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The Effect of a Pyrrolizidine Alkaloid, Monocrotaline, and a Pyrrole, Dehydroretronecine, on the Biochemical Functions of the Pulmonary Endothelium

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SUMMARY

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Monocrotaline was placed in the drinking water of rats at a concentration of 20 mg per liter for periods of up to three weeks. This method of administration produced pulmonary arterial hypertension and right ventricular hypertrophy without inflammatory changes. No changes were noted in liver weight or RNA or protein synthesis in the liver. Isolated, perfused lungs from treated rats were examined for changes in four endothelial cell functions: angiotensin converting enzyme (E.C.3.4.99.3), 5′-nucleotidase (E.C.3.1.3.5), serotonin uptake and metabolism, and norepinephrine uptake and metabolism. Monoamine oxidase (E.C.1.4.3.4) is involved in the metabolism of both transmitters. The activities per lung of the 3 enzymes were unaltered. Norepinephrine transport was also unaffected. Serotonin transport was specifically and markedly impaired. Extraction of serotonin from the perfusate was reduced to 33% of control.

Dehydroretronecine, a metabolite of monocrotaline, produced right ventricular hypertrophy following daily subcutaneous administration for 2 weeks at a dose of 4 mg/kg. Acid-fast activity remained in lungs following perfusion with [^{3}H]dehydroretronecine. Dehydroretronecine, coperfused through isolated lungs at 1 mM concentration, or injected subcutaneously 24 hours before sacrifice at a dose of 100 mg/kg, did not affect the activities of 5′-nucleotidase or angiotensin converting enzyme. However, the retention of serotonin by the lungs was decreased, and the release of metabolites was increased. The different effects observed between monocrotaline and dehydroretronecine may indicate that the pulmonary toxicity of monocrotaline is not mediated through dehydroretronecine. Alternatively, the differences may be due to increased permeability of the endothelial cell caused by the high doses of dehydroretronecine used relative to the levels of monocrotaline. Dehydroretronecine dosages were chosen to be consonant with work by others on this metabolite.

These data indicate that the slow release of metabolites from the liver into the circulation following low-level exposure to monocrotaline results in a specific inhibition of an endothelial cell function in the lung. As well as providing insight into the molecular mechanism of action of pyrrolizidine in the lung, this information suggests that human populations exposed to low levels of pyrrolizidines may have impaired ability to regulate circulating vasoactive substances.

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INTRODUCTION

Monocrotaline is a pyrrolizidine alkaloid isolated from the seeds of Crotalaria spectabilis (1). In mammals it produces a range of toxic effects, including tumors of various cell types, portal hypertension, veno-occlusive disease of the liver and pulmonary arterial hypertension (2, 3). The latter may progress to right ventricular hypertrophy and cor pulmonale. Among the earliest changes seen in the lung when animals are fed monocrotaline are platelet adhesion, the formation of thrombi in the small capillaries, and increased permeability of the capillary endothelial cells (4). This suggests that the cardiopulmonary damage caused by monocrotaline is a result of effects on endothelial cells (3, 5, 6). The effects are not produced directly by monocrotaline, but by the pyrrole metabolites dehydromonocrotaline and dehydroretronecine. These compounds are formed by hepatic metabolism of monocrotaline (7, 8).

The mechanism whereby the toxic effects of the metabolites of pyrrolizidine alkaloids are mediated is unknown. The responses of the endothelial cell are an expression of molecular interactions. This paper is an attempt to develop a mechanism of action at the endothelial cell to explain the toxic effects of pyrrole metabolites on the basis of their reaction with membrane constituents. To this end, we have examined the effect of monocrotaline and dehydroretronecine on four enzymatic or transport processes occurring at the endothelial cell membrane. An important nonrespiratory function of the lung endothelial cell is the regulation of circulating vasoactive substances (9). This function is closely involved in the maintenance of systemic blood pressure (10). The four processes examined are all involved in removing vasopressor agents from the pulmonary output. In addition, the four processes are endothelial cell functions that may be examined in the perfused lung relatively free of interference from the activities of other cell types. The two enzymatic processes are angiotensin converting enzyme (E.C.3.4.99.3) and 5′-nucleotidase (E.C.3.1.3.5), both of which occur on the luminal side of the endothelial cell membrane. The two transport processes are serotonin and norepinephrine uptake. The metabolism of both substances occurs within the cell and involves a common enzyme, MAO[2] (E.C.1.4.3.4). MAO activity was also examined in these experiments.

The cardiopulmonary effect of pyrrolizidine alkaloids are of public health interest in the light of findings that certain populations have been exposed to such alkaloids as a result of herbal tea consumption, and pyrrolizidines may also be present in food supplies (11–13).

METHODS

Radiochemicals. [4,5-^{3}H]Leucine, [1,2-^{3}H(N)]serotonin, [^{3}H(G)]AMP, and [7-^{14}C]norepinephrine were purchased from New England Nuclear, and [5-^{3}H]orotic acid from Amersham-Searle Corp.

Monocrotaline was isolated from the seeds of Crotalaria spectabilis, collected in Alabama (1).

Statistical comparisions are based on the Student's unpaired *t*-test.

Rats. Wistar strain male rats were housed four to a cage in a laminar flow hood, except during food and water consumption experiments, when rats were housed individually. If any rats in a group developed respiratory problems, the whole group was removed from the study. The rats used showed no histological signs of lung infection.

Monocrotaline administration. Monocrotaline (as the free base) was placed in drinking water at a concentration of 20 mg/liter. Animals were placed on monocrotaline at various intervals after attaining 50 g body weight such that, regardless of length of exposure to monocrotaline, all animals were the same age at sacrifice.

Organ dissection. Rats were sacrificed by decapitation. Blood was partially removed by perfusion of a saline solution (0.9% w/v) through the right ventricle, a small incision being made in the right atrium for outflow. Organs were then rapidly removed, and weighed immediately.

