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STUDIES ON POISONING BY *MACROZAMIA COMMUNIS*—I

BIOCHEMICAL DISTURBANCES IN THE LIVER

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Abstract—Oral administration to rats of kernels of *Macrozamia communis* induced changes in liver lysosomes and mitochondria prior to the onset of periacinar necrosis.

Within 3 hr of poisoning with macrozamia the rate of release of β-glycerophosphate phosphatase from light mitochondrial fractions increased. This change was more pronounced after 6 hr. A similar change was induced by carbon tetrachloride and ethionine, but not by lasiocarpine or thioacetamide.

Mitochondria isolated from livers of rats 6 hr after administration of macrozamia showed inhibition of NAD-dependent enzymes and an increased rate of swelling in the absence of substrate.

Secretion of triglyceride from the liver of poisoned rats was inhibited, and triglyceride accumulated in the liver. Serum non-esterified fatty acid levels rose and serum triglyceride levels fell after poisoning with macrozamia.

Protein synthesis was inhibited in the liver of rats treated with macrozamia. Density gradient centrifugation of post-mitochondrial and ribosomal fractions revealed a breakdown in polysomes after poisoning.

The results are discussed in relation to the pathogenesis of lesions induced in the liver by macrozamia.

INGESTION of the kernels of members of the order Cycadales by humans and domestic animals results in hepato-gastrointestinal disease.[1] The effects are due to the presence of a number of azoxyglycosides in the kernels.[2, 3] Recent studies have demonstrated that methylazoxymethanol, the common aglycone of the cycad azoxyglycosides, is the agent responsible for these changes.[4]

Oral administration to rats of aqueous homogenates or extracts of the kernels of *Macrozamia communis* or macrozamine, the major azoxyglycoside of *Macrozamia* spp., induces periacinar necrosis and accumulation of lipid in the liver.[5]

The present communication describes some effects of oral administration of aqueous extracts of the kernels of *Macrozamia communis* upon hepatic cell organelles and upon lipid and protein metabolism in the liver. The relationships between these changes necrosis and lipid accumulation are discussed.

EXPERIMENTAL

Female Wistar rats weighing between 125 and 200 g were used. Aqueous extracts of the kernels of *M. communis* were administered, by gastric intubation, at a dose equivalent to 1·5 g of fresh kernel/100 g.[5]

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Subcellular fractions of livers were prepared according to Schneider.[6] Oxygen uptake by mitochondrial preparations was measured in Warburg respirometers. ATP concentration in liver homogenates and mitochondrial preparations was determined by the method of Crane and Lipman.[7]

β-Glycerophosphate phosphatase and β-glucuronidase activities of liver and serum were determined according to Gianetto and de Duve[8] and Fishman[9] respectively. The rate of release of β-glycerophosphate phosphatase from light mitochondrial fractions was determined by the method of Roels *et al.*[10]

Lipid was extracted from serum and liver by the method of Folch *et al.*[11] Triglycerides, separated by chromatography on acid-washed florisil,[12] were estimated by the procedure of Blankenhorn *et al.*[13] The fatty acid composition of triglycerides was estimated by the method of Horning *et al.*[14] Serum non-esterified fatty acid concentration was determined by the procedure of Rees and Shotlander.[15] Triton WR-1339, as a 25% w/v solution in isotonic saline, was administered to rats anaesthetised with ether 3 hr before destruction; control rats, anaesthetised with ether, were injected with isotonic saline.

Incorporation of DL-1-^{14}C leucine and DL-1-^{14}C phenylalanine into TCA-insoluble material was measured by the method of Mizrahi and Emmelot.[16] TCA-insoluble material, prepared according to Siekevitz,[17] was counted in scintillator gels containing 0·1% thixin.[18]

Post-mitochondrial fractions of liver treated with 1·0% DOCA, and ribosomes isolated from such fractions, were subjected to density gradient centrifugation as described in Fig. 3. Gradients were analysed by puncturing the tubes and measuring the optical density, at 260 mμ, of 10 drop fractions diluted with 3 ml of water.

