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Metabolism of Verruculogen in Rats

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Radiolabeled verruculogen was detected in a wide range of body tissues 6 min after intravenous administration, but after a further 20 min it was mainly being excreted via the biliary route. In isolated liver perfusion, [^{14}C]verruculogen was rapidly taken up by the liver and metabolized completely, principally to the related tremorgen TR-2 but also to a desoxy derivative of verruculogen. In addition, a smaller amount of an isomer of TR-2 was detected. These metabolic products were excreted in the bile.

Tremorgenic mycotoxins are a group of about 20 metabolites, mainly of common saprophytic molds, which have been discovered or characterized only in the past decade. They cause tremors in animals and some have either been shown to be the cause of certain naturally occurring neurological disorders of farm animals or are putative causal agents for some hitherto idiopathic syndromes (8). The most potent tremorgenic mycotoxin is verruculogen (Fig. 1), intravenous doses being tremorgenic in, for example, the sheep or pig in the range 5 to 15 μg kg^{-1}. Even doses in the upper part of this range may be lethal; thus, studies on distribution and metabolism of such small amounts of substance in the whole animal are precluded unless radiolabeled tremorgen is available. Further, the severity of symptoms usually begins to decline within 1 h of intravenous administration, suggesting that the toxin may be eliminated quite rapidly.

Investigation of the biosynthesis of verruculogen by a strain of *Penicillium simplicissimum* and optimization of the incorporation of radiolabeled precursors have recently facilitated the production of [^{14}C]verruculogen of high specific activity (2), the quality of which compares very favorably with biosynthetically radiolabeled potent mycotoxins used in other contemporary studies of their distribution and metabolism in animals (4, 5).

The present paper therefore first briefly describes the gross distribution of verruculogen in rats and then concentrates on a study of the metabolic elimination of the toxin by the liver. This is the first report on the metabolism of a tremorgenic mycotoxin in animals.

MATERIALS AND METHODS

Verruculogen and related compounds. Unlabeled and ^{14}C-labeled verruculogen was produced by *P. simplicissimum* in pilot plant fermentors (3) and in Erlenmeyer flasks (2), respectively. The unlabeled verruculogen was prepared to >99% purity by the application of high-performance liquid chromatography (HPLC) on reversed-phase silica (Ultrasphere ODS; 5-μm particle size) in a preparative (1- by 25-cm) column with a methanol-water (5:1) solvent mixture, in which also the verruculogen purified by thin-layer chromatography (TLC) was injected (250-μl amounts). Eluates were monitored by UV absorbance at 235 nm, and appropriate fractions were collected and taken to dryness in vacuo.

The labeled verruculogen gave one homogenous spot on TLC, but a minor (approximately 10%) component of fumitremorgin B (Fig. 1) could be detected by HPLC. However, since the carbon skeletons of verruculogen and fumitremorgin B are identical, no attempt was made to eliminate [^{14}C]fumitremorgin B on account of the inevitable and undesirable processing losses of [^{14}C]verruculogen. The specific activity of the labeled tremorgen was 5.89×10^2 μCi mmol^{-1}.

An authentic sample of the structurally related compound TR-2 (Fig. 2) was prepared by catalytic hydrogenation of pure verruculogen over a paladium carbon catalyst for 15 min at atmospheric pressure, a modification of the method previously described (1). This treatment period was chosen as giving the maximum yield of TR-2, characterized by mass spectrometry, since prolonged hydrogenation resulted also in the formation of a tetrahydrogenated (M^+ = m/z 515) derivative of verruculogen.

Rats. Female Sprague-Dawley rats (200 to 250 g) were used. Before distribution studies, animals were given the tremorgen intravenously by using a formulation prepared by dissolving the tremorgen in ethanol (100 μl) and diluting with water (900 μl). Tissue samples were taken after stunning and exsanguination, and the samples were then macerated and freeze-dried.