[2] The abbreviations used are: AMP, adenosine 5′-monophosphate; HHL, hippuryl histidyl leucine; i.v., intravenous; MAO, monoamine oxidase; RNA, ribonucleic acids; s.c., subcutaneously; TCA, trichloroacetic acid.

The septum and left and right ventricular walls of the heart were dissected separately at ambient temperature, and weighed immediately.

Preparation of lungs for histological examination. The lungs were removed as described under "perfusion." They were placed in 10 times v/w of 10% neutral formalin for four days. The mainstem bronchi were then transected flush with the lungs. Slices (3 mm thick) were cut perpendicular to the hilar vessels, and paraffin embedded. Sections (4 microns) were cut and stained with hematoxylin and eosin by the Verhoeff method (14). Pulmonary arteries were evaluated by means of the Heath-Edwards classification (15).

Leucine incorporation. Rats were injected intraperitoneally with [4,5-^{3}H]leucine (specific activity 60 Ci/mmole) at a level of 10 μCi per 100 g body weight. Four hr following injection, the rats were sacrificed. The tissues of interest were homogenized in four volumes of water, and a sample taken for protein determination. An equal volume of 10% TCA was added to the remaining homogenate, and the sample centrifuged to precipitate insoluble material. The supernatant was removed, and an aliquot prepared for liquid scintillation counting. The precipitate was resuspended in 5% TCA, and reprecipitated. This procedure was repeated. The resulting precipitate was dissolved in 2 ml of Protosol and prepared for liquid scintillation counting. The radioactivity in the supernatant was taken as representing unbound leucine, whereas that in the final precipitate was taken as representing protein-incorporated leucine. Under the experimental conditions used, the major portion of the radioactivity in organs was in a bound form.

Orotic acid incorporation. The incorporation of [5-^{3}H]orotic acid was taken as an index of RNA biosynthesis. Rats were injected intraperitoneally with orotic acid (specific activity 0.5 Ci/mmole) at a dose level of 100 μCi/100 g body weight. The animals were sacrificed 4 hr later as described above.

Measurement of blood pressure. Rats were anesthetized with chloral hydrate, intubated, and placed on a respirator. The chest was opened and retracted, and 20 gauge teflon i.v. catheters inserted into the ventricles. The catheters were connected via a transducer to a photorecording physiograph, and the pressures were recorded. The catheter in the right ventricle was then gently moved into the pulmonary artery, and the pressures similarly recorded.

Isolation of lungs for perfusion. Each rat was anesthetized with Nembutal (50 mg/kg). The animal was secured to a rat board, and a tracheostomy performed. The cannula was attached to a respirator ventilating the animal at a rate of 45 per minute and a stroke volume of 2 ml. A midline incision through the peritoneum was made, and two incisions parallel to the costal margins to the flank. The midline incision was continued to the neck. The chest wall was retracted, and the thymus blunt-dissected free. A 3-0 suture was placed around the pulmonary artery. Tubing (PE 190) was inserted through the right ventricle and into the pulmonary artery, where it was ligated in place. The heart was then cut away, the lungs removed, and placed in the perfusion apparatus.

Perfusion apparatus. Krebs-Ringer bicarbonate buffer was used, containing 5.5 mM glucose and 5% albumin in addition to the normal salts. Lungs were ventilated with room air, and maintained in a humidified chamber. The lungs were perfused at 22° and a single pass system was employed, at a rate of 7 ml/min.

Perfusion procedure for AMP, serotonin, and norepinephrine. Radioactive substrates were used at a level of 0.05 μCi/ml buffer. Lungs were perfused with substrate-free buffer for 5 min, then with the buffer containing radiolabeled substrate at a concentration of 0.1 μM. Perfusion was continued for a further 10 min and 0.5 min fractions of lung eluate collected. At this time, perfusion medium was changed back to the substrate-free Krebs-Ringer solution. Dehydroretronecine perfusions were run in a similar manner, except tht dehydroretronecine (1 mM) was present in the buffer along with the substrate, and substrate perfusion was continued for 20 min.

Analysis of fractions. Aliquots of fractions were counted directly in a liquid scin-

tillation counter. In addition, substrate and metabolites were separated by ion-exchange chromatography. One ml aliquots of fractions from AMP perfusion were placed on an AG 1 chloride form anion exchange column (0.5 cm × 6 cm) and eluted with 4 ml water. Material eluting was counted. Preliminary experiments established, that under these conditions, [^{3}H]AMP was completely retained on the column and [^{3}H]adenosine quantitatively eluted.

Serotonin perfusions were analyzed in a similar manner, except that unchanged serotonin eluted through the column, while 5-hydroxyindolyl acetic acid was retained. Norepinephrine perfusions were analyzed on an AG 50 H^+ cation exchange column. Unmetabolized norepinephrine and normetanephrine were quantitatively retained on the column under these conditions, whereas mandelic acid was quantitatively eluted.

Scintillation counting data was obtained on punched tape, and fed into a Wang programmable calculator for data reduction. A partial format of the data obtained from each perfusion is given in Table 1. Column B is total concentration of substrate plus metabolites perfusing from the lungs. Column C is the concentration of metabolite. From this print-out, the average rate of metabolism may be derived, as well as the variation in metabolism with time.

Perfusion procedure for angiotensin converting enzyme. Hippuryl histidyl leucine was employed as substrate for assay of this enzyme, and the rate of reaction followed by spectrophotometric measurement of hippuric acid.

Lungs were perfused with buffer containing 0.5 mM HHL in the manner described above. Eluate fractions were placed in a boiling water bath for 5 min, and centrifuged at 40,000 × *g* for 30 min (to remove albumin). Supernatants were then extracted with ethyl acetate (1.5 ml). The extract was blown off under nitrogen, and the residue dissolved in water (1 ml) and

TABLE 1

Example of data reduction obtained from lung perfusion

This is an example of the partial data obtained from the perfusion of one pair of lungs. The rate of metabolism is obtained by averaging C/W × flow rate for the various time periods, the first and final two minutes of perfusion being ignored. This perfusion was run for 20 min. W is lung dry weight (mg).

