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HEPATIC METABOLISM AND PULMONARY TOXICITY OF MONOCROTALINE USING ISOLATED PERFUSED LIVER AND LUNG

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Abstract—Monocrotaline is a pyrrolizidine alkaloid obtained from the seeds of *Crotalaria spectabilis*. When perfused through an isolated liver, monocrotaline is metabolized to Ehrlich reactive (E+) metabolites. Metabolism of monocrotaline was faster in livers from male rats than female rats, was inducible with phenobarbital pretreatment, and was inhibited by coperfusion with the P-450 mixed-function oxidase inhibitor SKF-525A, anoxic perfusion conditions, and low temperatures. When metabolites generated by an isolated liver were perfused through isolated lungs in a recirculatory manner, serotonin transport by the pulmonary endothelium was reduced in correlation with the amount of E+ material contained in the perfusion medium. When metabolism of monocrotaline by the liver was inhibited with SKF-525A, low temperature perfusions or anoxic conditions, serotonin transport by the pulmonary endothelium was unchanged from controls. Monocrotaline alone had no effect on the lung. Thus, isolated perfused livers metabolized monocrotaline to chemical species which produced pulmonary damage *in vitro*. This provides direct evidence that liver metabolites can cause one of the pneumotoxic effects of monocrotaline observed *in vivo*.

Monocrotaline is a pyrrolizidine alkaloid obtained from the seeds of *Crotalaria spectabilis*. It can cause pulmonary hypertension and right heart hypertrophy, eventually leading to right heart failure. The mechanism of these actions of monocrotaline is unknown, but it has been proposed that hepatic biotransformation of monocrotaline to reactive pyrroles is a necessary step in the development of monocrotaline-induced pulmonary damage [1].

The evidence assembled so far suggests that it is the hepatic cytochrome P-450 monooxygenase that biotransforms monocrotaline to a reactive pyrrole. Most of this evidence is indirect. Metyrapone, an inhibitor of cytochrome P-450 type II, attenuates the pulmonary effects of monocrotaline (i.e. pulmonary hypertension, pulmonary and right ventricle increases in mass) in rats [2]. As the lung possesses little or no measurable cytochrome P-450 type II monooxygenase, the primary effects of metyrapone are, by inference, in the liver. In addition, phenobarbital, a P-450 type II monooxygenase inducer, potentiates the toxicity of monocrotaline. The LD_{50} of monocrotaline in male rats pretreated with phenobarbital has been reported to decrease 30% compared to rats not receiving pretreatment [3].

More direct evidence for the importance of the liver in activating monocrotaline is derived from other work by Mattocks and White [4]. Hepatic microsomes metabolize monocrotaline and other pyrrolizidine alkaloids to N-oxides and pyrroles. The latter are detectable by reaction with Ehrlich reagent and detection of a characteristic absorption spectrum.

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In this report, we study the hepatic metabolism of monocrotaline to E+ metabolites using isolated perfused organs. The toxic potential of these metabolites was determined by monitoring the metabolite-induced changes in a number of pulmonary endothelial cell functions of isolated perfused lungs. Our results indicate that some of the pulmonary damage caused by monocrotaline is mediated by hepatic biotransformation of this alkaloid.

MATERIALS AND METHODS

Monocrotaline was isolated from the seeds of *C. spectabilis* [5]. [^{3}H]Serotonin, [^{14}C]norepinephrine and [^{3}H]AMP were all purchased from the New England Nuclear Corp., Boston, MA. Hippuryl-histidyl-leucine (HHL) was purchased from Vega Biochemicals, Tucson, AZ. Ehrlich reagent (2 g *p*-dimethylaminobenzaldehyde in 100 ml of absolute ethanol acidified with 1.4% perchloric acid) was made fresh each day.

Animals. Sprague–Dawley rats (weight range 200–300 g) were obtained from the University of Arizona Division of Animal Resources and were housed five to a cage in daily 12 hr cycles of light and dark. Those animals that displayed respiratory distress (wheezing, difficulty in breathing, abnormal weight gain) were not included in any of the experiments.