Liver perfusion. The liver perfusion technique was essentially that described previously (6). A rat was anaesthetized with a diethyl ether-air mixture, the abdomen was opened, and the hepatic portal vein (inflow) was cannulated, and then the thorax was opened and the inferior vena cava (outflow) was cannulated. The preparation was connected via the input cannula to an apparatus incorporating a roller pump, a constant pressure head device, a multi-bulb

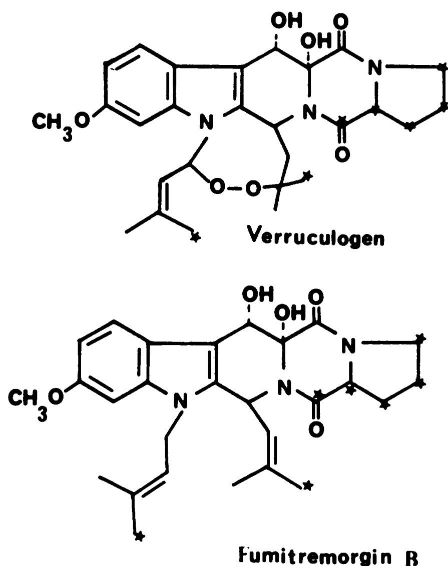


FIG. 1. Structure of [^{14}C]verruculogen and [^{14}C]fumitremorgin B. Stars indicate expected positions of ^{14}C.

glass oxygenator, and a reservoir for the continuous circulation (17 ml min^{-1}) of the perfusate through the isolated liver. The perfusion medium consisted of Krebs-Ringer bicarbonate buffer (7) containing 5 mM glucose and 2.5% (wt/vol) bovine serum albumin. The first 10 ml of venous blood was discarded, and fresh defibrinated rat erythrocytes (12% haematocrit) were added. The duodenum was ligated at the pylorus, above the insertion of the bile duct, and a separate cannula (a 4-cm piece of narrow-bore polythene tube) was inserted into the duodenum to channel the bile into a receiving vessel. The apparatus was contained within a constant-temperature (37°C) cabinet, and the exposed viscera were maintained humid with dampened tissues supported on a gauze bridge. After equilibration for about 30 min, [^{14}C]verruculogen, dissolved in acetone (100 μl mg^{-1}) and diluted to 2 ml with distilled water, was injected into the perfusate reservoir. The perfusate was sampled at intervals to follow uptake of [^{14}C]verruculogen by the liver. In the first perfusion 2-ml samples were taken, and the erythrocytes and the supernatant were separated by centrifugation. They were then analyzed for radioactivity separately by tissue oxidation. In the second perfusion 100-μl samples were dried onto filter paper disks before tissue oxidation. At the end of the perfusion the liver was flushed with Krebs bicarbonate buffer, homogenized, and freeze-dried. Bile was collected as two fractions, the first being that which emerged from the cannula during the first 1.5 h, and the second being that which emerged during the next 1.5 to 2 h together with the cannula washings at termination.

Liver slice incubation. Fresh liver slices from two rats were incubated on a shaker at 37°C in 25 ml of physiological saline containing glucose (0.1% wt/vol) and verruculogen (1 to 10 mg) added as a solution in acetone (100 μl). After 3 h the liver slices and solution were homogenized and freeze-dried.

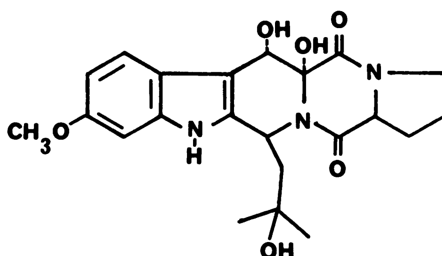


FIG. 2. Structure of TR-2.

Extraction and analysis. In preliminary distribution experiments freeze-dried samples, often rich in lipids, were extracted by stirring in solution for over 4 days with chloroform-methanol (3:1). Extracts were evaporated, taken up in chloroform, and applied to silica gel TLC plates (developed in chloroform-acetone [93:7], sometimes having previously been processed through a short preparative layer step to remove lipid). Freeze-dried samples from liver perfusions and liver slice incubations were extracted overnight by stirring in solution with acetone followed by treatment with methanol. Bile was extracted directly with chloroform. Verruculogen is stable in these extraction conditions.

Solvent extracts were evaporated to dryness, and the residual solids were taken up in methanol and applied to silica gel (F60-254; Merck & Co., Inc., Rahway, N.J.) preparative layer (2-mm) chromatography plates. Chromatograms were developed in chloroform-acetone (80:20) or, for more polar metabolites, chloroform-methanol (85:15). In the former solvent system verruculogen migrated near the solvent front, and verruculogen metabolites, indicated as such by autoradiography, could be seen as fluorescence (254 nm) quenching zones having R_f values of 0.58, 0.45, and 0.25 (metabolites A, B, and C respectively). Metabolite C was better resolved from other polar components by using the chloroform-methanol system. Metabolites were desorbed from silica with methanol and, where appropriate, were further purified by the HPLC system described above. Analytical TLC was performed by using precoated plates (silica gel F60-254). Verruculogen and its metabolites were visualized as orange-brown areas in visible light and mustard fluorescent areas under long-wave (350-nm) UV light, by spraying with 50% ethanolic H_2SO_4 followed by gentle heating (3).