Date: 2-15-77 Experiment: Serotonin perfusion
Rat: #28 Flow rate: 7.5 ml/min. Initial pressure: 17.4 cm Final pressure: 16 cm
Treatment: Dehydroretronecine perfusion (1 mM)
Body weight: 145 g RV/LV: 0.282 RV/body weight: 1.958×10^{-4} Lung dry weight (w): 158 mg
Specific activity: 356465 dpm/nmole
Concentration: 0.1 μM

Tube #	Time	B	C	B/W × flow rate	C/W × flow rate	C/B × 100
	min	*nmole/ml in eluent*	*nmole/ml metabolites in eluent*			
21	1	0.0030	0.0020	0.1447	0.0952	65.75
22	2	0.0498	0.0286	2.3677	1.3582	57.38
23	3	0.0656	0.0363	3.1162	1.7257	55.37
24	4	0.0705	0.0368	3.3465	1.7505	52.31
25	5	0.0731	0.0392	3.4710	1.8645	53.72
26	6	0.0748	0.0424	3.5520	2.0137	56.69
27	7	0.0759	0.0410	3.6060	1.9462	53.99
28	8	0.0802	0.0409	3.8257	1.9440	50.82
29	9	0.0805	0.0427	3.8070	2.0302	53.32
30	10	0.0805	0.0452	3.8257	2.1465	56.12
31	11	0.0841	0.0438	3.9922	2.0790	52.08
32	12	0.0850	0.0418	4.0387	1.9867	49.19
33	13	0.0850	0.0442	4.0365	2.1000	52.03
34	14	0.0859	0.0455	4.0793	2.1622	53.00

absorbance at 228 nm determined (extinction coefficient: 1.48×10^4). The extraction step removed 91% of the hippuric acid. In the calculation of rate, a correction factor was used.

Monoamine oxidase activity. Activity was determined on 600 g supernatant fractions of lung, with [^{3}H]serotonin as substrate. After deproteinization, reaction mixtures were analyzed by the chromatographic procedure described above.

Synthesis of dehydroretronecine. Retronecine was prepared by the method of Adams and Rogers (1).

Retronecine N-oxide (16). Retronecine (470 mg) was dissolved in ethanol and heated to 40° with 0.5 ml of 30% hydrogen peroxide for 45 min. On evaporation of the solution, an oil was obtained which solidified on the addition of dry acetone. Crystals were obtained by dissolving the solid in boiling ethanol, and leaving the solution to cool. The material was hydroscopic. Melting point 214° (decomp).

Dehydroretronecine. This was prepared by the method of Mattocks (17).

Synthesis of [^{3}H]dehydroretronecine. Formyl-7-hydroxy-6,7-dihydro-5H-pyrrolizine. Retronecine N-oxide (200 mg) in methanol was stirred with 1.66 g of active manganese dioxide for 3½ hr. At the end of this time the manganese dioxide was filtered off by suction and the methanol removed on the rotary evaporator. The crude produce was then dissolved as completely as possible in chloroform and eluted through a ¼ × 3" column of Bio-Sil A, 100–200 mesh. The eluent was decolorized by filtration through activated charcoal and taken to dryness. The produce crystallized from dichlormethane-ether. mp 35–38°.

[^{3}H] Dehydroretronecine. One hundred milligrams of the aldehyde obtained above was dissolved in 2.5 ml water. Sodium [^{3}H]borohydride was added (100 mCi, 15 Ci/mmole), followed by 200 mg of sodium borohydride. After 20 min, the solution was saturated with potassium carbonate and extracted with dichloromethane. Dehydroretronecine was crystallized from the extracts. Yield 52 mg, sp. act. 233 mCi/mmole.

Ehrlich color test for pyrroles. The sample was heated with Ehrlich reagent (1 ml) at 60° for four min. The reagent consists of 2 ml boron trifluoride etherate diluted to 100 ml with ethanol to which has been added 2 g 4-dimethylaminobenzaldehyde. Color intensity is read at 562 nm.

RESULTS

Model of Pulmonary Arterial Hypertension and Right Ventricular Hypertrophy Produced by Monocrotaline. Rats were maintained on drinking water containing monocrotaline (20 mg/liter). Over a 21 day period of exposure, total monocrotaline consumption was 7.6 ± 0.3 mg per rat (3.6 mg/kg/day).

Effect of monocrotaline on blood pressure. There is a progressive increase in pulmonary blood pressure with duration of exposure to monocrotaline (Table 2). After 22 days of treatment, systolic pressures in the pulmonary artery and right ventricle were markedly and significantly elevated. No change was observed in left ventricular systolic pressure relative to control animals.

Lungs were examined histologically. Medial hypertrophy of the pulmonary arteries was detectable by 14 days and was marked by 21 days. These changes developed without any signs of necrotizing arteritis or myocarditis. These inflammatory changes have been observed following single injections of monocrotaline in doses high enough to cause right ventricular hypertrophy (3).

Effect of monocrotaline on the right ventricle. Over a three-week period of monocrotaline administration, the lung/body weight ratio began to increase from the 9th day onward, approximately doubling after 20 days. Five days subsequent to the onset of increased lung mass, there was an increase in the right ventricular to body weight ratio. After two weeks of treatment, the right ventricular weight was significantly elevated, as shown in Fig. 1B. Thus, ventricular hypertrophy occurred after the increase in lung mass. There was no corresponding alteration in left ventricular to body weight ratio over the duration of monocrotaline treatment.

The alterations in weight ratios shown in Fig. 1 were not due to alteration in dry/wet tissue weight ratio. The percentage of water in tissues did not vary in either the right or

TABLE 2

Effect of Monocrotaline on Blood Pressure

Rats were maintained on monocrotaline (20 mg/liter drinking water) for the periods indicated.