Buffers. All organ perfusions were conducted with Krebs–Henseleit buffer containing 120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 5.5 mM glucose and 2% bovine serum albumin.

Perfused livers. Rats were administered sodium heparin (2000 units/kg) by a single intraperitoneal injection. Fifteen minutes later, 50 mg/kg intra-

peritoneal sodium pentobarbital was used to anesthetize the animals. A single midline incision was made to open the peritoneum, and the portal vein was cannulated with PE 200 tubing. The liver was removed and washed to remove blood and pentobarbital by perfusion with oxygenated 37° buffer at 60 ml/min for 5 min, and then it was perfused in a recirculatory manner from a 100 ml reservoir containing buffer, or buffer and monocrotaline as indicated. Unoxygenated buffers were used to produce anoxia, as bubbling with nitrogen led to precipitation of salts.

Determination of Ehrlich reactive metabolites. Samples (1 ml) from the perfusion reservoir were mixed with 1 ml of Ehrlich reagent and heated at 55° for 5 min. The absorbance at 562 nm was determined after cooling the reaction mixture to room temperature. As it was unknown what the pyrrolic metabolites of monocrotaline were that reacted with the Ehrlich reagent, concentrations were calculated assuming the same molar extinction coefficient as monocrotaline (45,000) and appear in figures and tables as μM equivalents.

Colorimetric detection of monocrotaline. Monocrotaline was measured according to the method of Mattocks [6]. Monocrotaline-containing samples were heated at 100° for 20 min or to dryness. The residue was oxidized with 1 ml of a solution containing 25 ml of methanol with 250 μl glycol and 250 μl H_2O_2 which had been stabilized previously with 5 mg/ml sodium pyrophosphate. After heating the contents of the tube to dryness, the residue was dissolved in 1 ml of diglyme (redistilled; containing 1 mg/ml butylated hydroxytoluene). To this was added 200 μl acetic anhydride, and the solution was mixed and heated at 100° for 5 min. After cooling, this solution was mixed with 1 ml of Ehrlich reagent, heated at 55° for 5 min, cooled to room temperature, and read at 562 nm.

Perfused lungs. Rats were treated with sodium heparin and sodium pentobarbital as described for perfused livers. The anesthetized rat was placed on a flat dissection board and the trachea cannulated. The cannula was attached to a rodent respirator which ventilated the animal at a rate of 60 strokes/min with a volume of 2 ml. The thoracic cavity was opened and the pulmonary artery cannulated through the right ventricle with PE 200 tubing. The heart was cut away, and the lungs removed, attached to a perfusion apparatus containing air at room temperature, and perfused with oxygenated 37° Krebs–Henseleit buffer at 10 ml/min. Lungs that displayed hemorrhagic areas, or those that were not cleared of blood elements within 5 min of initiation of perfusion, were discarded.

Polyethylene glycol retention. Lungs perfused for 20 min with E+ metabolite as described above were perfused for 10 min with fresh buffer containing [1, 2-^{3}H]polyethylene glycol (PEG, molecular weight 900, 0.5 μCi/ml). This was followed by a 1-min perfusion with fresh, PEG-free, buffer. The lungs were then homogenized in ice-cold 10% trichloroacetic acid (TCA), and the homogenate was centrifuged at 5000 *g* for 20 min. The supernatant fraction was assayed for radioactivity, and the retention of PEG per g of precipitate was calculated.