Radioisotope assay. ^{14}C-radiolabel in tissue samples was measured by first processing in a tissue oxidizer (Intertechnique model IN 4101) in which derived $^{14}CO_2$ is absorbed in a scintillation cocktail (toluene, 400 ml; methanol, 220 ml; phenylethylamine, 330 ml; distilled water, 50 ml; butyl PBD [2-(4'-*tert*-butylphenyl)-5-(4''-biphenylyl)-1,2,4-oxadiazole], 7 g) and subsequently measured in a liquid scintillation counter (Beckman LS230). Radiolabeled products of chromatography were also measured in the same scintillant. Chromatograms were autoradiographed by exposing the plates to sensitized nonscreen X-ray film (Fuji) for 2 to 5 days.

RESULTS

Distribution of verruculogen. (i) Unlabeled verruculogen. In a pilot study we attempted to

TABLE 1. Distribution of radioactivity in rat tissues after intravenous administration of [^{14}C]verruculogen

Tissue	^{14}C radioactivity (dpm/100 mg [dry wt] of tissue)	
	5 min[a]	25 min[a]
Liver	348	384
Kidney	231	113
Bile duct and duodenum		248
Small intestine		297
Heart	222	162
Diaphragm	131	86
Skeletal muscle	130	24
Fat pad		20
Cerebral cortex	81	38
Cerebellum	57	29
Brain (except cortex and cerebellum)	48	26

[a] Time after administration.

analyze the distribution of unlabeled verruculogen by using TLC analysis. Lightly anesthetized rats were given verruculogen (2.5 mg kg^{-1}) by tail vein injection and allowed to recover consciousness and to respond to the tremorgen before being sacrificed after 6 or 25 min. Both animals displayed acute tremor and incoordination, although by 25 min the symtomatology was becoming less severe. Samples of liver, kidney, brain, spinal cord, heart and diaphragm, fat pads, serum, and erythrocytes were freeze-dried and extracted with chloroform-methanol. Several of these tissues were particularly rich in lipids, thus presenting problems in TLC. However, approximately 20% of the dosed verruculogen was accounted for in the extract of the erythrocyte fraction of the animal sacrificed after 6 min. None was detected in the serum at 6 min or in erythrocytes or serum at 25 min. Kidney was the only other tissue in which verruculogen was clearly evident; fluorescent compounds in the liver extract interfered with chromatography.

(ii) ^{14}C-labeled verruculogen. The above procedure was repeated with radiolabeled tremorgen (1.73 mg kg^{-1}). Freeze-dried tissues (100 mg) were processed in a tissue oxidizer, followed by scintillation counting. The relative distributions of radiolabel at 5 min and 25 min (Table 1) indicate that, although label can be located in a wide range of tissues, verruculogen appears to be accumulated preferentially by the liver and thence excreted via the bile. This mode of elimination and associated metabolism was therefore subsequently explored by using the isolated perfused rat liver.

Isolated liver perfusions. Two similar liver perfusions were undertaken (Tables 2 and 3), the protocols differing only in that the second perfusion was given twice the amount of verruculogen. About 80% of the ^{14}C-radiolabel used in each experiment was accounted for by summation of the analyses of perfusate, liver, and bile. The second perfusion was somewhat less efficient in absorbing verruculogen from the perfusate, although uptake during the first 15 min was rapid (Fig. 3). It is possible that the larger amount of mycotoxin used here may have saturated the detoxification mechanism of the liver.