Treatment	Duration	Pulmonary arterial pressure		Right ventricular pressure (mm Hg), systole	Pulse rate	Lung perfusion pressure
		Systole	Diastole			
	days	*mmHg*		*mmHg*	*beats/min*	*mmHg*
Control	14	18.4 ± 1.7	10.8 ± 1.0	21.8 ± 1.0	330 ± 14	
	22	20.2 ± 1.9	14.7 ± 1.4	24.4 ± 5.6	350 ± 59	14.68 ± 0.58[a]
Monocrotaline	14	26.5 ± 3.6	14.1 ± 2.8	28.3 ± 3.2	378 ± 19	
	22	34.7 ± 4.6*	20.4 ± 3.0	42.2 ± 1.8*	286 ± 21	16.78 ± 0.83[b]

* $p < 0.05$ relative to control group for day 22. No alterations occurred in left ventricular pressures. Right ventricular diastolic pressures were not significantly elevated in treated groups. 6 animals per group.

[a] Average of 102 animals.

[b] Average of 43 animals.

left ventricle or in the lungs over the period of monocrotaline treatment. Although we have reported changes in organ size in terms of organ/body weight ratios, absolute weights vary in the same manner as the ratios plotted in Fig. 1.

No alteration in liver/body weight ratio occurred over the course of monocrotaline administration (Fig. 1A). No significant changes occurred in RNA (Table 3) or protein synthesis in the liver (Fig. 2).

Monocrotaline-induced Changes in Endothelial Cell Membrane Function

Methodology. Enzymatic and transport functions were examined in isolated perfused lungs of animals pretreated with monocrotaline. Radiolabeled substrates were perfused through the lung, and the effluent analyzed for substrates and metabolites.

The perfusion apparatus and procedure has been described in the experimental section. Part of the results of one experiment are shown in Table 1, to illustrate the data format obtained. Lungs from monocrotaline-treated animals were examined for their ability to metabolize norepinephrine, serotonin, AMP, and HHL. Rats were treated with monocrotaline for either 10 or 21 days. The characteristics of these animals are summarized in Table 4. Rats treated for 21 days gained weight more slowly than did the control group.

Norepinephrine. Norepinephrine, like serotonin, is metabolized intracellularly, and must be transported across the cell membrane. A pool of norepinephrine plus metabolites is formed within the cell, and release into perfusate occurs from this pool.

After 10 days of exposure to monocrotaline there was no impairment of the lungs' ability to metabolize norepinephrine (Table 5). After 21 days of treatment, however, a more complex situation arose (Table 6). The percentage of the perfused substrate that underwent metabolism remained unchanged, but the rate of metabolism—that is to say the rate of conversion per gram of lungs—was markedly decreased. The monocrotaline-treated animals had a rate of metabolism only 65% of that of the controls. Therefore, although metabolism per total lung was unchanged, the metabolism per gram of lung was decreased due to the increase of tissue mass. Furthermore, the retention of norepinephrine and its metabolites by the lung was unaltered (Table 6), and MAO activity in lung homogenates was not depressed after 21 days of monocrotaline pretreatment.

Serotonin. When serotonin was perfused, the concentration of metabolites in the perfusate was decreased along with the rate of metabolism per gram of lung (Table 6). Thus, the ability of the whole lung to metabolize serotonin was decreased by the monocrotaline pretreatment. Total organ activity was reduced to 65% of control, and the rate of metabolism per gram of lung was reduced to 50% of control. Retention of serotonin and its metabolites by the lung was reduced to one third of control.

AMP and HHL. Changes in metabolism in the monocrotaline-treated lung were

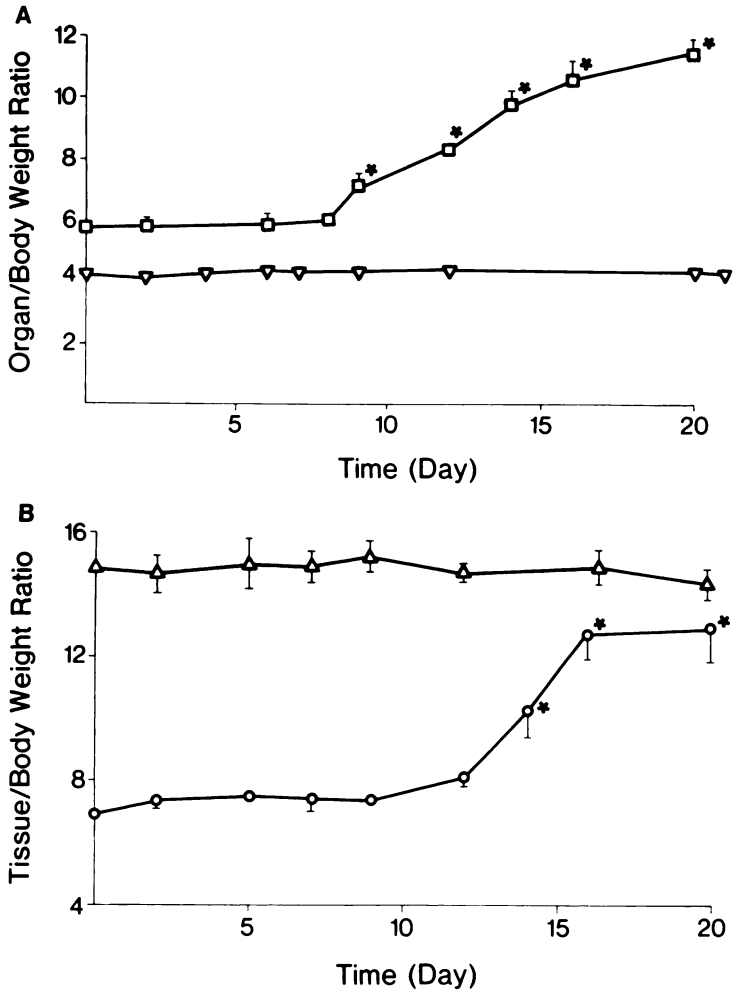

FIG. 1. *Effect of monocrotaline on liver and lung weights (A) and on right and left ventricular weight ratios (B)*