Determination of serotonin, norepinephrine and AMP removal by the lung. Isolated lungs were perfused by a single pass technique at 10 ml/min with substrate-free buffer for 10 min and then for 10 min with buffer containing 0.1 μM substrate (0.05 μCi/ml). Five-minute fractions were obtained of the perfusate draining from the lungs. After 10 min the buffer was changed back to substrate-free medium for 1 min and the perfusate was collected. Total counts were obtained by liquid scintillation spectroscopy of all the fractions collected. Substrate and metabolites were separated by ion exchange chromatography. For serotonin and 5′-nucleotidase assays, 1-ml samples were placed on AG 1 chloride ion form anion exchange columns (0.5 × 6 cm) and eluted with 6 ml of water. Eluted material was counted. Under these conditions, serotonin was eluted from the column and 5-hydroxyindolylacetic acid was retained. In the assay for 5′-nucleotidase, AMP was retained on the column and adenosine was eluted. Norepinephrine was analyzed in a similar manner using an AG 50 H^+ cation exchange column. Unmetabolized norepinephrine was retained on the column and mandelic acid was eluted. Both serotonin and norepinephrine metabolism were assumed to be dependent upon transport. Rates of metabolism were calculated from the metabolites generated over the course of the perfusion. In assays for norepinephrine and serotonin, the lungs were homogenized in ice-cold 10% TCA and centrifuged, and the supernatant fraction was assayed for parent compound and metabolites. This procedure determined the amount of substrate and metabolites remaining in the lung after perfusion. Transport was calculated as substrate and metabolites retained in the lung plus metabolites obtained during perfusion. All values were expressed as nmoles per min per g dry TCA precipitate.

Angiotensin converting enzyme assay. Angiotensin converting enzyme activity was assayed in perfused lungs by a modification of the method of Cushman and Cheung [7]. Isolated lungs were perfused with buffer containing 0.5 mM HHL in the same manner as the radiolabeled substrates described above. The 5-min fractions were placed in a boiling water bath for 5 min and centrifuged at 40,000 *g* for 30 min to remove proteins. The supernatant fractions were extracted with 50 ml of ethyl acetate. The ethyl acetate was separated from the aqueous phase and evaporated to dryness in a boiling water bath under N_2. The residue was dissolved in 1 ml of water and the absorbance at 228 nm was determined.

Tandem lung–liver perfusions. Isolated livers were perfused with 300 μM monocrotaline in buffer for 90 min in a recirculatory system. After the perfusion, the liver was discarded and the buffer was assayed for E+ metabolites. A fraction of this buffer was diluted with fresh buffer to obtain the final concentration of metabolite necessary for the experiment. One hundred milliliters of this preparation was perfused through an isolated lung in a recirculatory manner for 20 min, and then various endothelial cell functions were determined as described. The period of time between the end of the liver perfusion and the start of lung perfusions was 15–30 min. The buffer was maintained at 37° during

this period. For every experiment, three sets of controls were required. The first resulted from perfusing the lungs with fresh buffer. The second was obtained by perfusing the lungs with fresh buffer containing 300 μM monocrotaline. The final control consisted of perfusing the lung with perfusate obtained from an isolated liver which was perfused with buffer but no monocrotaline.

Phenobarbital pretreatment. Animals received a single intraperitoneal injection of phenobarbital (100 mg/kg) and were administered 0.1% phenobarbital in their drinking water for 3 days. Animals were given tap water 1 day prior to use.

Statistics. All direct comparisons to a single control were conducted with the unpaired Student's *t*-test. Multiple comparisons to a single control were achieved with Dunnett's *t*-test [8]. Determination of homogeneity between groups was accomplished using analysis of variance and Scheffe analysis of posteriori contrasts.

RESULTS

Liver metabolism of monocrotaline. Isolated perfused livers metabolized monocrotaline to an E+ material in a dose-dependent manner that was inducible with phenobarbital pretreatment (Figs. 1, a and b) and was greater in males than in females. When perfused in a recirculatory system with 300 μM monocrotaline, measurable amounts of E+ material appeared in the perfusate after 15 min and the rate of appearance of E+ material became linear with time after 30 min (Fig. 2), while the levels of monocrotaline decreased. The generation of E+ material was eliminated by preperfusing the liver with 100 μM SKF-525A and was reduced by anoxic conditions and low temperatures (Table 1).

