.4	—†
NADH	5.4	—†

* The moniliformin concentrations causing 50 per cent inhibition were determined from the data represented in Fig. 4.

† Oxygen uptake resulting from the oxidation of succinate and NADH was only negligibly inhibited by moniliformin even at high moniliformin concentrations.

α-ketoglutarate, iso-citrate and citrate were 50 per cent inhibited by extremely low concentrations of moniliformin (< 10 μM). Secondly, much higher concentrations were necessary to cause a 50 per cent inhibition of the oxidation of malate and oxaloacetate. Thirdly, the oxidation of NADH and succinate was only negligibly affected by moniliformin.

The maximum inhibition of the rate of oxidation which could be brought about by increasing the moniliformin concentration was determined for each individual substrate (Table 2). The oxidation of pyruvate and α-ketoglutarate could be completely inhibited by increasing the concentration of moniliformin while the inhibition of the oxidation of NADH and succinate remained negligible even at concentrations exceeding 3 mM.

DISCUSSION

The salient results obtained from the inhibition experiments were the extreme sensitivity of the oxidation of pyruvate and α-ketoglutarate to the inhibitory effect of moniliformin. Exceedingly low moniliformin concentrations caused 50 per cent inhibition of oxygen uptake (Table 1) and the oxidation of both could be virtually completely inhibited by increasing the moniliformin concentration (Table 2).

The inhibition of the oxidation of pyruvate can be caused in three possible ways. Firstly, moniliformin may inhibit one of the steps involved in the incorporation of pyruvate via acetyl CoA into the tricarboxylic acid cycle. Secondly, moniliformin could inhibit one of the enzymes in the tricarboxylic acid cycle or thirdly, moniliformin may be an inhibitor of the electron transport system. The third possibility can be excluded since a relatively high concentration of moniliformin (3600 μM) failed to inhibit oxygen uptake with NADH as substrate (Table 2). The mitochondrial membrane is impermeable to NADH *in vivo*, but is known to become permeable during isolation procedures, permitting the use of NADH as a substrate with isolated mitochondria. The addition of an equimolar quantity of oxaloacetate did not cancel the inhibitory effect of moniliformin on pyruvate oxidation (Table 2). Under these conditions the oxidation of pyruvate would be independent of the replenishment of oxaloacetate through the tricarboxylic acid cycle, hence the second possible explanation can also be eliminated. It therefore appears that moniliformin inhibits the incorporation of pyruvate into the tricarboxylic acid cycle. As oxygen uptake during pyruvate oxidation can be inhibited completely, it seems that moniliformin blocks the conversion of pyruvate to acetyl CoA, a step which proceeds with the utilization of oxygen due to the oxidation of NADH formed in the reaction. (Fig. 5).

Apart from its inhibitory effect on pyruvate oxidation, moniliformin inhibited the oxidation of intermediates of the tricarboxylic acid cycle in three distinctly different ways. Whereas negligible inhibition was observed with succinate as substrate (Fig. 4 and Table 2), the oxidation of all substrates preceding succinate in the normal sequence of the cycle was depressed 50 per cent at less than 10 μM moniliformin while those substrates following succinate (fumarate and malate) needed much higher moniliformin concentrations to achieve 50 per cent inhibition. This indicates a selective inhibition of the oxidation of α-ketoglutarate.

Furthermore, by increasing the concentration of moniliformin (Table 2), 100 per cent inhibition could be obtained with α-ketoglutarate as substrate while