(Panel A) Data are shown as wet weight ratios. ▽ = liver/body weight ratio × 100. 9–10 samples per point, standard errors too narrow to depict. □ = lung/body weight ratio × 10^3. 6 samples per point, shown as mean ± S.E. * $p < 0.05$ compared to untreated group. (Panel B) Effect of monocrotaline on right and left ventricular weight ratios. Data shown as free ventricular wall/body weight ratio × 10^4, means ± S.E., 6 samples per point. * $P < 0.05$ compared to untreated groups. △ = left ventricle; ○ = right ventricle. The data are typical of a number of similar experiments.

closer to those found with norepinephrine than serotonin. The percentage of conversion of substrate to metabolite was unchanged by the alkaloid treatment, although the rate of metabolism per gram of lung was decreased. Thus, total converting ability in the whole lung was unchanged.

In all four sets of experiments, perfusion pressures in the monocrotaline-treated animals were slightly but not significantly higher than in the control animals.

Changes over the course of perfusion. The data presented above were derived by considerations of the average rate of metabolism between 2 and 10 min of perfusion. However, further information may be gleaned by an examination of the time course of perfusion (Fig. 3). Metabolite production from AMP showed the pattern to be expected for a substrate which is metabolized at the endothelial cell surface. By 2 min, a constant rate of metabolite produc-

tion had been established in the perfusate, which remained unchanged for the rest of the perfusion period.

With serotonin, the pattern conformed to that of a substrate that undergoes transport within the endothelial cell before metabolism may occur. Thus, the rate of release of metabolites into the perfusate increased monotonically over the period of perfusion. Norepinephrine, a compound that is also metabolized intracellularly, showed yet a third pattern of behavior. That is, an apparent bimodal dependence on time in the rate of metabolite appearance. Norepinephrine has a more complex pattern of metabolism than serotonin, and variation in efflux of metabolites may be due to this complexity.

TABLE 3

Uptake and incorporation of [^{3}H]orotic acid into liver

1 μCi/g body weight of [5-^{3}H]orotic acid (specific activity 0.5 Ci/nmole) was injected i.p. and animals sacrificed 4 hours later. Values are dpm/g wet weight × 10^{-3}, and are reported as mean ± S.E.M. for 5 animals per group. No values are significantly ($p < 0.05$) different from control (day zero).

Days on monocrotaline	Uptake	Incorporation
0	18,750 ± 7,120	726 ± 174
2	22,480 ± 6,930	761 ± 243
4	16,530 ± 4,220	642 ± 220
6	17,910 ± 4,150	873 ± 314
9	13,190 ± 6,860	1304 ± 985
12	22,830 ± 2,210	2287 ± 1403
20	21,690 ± 3,560	4116 ± 3220

Data on the appearance of radioisotope in the perfusate are illustrated in Fig. 4. For AMP, no difference was seen in the concentrations of AMP plus adenosine appearing from monocrotaline and control lungs. Furthermore, steady-state conditions were achieved within 2 min of initiating perfusion. With serotonin, a difference was observed between monocrotaline-treated and control animals. Both groups showed an increase with time in the concentrations of

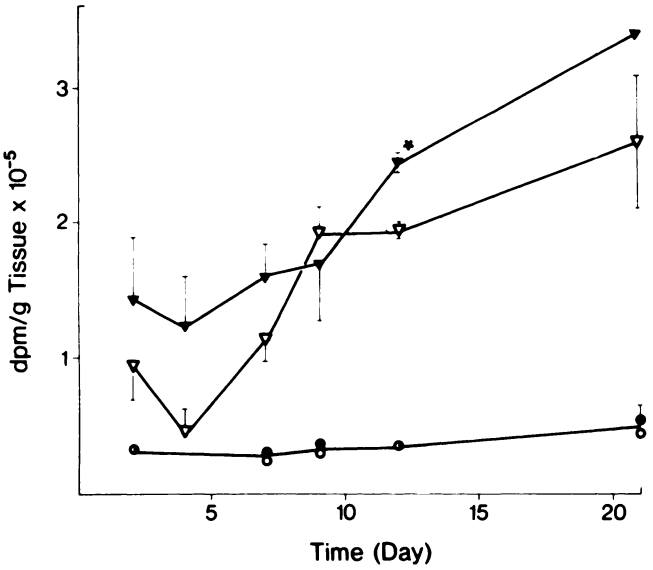

FIG. 2. *Leucine uptake by liver and incorporation into protein*

Animals were placed on monocrotaline on attaining 50 g body weight, and leucine incorporation determined at various intervals thereafter. [^{3}H]Leucine (100 μCi/kg body weight) was injected i.p. and the animals sacrificed 4 hr later. Filled symbols represent monocrotaline-treated animals, and unfilled symbols control. 5–6 animals per point. * $p < 0.05$. ▽ = acid-precipitable activity; taken to indicate incorporation into protein. ○ = supernatant activity. This is taken as a measure of the uptake of leucine by the liver. The similarity in supernatant activity between experimental and control animals indicates that alterations in incorporation are not due to differences in the availability of [^{3}H]leucine within the cell. This is one of four similar experiments.

TABLE 4

Effect of monocrotaline on organ weights

Data shown as means ± S.E.M.

	Days on monocrotaline		
	0	10	21
Number of animals	28	9	36
Body weight (g)	183 ± 4	170 ± 9	151 ± 3*
Lung dry weight (mg)	174 ± 6	192 ± 13	286 ± 7*
Right ventricular/body weight[a]	1.63 ± 0.06	1.68 ± 0.18	3.41 ± 0.11*
Left ventricular/body weight[a]	5.74 ± 0.11	5.78 ± 0.22	5.82 ± 0.12
Right/left ventricle	0.285 ± 0.010	0.308 ± 0.029	0.561 ± 0.024*
Dry lung/wet lung weight	0.184 ± 0.008	0.185 ± 0.032	0.182 ± 0.015
Liver/body weight[b]	4.514 ± 0.140	4.014 ± 0.270	4.184 ± 0.361

[a] Dry ventricular weights have been used. Ratios are $\times 10^4$.