The chemical natures of the E+ metabolites remain to be elucidated. Thin-layer chromatography revealed the major metabolite to be a polar substance, chromatographically distinct from dehydromonocrotaline and dehydroretronecine. Its half-life in aqueous solution at 4° was in excess of 6 days, indicating it to have a greater chemical stability than dehydromonocrotaline.

Assay of endothelial functions altered by monocrotaline metabolites. The effluent from an isolated liver perfused with monocrotaline containing buffer caused a significant and dose-dependent decrease in serotonin transport in the isolated perfused lung (Fig. 3). Monocrotaline, perfused directly through the lungs, had no effect on serotonin transport. Monocrotaline metabolites from the perfused liver had no effect on norepinephrine transport, 5′-

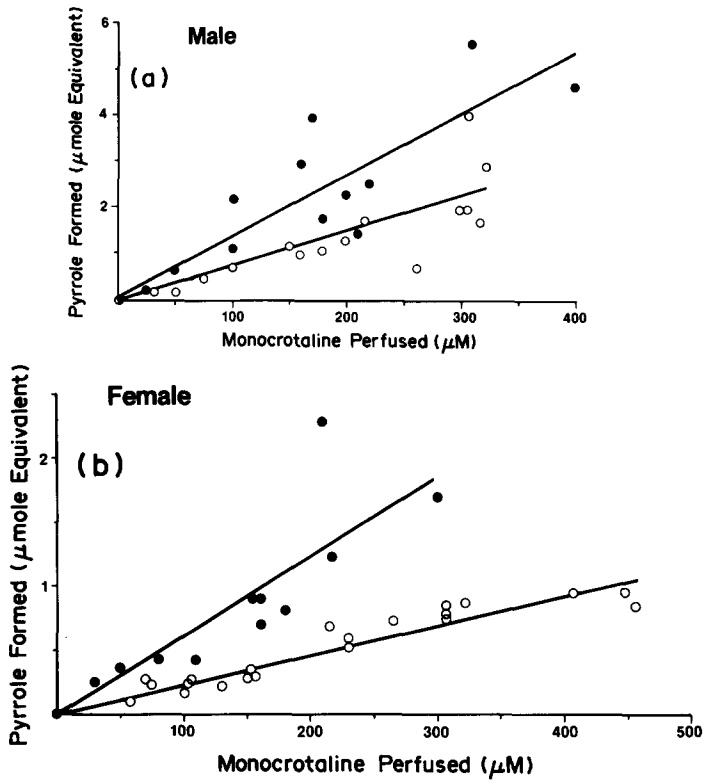

Fig. 1. Metabolism of monocrotaline by isolated perfused liver. Livers from Sprague–Dawley rats (200–300 g) were perfused in a recirculatory system at 60 ml/min with Krebs–Henseleit buffer containing 2% bovine serum albumin and the indicated concentrations of monocrotaline. After 60 min of recirculatory perfusion, the buffer in the perfusion reservoir was analyzed for Ehrlich reactive material with *p*-dimethylaminobenzaldehyde in acidified ethanol. The y-axis indicates the amount of Ehrlich reactive material generated in 60 min liver. The units are μmole equivalents based on the extinction coefficient of monocrotaline (45,000). (a) Male rats: (○) untreated; and (●) phenobarbital-pretreated. (b) Female rats: (○) untreated; and (●) phenobarbital-pretreated. The regression lines generated in all the plots were analyzed for correlation by linear regression. All plots shown had a correlation coefficient of greater than 0.90.