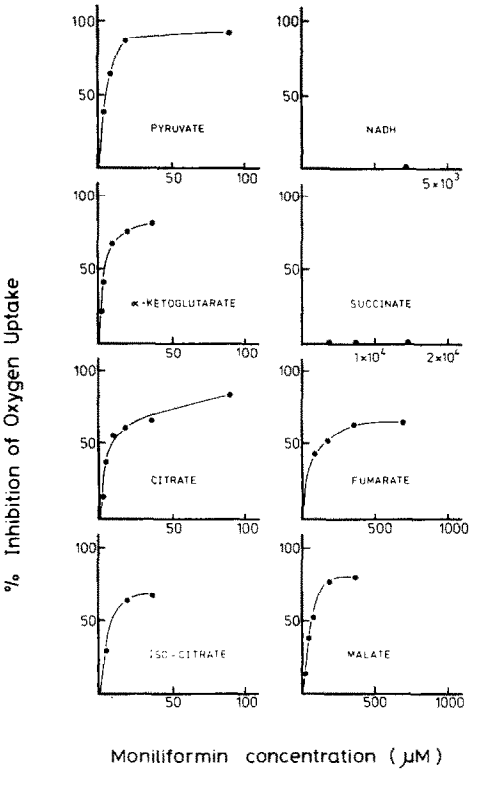

Fig. 4. The effect of moniliformin concentration on oxygen uptake by isolated rat liver mitochondria oxidizing different substrates. Assay mixtures as well as techniques are described in Methods. The concentrations of substrates are given in Table 1.

Table 2. Effect of high concentrations of moniliformin on the rate of oxygen uptake for different substrates and combinations of substrates

Substrate	Moniliformin concn (μM)	Inhibition of oxygen uptake (%)
8.0 mM Pyruvate	90	91
	360	96
5.4 mM α-Ketoglutarate	180	95
	360	96
	3600–7200	100
8.0 mM Citrate	36	67
	90	86
	360	85
16.0 mM Iso-citrate	36	68
	360	79
	3600	78
8.0 mM Fumarate	360	65
	700	66
	900–7200	60
5.4 mM Malate	180	78
	360	80
21.4 mM Succinate	3600	<1
	7200	<1
	14400	<1
5.4 mM NADH	3600	2.2
10.8 mM Pyruvate + 10.8 mM Oxaloacetate	3600	95

the rate of oxygen consumption could not be inhibited to near 100 per cent with any of the other tricarboxylic acid cycle intermediates as substrates. This can also be explained by a selective effect of moniliformin on the oxidation of α-ketoglutarate. Even in the presence of high concentrations of moniliformin, the oxidation of citrate would still proceed up to the formation of α-ketoglutarate, thus including one oxidative step (iso-citrate dehydrogenase) resulting in oxygen uptake.

Moniliformin may be a metabolic inhibitor by virtue of its specific inhibition of the oxidation of pyruvate and α-ketoglutarate (Fig. 5). The unusual potency of moniliformin as a metabolic inhibitor may be judged from the extremely low concentrations which cause 50 per cent inhibition of pyruvate and α-ketoglutarate oxidation (5 and 4 μM respectively). The enzyme systems responsible for the oxidative decarboxylation of pyruvate to acetyl CoA and α-ketoglutarate to succinyl CoA are remarkably similar. Both consist of a multi-enzyme complex containing 3 different enzymes and 5 coenzymes [12]. Both these systems are characteristically inhibited by arsenicals or by arsenite [12] through inactivation of lipoate, thus preventing the transfer of acyl groups to coenzyme A as well as the uptake of oxygen for the reoxidation of reduced lipoate [13]. The possibility that moniliformin exerts its inhibitory effect on these two systems in a manner similar to arsenite should be investigated.

The rapid lethal action of moniliformin resembles the action of other inhibitors of the tricarboxylic acid cycle such as fluoroacetate [14] and of electron transport such as cyanide [15]. It therefore seems possible

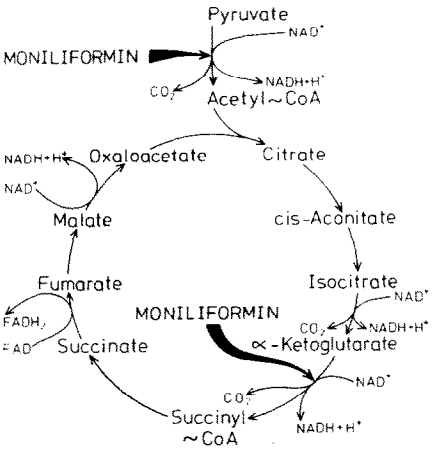

Fig. 5. Schematic representation for the possible mechanism of action of moniliformin.

that the inhibitory effect of moniliformin on pyruvate and α-ketoglutarate oxidation could constitute the major or possibly the sole molecular mechanism for its toxic action.

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