[b] Wet liver weight has been used. Four animals per group. Ratios are $\times 10^2$.

* $p < 0.001$ relative to day 0 values.

substrate plus metabolites eluting from the lung, as would be expected for a substance that is transported within the endothelial cells, and subsequently released either unchanged, or after metabolism. However, the radioactivity perfusing from the control lungs was lower than in treated lungs at all time points. This indicated that at any given time less serotonin was retained by treated lungs, a finding that conforms with the findings in Table 6 on residual activity in the lungs at the termination of perfusion.

Dehydroretronecine and Endothelial Cell Function

Stability of dehydroretronecine. The half-life of dehydroretronecine in distilled water or Krebs-Ringer bicarbonate buffer was in the range of 39–54 hours. The substance was extremely sensitive to acid. Addition of a few drops of 2 N HCl to an aqueous solution destroyed Ehrlich reactivity in less than 1 min.

Effect of dehydroretronecine on organ weights. Rats were given daily subcutaneous injections of 4 mg/kg dehydroretronecine (Table 7). Effects observed were generally similar to those seen with monocrotaline, except the changes were more severe. A marked inhibition in body weight gain occurred after 2 weeks of treatment, and right ventricular to left ventricular weight ratio doubled. In addition, increased mass of lung dry weight was seen.

Distribution of [^{3}H]dehydroretronecine. Tritiated dehydroretronecine was injected via the tail vein at a dose of 4 mg/kg body weight. The organ distribution of total and acid stable activity is shown in Table 8 for 1, 2 and 7 days after injection. Marked variations in distribution with time were seen, possibly as a result of the changing chemical identity of the labeled species. At 24 hour, the highest radioactivity was found in red blood cells, followed by brain, pulmonary trunk and liver. By days 2 and 7 the order changed to liver, small intestines, brain and right atrium, and pulmonary arterial trunk, right atrium, right ventricle, and liver, from highest to lowest respectively. Acid-fast activity—which includes

TABLE 5

Effect of 10 day monocrotaline treatment on norepinephrine metabolism

Data are mean ± S.E.M. Treated rats (120 g body weight) were exposed to monocrotaline for 10 days before lungs were removed and perfused with 0.1 μM norepinephrine. No significant differences between control and treated groups were found.

	Control	Treated
Number of animals	9	8
Concentration of metabolites (μM)	0.032 ± 0.002	0.040 ± 0.002
Rate of metabolism (nmole/min/g dry weight lung)	1.350 ± 0.126	1.514 ± 0.096
Perfusion pressure (cm H_2O)		
initial	20.8 ± 4.3	19.9 ± 2.6
final	22.8 ± 4.5	21.7 ± 2.8

TABLE 6

Handling of vasoactive materials after 3 weeks of monocrotaline treatment

Lungs were perfused with 0.1 μM [^{3}H]AMP, [^{3}H]serotonin or [^{14}C]norepinephrine or with 0.5 mM HHL.

	Norepinephrine		Serotonin		AMP		HHL	
	control	treated	control	treated	control	treated	control	treated
Number of animals	10	10	9	9	6	6	4	4
Concentration of metabolites (μM)	0.040 ± 0.001	0.041 ± 0.002	0.029 ± 0.001	0.020 ± 0.000**	0.029 ± 0.001	0.029 ± 0.001	18.72 ± 0.73	17.68 ± 0.72
Rate of metabolism (nmole/min/g dry weight lung)	1.722 ± 0.092	1.117 ± 0.045**	1.265 ± 0.019	0.629 ± 0.010**	2.612 ± 0.070	1.580 ± 0.036**	950 ± 73[a]	705 ± 92[a]*
Metabolism (% control)		64.9		49.7		60.5		74.2
Rate/lung	0.327 ± 0.017	0.298 ± 0.012	0.221 ± 0.003	0.165 ± 0.003**	0.376 ± 0.010	0.431 ± 0.010	165 ± 13	153 ± 20
Residual in lung (dpm/g dry weight × 10^{-3})	306 ± 112	263 ± 66	447 ± 65	148 ± 30**				
Perfusion pressure (cm H_2O)								
initial	23.0 ± 3.4	26.5 ± 4.4	16.6 ± 2.1	21.2 ± 1.1	20.2 ± 2.7	24.2 ± 2.0	15.0 ± 2.4	19.5 ± 4.2
final	24.6 ± 3.4	31.4 ± 5.6	15.6 ± 4.6	21.4 ± 1.0	18.0 ± 2.4	24.0 ± 2.2	15.8 ± 2.3	23.5 ± 5.7

** $p < 0.001$.

* $p < 0.05$.

[a] units are μmole/min/g dry weight lung.

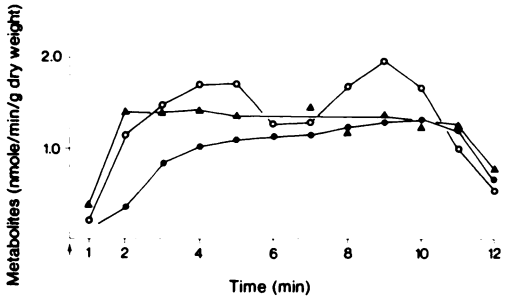

FIG. 3. *Rate of appearance of metabolites in perfusate*

Perfusion with radioactive substrate was initiated at 0 min, and changed to substrate-free buffer at 10 min. Substrate perfused: 0–0 0.1 μM norepinephrine (means of 20 experiments); ▲——▲ = 0.1 μM AMP (means of 10 experiments); ●——● = 0.1 μM serotonin (means of 10 experiments). Standard errors are omitted for clarity. All animals are controls (i.e., none were treated with monocrotaline).