RESULTS

Effect upon mitochondria

The activities of NAD-dependent enzymes of liver mitochondria were inhibited 6 hr after poisoning. Addition of NAD, reduced glutathione and nicotinamide to the

TABLE 1. EFFECT OF MACROZAMIA UPON OXIDATION OF SUBSTRATES BY MITOCHONDRIA

Substrate	Addition	Oxygen uptake μl O_2/mg mitochondrial N/hr	
		Control	Poisoned 6 hr
L-Malate (10 mM)	None	241	163
	NAD, 0·5 mM; GSH 0·67 mM; nicotinamide 40 mM	281	274
L-Glutamate (10 mM)	None	297	250
	NAD, 0·5 mM; GSH 0·67 mM; nicotinamide 40 mM	302	284
Succinate (10 mM)	None	742	845
	NAD 0·5 mM	701	718

System: AMP 1 mM; $MgSO_4$, 6·7 mM; KCl 25 mM $NaKPO_4$ buffer, pH 7·4, 13·3 mM; cytochrome c 10 μM; enzyme for malate and glutamate equivalent to 100 mg of liver in 0·5 ml of 0·25 M sucrose, for succinate equivalent to 50 mg of liver; water to 3·0 ml; 0·1 ml of 20% KOH in centre well to absorb CO_2; gas phase, air; temperature 37·5°. Results are the means of four estimations, performed in duplicate.

reaction mixture reversed this inhibition. Although succinic dehydrogenase activity in mitochondria was not inhibited, the addition of NAD to the reaction mixture resulted in a greater inhibition of succinic dehydrogenase activity in mitochondrial preparations from poisoned livers than in similar preparations from control livers (Table 1).

Addition of 1 mM EDTA to the incubation medium, or 3·3 mM EDTA to the isolation and incubation media, failed to restore the activity of NAD-dependent enzymes in mitochondrial preparations from poisoned livers to levels equal to those in control preparations.

The rate of swelling of mitochondria in 0·25 M sucrose, 0·05 M Tris.-HCl, pH 7·3, in the absence of substrate, was increased 6 hr after poisoning.

ATP concentration in whole liver and in liver mitochondrial preparations was not altered after 6 hr. However, after 12 hr, ATP levels in liver and in liver mitochondrial preparations were only 60 per cent of those in control livers and in mitochondrial preparations from control livers.

Effect upon Lysosomes

The levels of β-glucuronidase and β-glycerophosphate phosphatase in serum rose rapidly after poisoning. However, estimation of the ratio of "free" to "total" activity

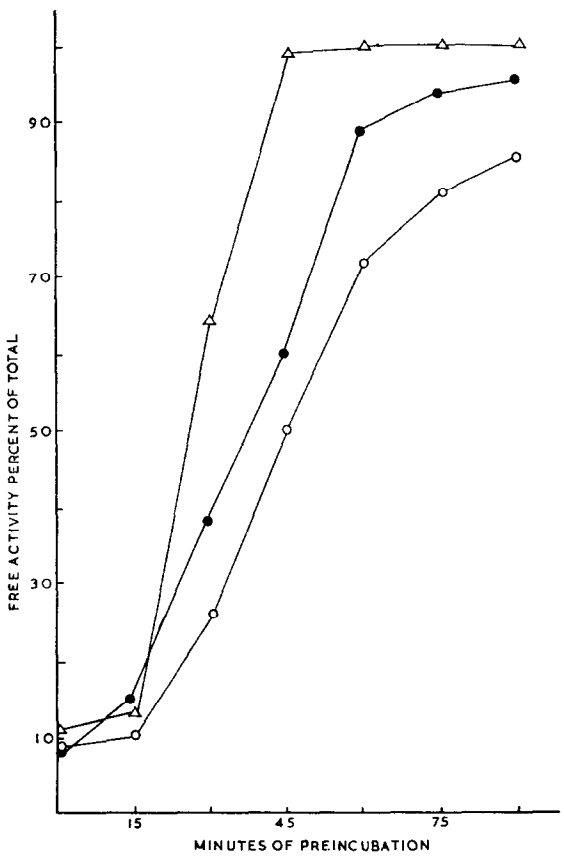

FIG. 1. Activation of β-glycerophosphate phosphatase in light mitochondrial fractions. Control O—O; poisoned 3 hr ●—●; poisoned 6 hr △—△. System: Roels *et al.*[10] Results are means of two experiments performed in duplicate.