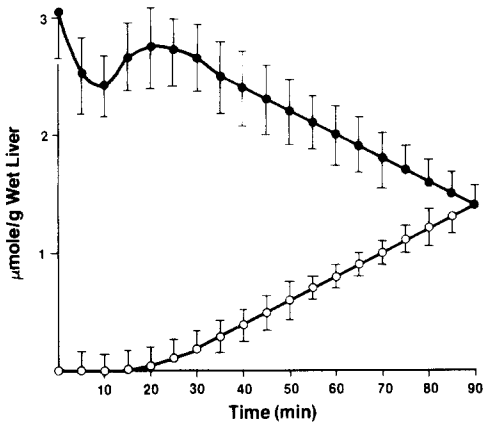

Fig. 2. Appearance of monocrotaline metabolites in liver perfusate. Female rats (200–300 g) were anesthetized with pentobarbital (50 mg/kg) and the livers were isolated and perfused with Krebs–Henseleit buffer containing 2% bovine serum albumin and 100 μg/ml monocrotaline. Samples (1 ml) of the perfusate were analyzed for Ehrlich reactive material every 5 min for 90 min (open symbols). Values are μmole equivalents (× 10) of Ehrlich reactive material in the perfusion reservoir per gram wet weight of the liver. The closed symbols represent the disappearance of monocrotaline from the perfusion reservoir. There were nine samples per point. Error bars represent standard deviations of the mean.

nucleotidase or angiotensin-converting enzyme activities at concentrations we were able to obtain with the isolated perfused liver preparation (data not shown).

Other types of lung damage were only seen at much higher concentrations of metabolite than those required to inhibit serotonin transport. Pyrrole-induced edema was produced at pyrrole concentrations of 600 μM or above (Fig. 4a). Retention of the 900 molecular weight polymer, PEG, can be used as an index of pulmonary permeability [9]. As shown by Fig. 4b, alteration in this criterion of lung damage is seen only at pyrrole concentrations several times those at which serotonin transport is inhibited.

Inhibition of endothelial cell damage. When isolated lungs were perfused with E+ metabolites of monocrotaline generated by an isolated perfused liver, there was a significant and dose-dependent decrease in serotonin transport. If the livers were perfused with a mixed-function oxidase inhibitor (SKF-525A), the generation of E+ metabolites of

Table 1. Inhibition of metabolism of monocrotaline*

Treatment	Rate (nmoles/min/liver)	% Change
Control	20 ± 6	
100 μM SKF-525A	0	−100†
Anoxic perfusions	6 ± 3	−70†
30°	17 ± 5	−15
25°	13 ± 6	−35†
20°	3 ± 2	−85†

* Livers were perfused for 1 hr with 300 μM monocrotaline with or without indicated treatments. The perfusate was analyzed for metabolites and rates were calculated for the last 10 min of perfusion. Values are mean rates ± standard deviations of the mean. N = 15 for control; N = 4 for all other groups.

† $P < 0.05$ compared to no treatment using Dunnett's *t*-test.