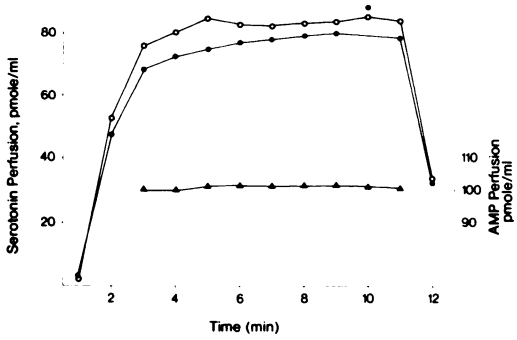

FIG. 4. *Effect of monocrotaline exposure on lung substrate retention*

Lungs from animals consuming monocrotaline in their drinking water (20 mg/liter) for 3 weeks were compared to untreated animals. Lungs were perfused with 0.1 μM substrate, and the combined concentration of substrate plus metabolites determined in the perfusate. ▲——▲ = AMP perfusion (control and treated curves overlay). ○——○ and ●——● = serotonin perfusion. Filled symbols represent control animals, and unfilled symbols monocrotaline-treated animals. Average slope 3–9 min: control 1.65 pmol/min; monocrotaline 0.78 pmol/min. 6–12 animals per point, shown as means. Error bars omitted for clarity.

activity covalently bound to protein and nucleic acids—after 7 days was highest in the right ventricle and the lung.

Effect of 3-weeks exposure to dehydroretronecine on AMP metabolism. Rats were injected with dehydroretronecine (4 mg/kg s.c.) daily for 20 days. AMPase activity at this point was decreased in perfused lungs, relative to control. Rate of metabolism was 0.683 ± 0.051 nmol/min/g dry weight on perfusion with 0.1 μM [^{3}H]AMP [mean ± S.E.M. of 10 determinations on 4 lungs].

TABLE 7

Effect of 2-week exposure to dehydroretronecine on organ weights

100 g rats were injected 4 mg/kg subcutaneously with dehydroretronecine each day for 14 days. Animals were sacrificed 24 hours after final injection. Three animals were used per group, and data are shown as mean ± S.E.M.

	Control	Treated with dehydroretronecine
Body weight (g)	191 ± 4	121 ± 9*
Right ventricle/body weight[a]	2.08 ± 0.22	4.54 ± 0.49*
Left ventricle/body weight[a, b]	5.20 ± 0.26	5.60 ± 0.18
Right/left ventricles	0.40 ± 0.05	0.82 ± 0.11*
Lung (mg)	186 ± 8	242 ± 7*

* $p < 0.025$, relative to control.

[a] Ratios × 10^4.

[b] Septum is included in left ventricle weight.

Effect of dehydroretronecine pretreatment on serotonin metabolism. Rats were injected with dehydroretronecine (100 mg/kg s.c.). Twenty-four hours later the lungs were removed and perfused with 0.1 μM serotonin. The rate of metabolism was increased to 2.195 ± 0.028 nmol/min/g dry weight of lung.

Effect of dehydroretronecine perfusion. The effects of coperfused dehydroretronecine on AMP, HHL, and serotonin metabolism are shown in Table 9. Metabolite production was increased 34% with serotonin, and decreased 18% with AMP and HHL. Retention of activity within the lungs was unchanged with AMP, but decreased 44% for serotonin.

Inactivation and binding of dehydroretronecine by lungs. Lungs were perfused in the standard way, with the addition of 1 mM dehydroretronecine. The mean retention of Ehrlich reactivity in the perfusate was 64.4 ± 4.2%. This is equivalent to an inactivation of the pyrrole chromophore by the lungs at a rate of 11.8 μmole/min/g dry weight. When lungs were perfused with 1

TABLE 8

Distribution of [^{3}H]dehydroretronecine

Activities are $\times 10^{-3}$. Animals were injected with 4 mg/kg pyrrole into the tail vein (8 μCi/100 g body weight) and sacrificed after 1, 2 or 7 days. Seven day results are the mean of 2 animals; other days, a single animal was used.

Organ	Total activity			Bound activity		
	Days			Days		
	1	2	7	1	2	7
	dpm/g wet weight			*dpm/g wet weight*		
Liver	59.9	33.8	15.7	42.07	3.38	0.43
Heart						
Right Atrium	45.5	25.9	70.1	ND[c]	ND	ND
Right Ventricle	26.7	23.8	40.2	11.65	1.21	5.17
Pulmonary trunk	67.3	15.1	88.3	ND	ND	ND
Lung	35.8	15.1	27.9	2.97	0.67	5.13
Heart						
Left Atrium	ND	ND	25.7	ND	ND	ND
Left Ventricle	44.2	24.0	13.4	12.82	0.56	1.07
Aorta	43.7	22.9	97.0	ND	ND	ND
Brain	69.9	27.2	14.5	2.74	1.63	1.35
Kidney	46.4	20.8	2.6	33.13	1.17	0.55
Intestine						
large	38.6	20.2	17.2	14.10	1.01	1.74
small	38.3	28.8	16.6	6.21	0.76	2.50
Spleen	29.9	21.0	46.0	1.96	0.61	1.82
Thymus	46.5	21.9	22.0	4.16	0.87	1.58
Serum[a]	45.3	20.0	24.2	ND	ND	ND
RBC[a, b]	476.1	111.0	24.2	ND	ND	ND
Urine[a]	3,400	25000				

[a] Activities per ml are reported.

[b] RBC is red blood cells.

[c] ND indicates not determined.

mM [^{3}H]dehydroretronecine, the mean retention of radioactivity in the lungs was equivalent to 0.456 ± 0.097 μmole/g dry weight/min (mean of 5 lungs). Of this activity, 0.258 ± 0.099 μmole/g dry weight/min remained bound after being washed with 10% TCA.