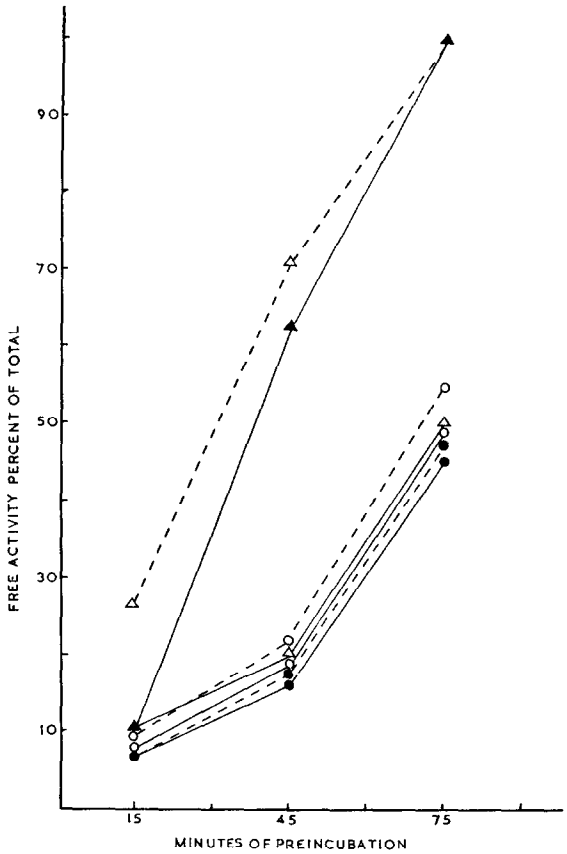

FIG. 2. Activation of β-glycerophosphate phosphatase in light mitochondrial fractions. Control ○—○; saline i.p. ●—●; paraffin oral 2·5 ml/kg △—△; CCl_4 2·3 ml/kg in paraffin 1; 1 v/v orally ▲—▲; thioacetamide, 200 mg/kg i.p. ○- - - ○; lasiocarpine 80 mg/kg i.p. ● - - - ● ethionine 1 g/kg i.p. △ - - - △. System: as Fig. 1. Results are the means of two experiments performed in duplicate.

of these enzymes in liver homogenates failed to reveal an increase in lysosomal "activation" until necrosis was evident.

The rate of release of β-glycerophosphate phosphatase from light mitochondrial fractions *in vitro* was increased in fractions isolated from rats poisoned for 3 and 6 hr (Fig. 1).

Experiments with other hepatotoxic agents showed that a similar response occurred after carbon tetrachloride and ethionine treatment but not after lasiocarpine or thioacetamide administration (Fig. 2).

Effect upon lipid metabolism

Administration of macrozamia resulted in an early increase in serum non-esterified fatty acid concentration and a fall in serum triglyceride levels (Table 2).

Levels of lipid in the liver rose rapidly after poisoning .Table 2 indicates that this increase was a consequence of an accumulation of triglyceride. The fatty acid composition of the accumulated triglyceride closely resembled that of adipose tissue triglyceride.

TABLE 2. SERUM AND LIVER LIPID LEVELS AFTER POISONING WITH MACROZAMIA

Hr after poisoning	Serum			Liver	
	NEFA μE/100 ml	Triglyceride mg/100 ml		Total lipid mg/100 mg fat free dry weight	Triglyceride mg/100 mg fat free dry weight
		—	Triton		
0	34·7 ± 4·2	102	1016	19·3	4·8
3	43·3 ± 7·1	79	1010	25·1	9·3
6	48·5 ± 3·6	77	468	30·1	14·0
12	47·7 ± 5·3	37	222	53·8	36·6

Results are means of at least four animals.

Estimation of the rate of secretion of triglyceride from the liver by the use of Triton WR-1339,[15] revealed that triglyceride secretion was inhibited within 6 hr of poisoning (Table 2).

Effect upon protein synthesis

Incorporation of ^{14}C-leucine into TCA-insoluble material of post-mitochondrial fractions of liver fell rapidly after poisoning (Table 3). In preparations containing microsomes from poisoned livers, ^{14}C-leucine incorporation was inhibited in the presence of supernatant fractions from either control or poisoned livers. However, ^{14}C-leucine incorporation was increased to a slight extent in preparations containing microsomes from control livers and supernatant from poisoned livers in comparison with that in preparations containing microsomes and supernatant from control livers (Table 4).

TABLE 3. EFFECT OF MACROZAMIA UPON ^{14}C-LEUCINE INCORPORATION INTO PROTEINS OF POST-MITOCHONDRIAL FRACTIONS

Hr after poisoning	Incorporation as a percentage of control
0	900
1·5	93
3	74
6	50
12	28

Results are the means of two experiments, each performed in duplicate.

TABLE 4. EFFECT OF MACROZAMIA UPON INCORPORATION OF ^{14}C-LEUCINE INTO PROTEINS OF POST-MITOCHONDRIAL FRACTIONS

Preparation	Incorporation as a percentage of control
Control supernatant + control microsomes	100
Control supernatant + poisoned microsomes	38
Poisoned supernatant + control microsomes	150
Poisoned supernatant + poisoned microsomes	56

Results are means of two experiments, each performed in duplicate. Rats destroyed 6 hr after poisoning.

Addition of polyuridylic acid to post-mitochondrial preparations from poisoned livers (6 hr) resulted in a greater stimulation of ^{14}C-phenylalanine incorporation than in similar preparations from control livers (Table 5).