The rate of bile production in liver perfusions is well known to be variable. In the present experiments bile flow was initially sluggish in the second perfusion, and this may reasonably have caused both the apparent delay in the delivery of radiolabeled metabolites from the duodenal cannula and the retention of metabolites in the liver. Nevertheless the pattern of three ^{14}C-metabolites, designated metabolites A, B, and C in increasing order of polarity, found in the bile in the first perfusion was confirmed by the second perfusion. Metabolites from the latter also provided chromatographic markers so that the same verruculogen metabolites from subsequent incubations of liver slices could be recognized. Thus, although liver slices metabolized verruculogen so that TR-2 was a more dominant product than in the perfused liver, it was possible to obtain metabolites B and C in amounts sufficient for mass spectrometric characterization. Some of the bile extract from the second perfusion was processed by TLC and HPLC to select pure fractions of metabolites A and B for confirmation of the retention of label. It was not possible to quantify the microgram amounts of each pure component, but the closely matched measured radioactivities of ^{14}C-metabolite A (4,229 dpm) and ^{14}C-metabolite B (3,371 dpm) were consistent with autoradiographic evidence that these two substances are the most important products of the metabolism of verruculogen by the rat liver. Metabolite C was much less abundant. Verruculogen was not excreted intact in the bile.

Characterization of metabolites of verruculo-

TABLE 2. Metabolism of [^{14}C]verruculogen in isolated perfused rat liver

Perfusion	[^{14}C]verruculogen added		% of added radiolabel	
	mg	dpm	Taken up from perfusate during perfusion	Accounted for at end of perfusion
1	0.9	2.28×10^6	98.5	77.9
2	1.8	4.56×10^6	85	80

TABLE 3. Distribution of radiolabel in isolated perfused rat liver

Prepn	% Distribution of recovered radiolabel		Radiolabeled components[a]
	Perfusion 1	Perfusion 2	
Perfusate			
Supernatant fraction	1.6 } 2.0	12.54 } 19.9	Verruculogen
Erythrocyte fraction	0.4	7.36	
Liver	4.9	21.2	84% $CHCl_3$ extractable: Metabolite C, Metabolite A
Bile			
0 to 1.5 h	60.1 } 93.1	0.03 } 58.9	98.7% $CHCl_3$ extractable: Metabolite A, Metabolite B, Metabolite C
1.5 to 3 h	33.0	58.87	

[a] Listed in order of decreasing abundance.

gen. Metabolites A, B, and C were obtained in milligram amounts by a combination of TLC and HPLC of extracts of rat liver slices incubated with verruculogen. The least polar metabolite, A, was identified as TR-2 by comparison with the authentic TR-2 standard prepared by reduction of verruculogen. Metabolite A was chromatographically inseparable from TR-2 in both TLC and HPLC systems and showed an identical mass spectrum fragmentation pattern and a similar UV absorption spectrum.

Metabolite B was shown by field desorption mass spectrometry to have a molecular ion (M^+, m/z 495) consistent with the composition $C_{27}H_{33}N_3O_6$. The molecular ion was not evident in the electron impact spectrum, although this spectrum showed evidence of fragmentations in common with verruculogen and TR-2. For example, all three compounds showed the important ion m/z 322 in the mass spectra, and all three spectra showed important ions at a mass of M-73, 73 mass units comprising the hydroxylated