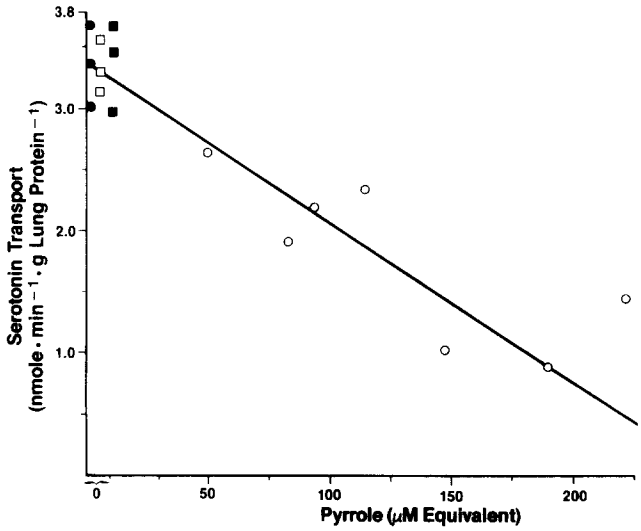

Fig. 3. Effect of monocrotaline metabolites on serotonin transport. Monocrotaline metabolites were obtained as described for Figs. 1 and 2. Lungs obtained from female rats were isolated and perfused with Krebs–Henseleit buffer as described in Materials and Methods. [^{3}H]Serotonin was perfused through the lungs as described in Materials and Methods, and the endothelial transport of serotonin was calculated. Key: (●) lungs perfused with parent alkaloid with no metabolism by liver; (□) lungs perfused with fresh buffer that had not been perfused through a liver prior to lung perfusion; (■) lungs perfused with effluent from livers that received no monocrotaline; and (○) lungs perfused with monocrotaline metabolites obtained from isolated, perfused livers. The regression line was analyzed by linear regression; correlation coefficient 0.91 and $P < 0.001$.

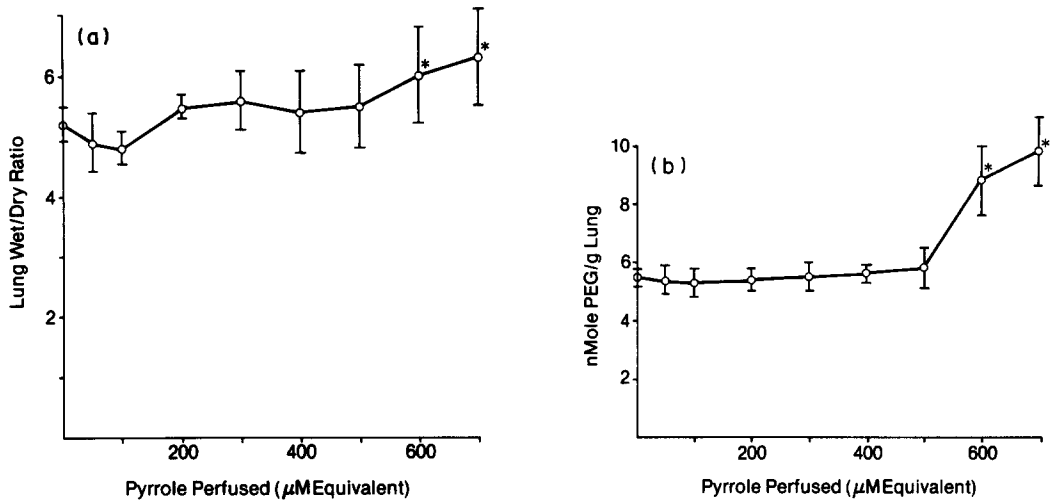

Fig. 4. Effect of monocrotaline metabolites on lung edema and permeability. Lungs were perfused as described for Fig. 3. (a) Lung wet:dry weight ratio at end of perfusion. (b) [^{3}H]Polyethylene glycol retention by lungs exposed to monocrotaline metabolites. An asterisk (*) indicates $P < 0.05$ as compared to lungs not perfused with metabolites (Dunnett's t-test). Points are means ± S.D. for an average of twelve lungs per determination.

monocrotaline was inhibited and the transport of serotonin was unchanged from that of control lungs (Table 2). Likewise, anoxic perfusion conditions or low temperatures reduced the ability of liver perfusate to alter serotonin transport in the lung.

DISCUSSION

Monocrotaline is pneumotoxic but its mechanism of action is unknown. Presumably it must be biotransformed by the liver to a chemically reactive species to cause pulmonary damage. Mattocks [10] first proposed that the toxicity of monocrotaline is due to the pyrrole or dehydro metabolites of monocrotaline. The pyrroles cause edema and hemorrhaging, pulmonary arterial hypertension, vascular smooth muscle hypertrophy and hyperplasia, endothelial cell proliferation and, eventually, right heart hypertrophy.