TABLE 5. EFFECT OF POLYURIDYLIC ACID UPON INCORPORATION OF ^{14}C-PHENYLALANINE INTO PROTEINS OF POST-MITOCHONDRIAL FRACTIONS

Amount of poly-U	Incorporation as a percentage of that without poly-U	
	Control	Poisoned
150 μg	108	127
300 μg	138	232
750 μg	147	314

Results are the means of two experiments, each performed in duplicate. Rats destroyed 6 hr after poisoning.

Density-gradient separation of ribosomes in post-mitochondrial fractions and ribosomal preparations revealed a breakdown in polysomes in these preparations from livers of rats poisoned for 6 hr with macrozamia (Fig. 3).

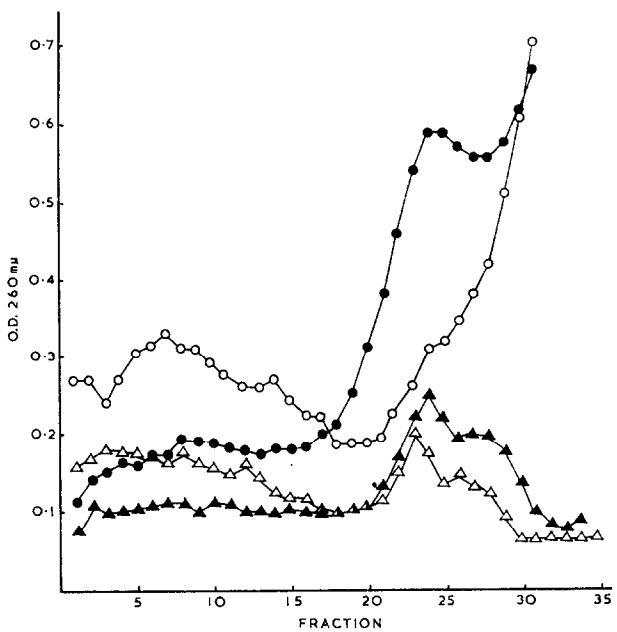

FIG. 3. Sucrose density gradient separation of ribosomes. System: Material equivalent to 600 mg of liver layered on 30 ml gradients of 0·3–1.OM sucrose in Tris salt buffer. Centrifuged in SW_{25} rotor or 30,000 *g* for 4 hr. Control rats, open symbols; poisoned rats (6 hr), closed symbols; post-mitochondrial fractions ◑—◑, ribosomal preparations. ▲—▲.

DISCUSSION

This report records the occurrence of changes in mitochondria isolated from the livers of rats, treated with kernels of *M. communis*, prior to the onset of periacinar necrosis. Similar changes in the permeability of mitochondrial membranes have been observed at or about the time of onset of necrosis after the administration of a number

of hepatotoxins.[19, 20] The nature of these changes and their importance in the pathogenesis of necrosis are undecided. They would appear to be non-specific in character, as a wide variety of agents have been shown to induce similar changes *in vivo* or *in vitro*.[20, 21] It has also been suggested that these changes may merely be a reflection of exposure of mitochondria to an adverse environment during homogenisation or isolation.[22]

A general role of lysosomal "activation" in the initiation of the pathogenesis of lesions induced by hepatoxic agents has been questioned.[23, 24] Observations reported in this study support this doubt. Some change is evident in the stability of lysosomal membranes after macrozamia poisoning. The change, however, is not a response common to all necrogenic agents, as lasiocarpine and thioacetamide failed to elicit such a reaction. Conversely, ethionine, an agent that does not induce hepatic necrosis, stimulated an even greater lability of lyosomal membranes than macrozamia.

Accumulation of lipid in the livers of rats poisoned with macrozamia appears to parallel the situation after carbon tetrachloride or dimethyl nitrosamine poisoning.[15] The lipid that accumulated is triglyceride and a major factor in the pathogenesis of the fatty change is a failure of triglyceride secretion from the liver. The role of an increase in mobilisation of fatty acids from adipose tissue in the genesis of fatty changes has been the subject of some controversy. However, a sophisticated study by Schotz *et al.*[25] has indicated that in the genesis of the fatty change in the liver of rats treated with carbon tetrachloride, mobilisation of adipose tissue fatty acids is not a major factor.

The early inhibition of protein synthesis in livers of rats treated with macrozamia adds support to the hypothesis that failure of triglyceride secretion is a major factor in the genesis of the fatty change, as proteins are the vehicles of triglyceride transport from the liver.[26] However some controversy exists concerning the rate of turnover of the protein moiety of lipoproteins responsible for the triglyceride transport from the liver.[27]

Post-mitochondrial fractions were used in both the synthetic mRNA and density gradient experiments in this study in order to take advantage of the presence of RNA-ase inhibitors in these fractions.[28] However, despite this precaution the possibility exists that the changes observed after macrozamia administration were "artefacts" created during preparation of the fractions.[29]

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