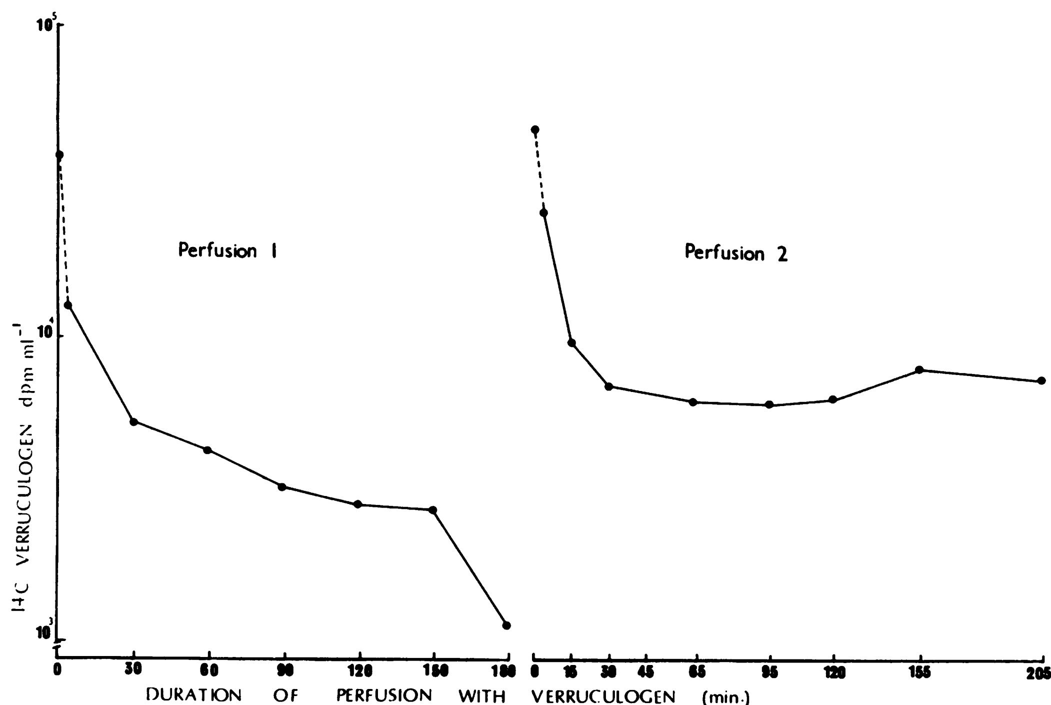


FIG. 3. Depletion of ^{14}C radiolabel from the perfusate during perfusion of the isolated rat liver.

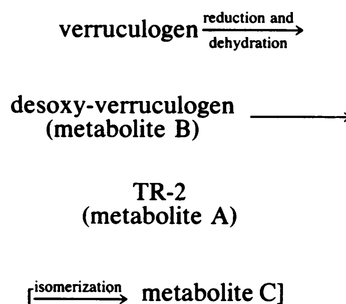
four-carbon (isoprene-derived) unit of TR-2. This strongly suggests that metabolite B is a desoxy derivative of verruculogen, possibly derived by reduction (dihydrogenation) together with the loss of the elements of water. Electron impact mass spectrometry of metabolic C gave an important ion *m/z* 395 ($C_{22}H_{25}N_3O_4$) which by the linked scanning (*B/E* constant) technique (9) was shown to be derived from the less intense ion *m/z* 429 (M^+). It is difficult to see how this mass difference can be anything other than the loss of H_2O_2. It is therefore deduced that metabolite C has the molecular formula $C_{22}H_{27}N_3O_6$ and is probably an isomer of TR-2.

DISCUSSION

In general, detoxification of foreign substances by the liver gives rise to more polar molecules (10, 11), and verruculogen is clearly no exception. One transformation of verruculogen appears essentially to involve the loss of one atom of oxygen, whereas another requires the additional loss of an isoprenoid unit. The third mechanism is isomerization. There was no evidence of O-demethylation such as is an important step of biodegradation in the liver of aflatoxin B1 to aflatoxin P1 before excretion in the bile (10).

The limits of detection achieved by the use of biosynthetically radiolabeled tremorgen were potentially of the same order, assuming 100 dpm above background as the minimal clear indication of radiolabel, as allowed by the fluorimetric assay devised previously (3). However, reliance on radiolabel saved the necessity to extract to make quantitative measurements and was therefore a considerable advantage. With location of ^{14}C expected to be uniform in the proline-derived part of verruculogen and additionally present in one of the gem-dimethyl carbons of each of the two isoprenoid substituents, the radiolabeled molecule was well designed to allow recognition in spite of the structural transformations to which it was subjected during passage through the liver. Indeed, since the formation of TR-2 involves the loss of label in one isoprenoid substituent of verruculogen, the radioactivities measured in the HPLC-pure samples of TR-2 and metabolite B from the second perfusion infer that in molar terms TR-2 is the more abundant metabolite. Consequently, since TR-2 is a less potent tremorgen than verruculogen (1), metabolism of the latter to TR-2 involves an important degree of detoxication.

These studies have been extended to include investigation in sheep (K. P. W. C. Perera, R. H. C. Penny, and P. G. Mantle, Res. Vet. Sci., in press), in which metabolism was qualitatively similar in many respects. The principal verruculogen metabolite excreted in feces was metabolite C. Consequently the following tentative metabolic scheme is proposed:



Metabolism of fumitremorgin B would not necessarily be expected to involve any of these verruculogen metabolites, but it is recognized that the present findings relate strictly to verruculogen in the presence of a small proportion of its associated mycotoxin, fumitremorgin B. The latter always seems to be produced concurrently with verruculogen, and therefore TR-2 may also be regarded as the principal biliary-excreted product of the metabolism of this naturally occurring tremorgen mixture by the perfused isolated rat liver.

The structure of metabolite C (the TR-2 isomer) is currently being investigated.

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