The cardiopulmonary effects of monocrotaline metabolites may be mediated by platelets. Platelet counts decrease in response to a single injection of monocrotaline and platelet aggregates appear in the lung [11–13]. This theory explains the apparent disparity between the supposed reactivity of the proposed toxic metabolite of monocrotaline, dehydromonocrotaline, and its survival of the transit to the pulmonary endothelium from the liver. Dehydro-

Table 2. Reduction of pulmonary damage by metabolic inhibitors

Treatment	Ehrlich reactive material* (μM)	Serotonin transport† (nmoles/min/g lung)
Control		
No preperfusion of lung		3.0 ± 0.4
Monocrotaline metabolites from untreated liver	215 ± 32	1.2 ± 0.5‡
Altered liver metabolism		
100 μM SKF-525A	0	3.3 ± 0.5
Liver perfusion at 20°	18 ± 13§	3.1 ± 0.3
Anoxic liver perfusion	40 ± 10§	2.8 ± 0.5

* Values are the mean amounts of Ehrlich reactive material ± S.D. generated by the isolated liver perfused for 90 min with 300 μM monocrotaline.

† Values are means ± S.D.

‡ Indicates statistically different from control and from all other conditions, $P < 0.05$ (Dunnett's t-test).

§ Indicates statistically different from concentrations of metabolites from untreated liver, $P < 0.05$ (Dunnett's t-test).

monocrotaline is chemically reactive, and it has been postulated that survival of this species from the liver to the lung in the blood is unlikely. Therefore, the binding of this metabolite to platelets or other blood elements may partially explain how the reactive metabolites of monocrotaline reach the lung endothelium. Once the platelets reach the pulmonary endothelium, the metabolites that are associated with the platelets react with the pulmonary endothelium causing damage.

Another possibility is that the platelets are themselves reacting with the metabolites of monocrotaline in the liver. The damaged platelets then pass out of the liver and to the lung where, because of their altered surface membranes, they lyse in the turbulent flow of the lung, releasing vasoactive substances and causing the pulmonary damage observed.

We tested these theories with *in vitro* methods. When monocrotaline metabolites generated by an isolated liver were perfused through an isolated lung, pulmonary damage in the form of decreased serotonin transport could be produced (Fig. 3), thus establishing that monocrotaline-induced pulmonary damage could occur without the involvement of platelets or other blood elements. Further, the effect of the metabolites generated by perfused livers can be reduced *in vitro* using inhibitors of liver biotransformation of monocrotaline (Table 2). These experiments demonstrate that not only can the pulmonary effects of monocrotaline that are seen after 3 weeks exposure *in vivo* [13] be produced *in vitro*, but that the effects can be manipulated with pharmacological agents. A useful aspect of this finding is that the inhibition of serotonin transport by the perfused lungs can be used as an endpoint or a marker of toxicity for monocrotaline metabolite damage to the lung. With this as a tool, it will be possible to conduct further investigations *in vitro* to study the pulmonary response to monocrotaline metabolites in detail.

However, the experiments which demonstrate decreased serotonin transport *in vitro* do not eliminate the involvement of platelets in monocrotaline-induced lung damage *in vivo*. These experiments only indicate that lung damage caused by monocrotaline metabolites may occur without blood-borne elements.

The major metabolite produced by the perfused liver system is clearly not dehydromonocrotaline. This suggests that, if dehydromonocrotaline is the initial metabolite of monocrotaline, then it is rapidly converted to a secondary, more stable, material that is then released from the liver into the circulation.

It is surprising that serotonin transport was the only endothelial cell function which was altered by monocrotaline metabolites. Pyrrole derivatives of monocrotaline have been shown to bind covalently on passage through the lung [14]. Norepinephrine transport is also an endothelial membrane function but it appears to be an insensitive marker for monocrotaline damage. When animals were administered monocrotaline for 21 days in their drinking water at a concentration of 20 mg/l, serotonin transport is inhibited but norepinephrine transport is unaffected, as are angiotensin converting enzyme and 5′-nucleotidase activities [14, 15]. However, serotonin transport sites appear to be evenly distributed throughout the pulmonary vasculature [16], whereas norepinephrine transport may occur primarily in pre- and postcapillary vessels [17]. The anatomical placement of these sites may, therefore, determine the susceptibility to chemical damage.

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