DISCUSSION

A model of monocrotaline-induced lung damage. Single injections of large doses of monocrotaline (95 mg/kg i.p.) produce pulmonary hypertension and right ventricular hypertrophy. In addition, severe liver damage and pulmonary edema is caused (18). The liver damage is accompanied by collagenization and portal hypertension. Multiple thrombi occur in the lungs and a high percentage of rats develop pulmonary arteritis and a myocarditis affecting the right septal wall (3, 9, 19).

We now report a method of monocrotaline administration that produces right ventricular hypertrophy without accompanying inflammatory changes. Young rats given free access to drinking water containing monocrotaline at a concentration of 20 mg/liter develop a mild right ventricular hypertrophy and a pulmonary hypertension over a two to three week period. After five weeks of receiving monocrotaline in this manner, the animals have no signs of myocarditis or arteritis. Hepatomegaly does not occur. Protein synthesis and RNA synthesis in the liver are not altered significantly, although individual animals may show large deviations from the mean. In contrast, single doses of monocrotaline large enough to cause pulmonary changes produce marked liver damage and hypertrophy (3, 18). The ventricular hypertrophy and pulmonary hypertension produced by

TABLE 9

Effect of dehydroretronecine coperfusion on the metabolism of AMP, serotonin, and HHL

Lungs were perfused with [^{3}H]AMP, or [^{3}H]serotonin (0.1 μM) or 1 mM HHL in the presence of 1×10^{-3} dehydroretronecine for 20 min.

	Serotonin		AMP		HHL	
	control	treated	control	treated	control	treated
Number of animals	7	7	7	10	3	3
Rate of metabolism	1.221 ± 0.067[a]	1.633 ± 0.124[a]*	2.677 ± 0.230[a]	2.205 ± 0.181[a]	857 ± 90[b]	720 ± 87[b]
Percent of control		133.7		82.4		84
Residual activity in lungs (dpm/g dry weight × 10^{-5})	14.64 ± 3.09	8.24 ± 2.19	1.12 ± 0.33	1.20 ± 0.21		
Perfusion pressure (cm H_2O)						
initial	20.2 ± 4.3	22.3 ± 1.7	19.5 ± 1.8[c]	19.3 ± 2.5[d]	16.0 ± 4.5	10.7 ± 0.6
final	17.6 ± 3.3	23.0 ± 2.0	19.1 ± 1.8	18.7 ± 2.2	16.0 ± 3.6	9.7 ± 0.6

* $p < 0.001$.

[a] Units: nmole/min/g dry weight lung.

[b] Units: μmole/min/g dry weight lung.

[c] Group size, 17 animals.

[d] Group size, 14 animals.

our procedure are highly reproducible: our finding that in the rat continual exposure to small quantities of monocrotaline over a 3-week period produces primarily cardiopulmonary damage, unaccompanied by edema, whereas a large single dose mainly produces liver damage (18) is understandable in terms of the hypothesis that monocrotaline is converted in the liver to pyrrole metabolites responsible for the toxic effects (7). Production of large quantities of pyrroles lead to profound impairment of liver function because of the ability of pyrroles to form covalent bonds with nucleophilic sites, such as sulfhydryl (7). Lower levels of pyrroles might be minimally damaging to the liver, and be released into the blood stream to affect the next organ in line—the lung.

Endothelial cell functions. We examined the hypothesis that the cardiopulmonary changes produced by monocrotaline were due to the reaction of pyrrole metabolites with the endothelial cells of the lung vasculature. To do this, we studied in the isolated perfused lung four metabolic functions of endothelial cells. Two of these functions, angiotensin-converting enzyme and 5′-nucleotidase, exist on the luminal surface of the endothelial cell (4). Substrate is metabolized without prior transport into the cell, and is perfused through the lung without retention. HHL was used as substrate for the first enzyme (20), and AMP as substrate for the second. The other two enzymatic activities examined are intracellular. These were the metabolism of norepinephrine and serotonin (4). With these two substances, transport into the cell is required prior to metabolism. After metabolism, metabolites are released over a period of time back into the circulation.

We are able to use these probes of endothelial cell function in the isolated perfused lung. Angiotensin-converting enzyme activity is present only in the endothelium (21). 5′-Nucleotidase occurs also in muscle cells, but only endothelial activity is expressed when lungs are perfused with AMP (22). Serotonin uptake has been shown radioautographically to occur almost entirely in the endothelium (23). In the perfused lung, norepinephrine is also taken up by endothelial cells and not by adrenergic neurons (dis-

cussed in reference 24).

Monocrotaline and endothelial metabolism. After three weeks of monocrotaline treatment, gross changes have occurred in the lungs. Among these changes is endothelial proliferation (3). Of the four biochemical markers of endothelial cell function we have examined, the only one to be specifically impaired is serotonin transport. This is shown by the findings that monocrotaline-treated lungs are less efficient in extracting serotonin from perfusate (Fig. 4). Radioactivity within the lung after serotonin perfusion is down to 33% of control, and MAO activity within the lung is unchanged. Both serotonin and norepinephrine are degraded by MAO (see reference 4 for review).

The transport system for norepinephrine remained unimpaired, and the activities of the cell surface enzymes, 5′-nucleotidase and angiotensin-converting enyzme, were unaffected. Modification of cell membrane function involves a chemical interaction between the plasma metabolites of monocrotaline and structures on the endothelial cell. Our findings indicate the neighborhood of the serotonin transport site fulfills the chemical requirements for interaction more closely than the norepinephrine site.

Dehydroretronecine and endothelial metabolism. The toxic effects of pyrrolizidine alkaloids have been suggested to be due to the production of pyrrrole metabolites (7). The major such metabolite of monocrotaline has been reported to be dehydroretronecine (8), although this has been disputed by White and Mattocks, who feel pyrrolic esters may be more important metabolites (25). When given intravenously to rats daily for 14 days, dehydroretronecine produced the same effects on the cardiopulmonary system as did monocrotaline (Table 6). The distribution of tritiated dehydroretronecine following intravenous injection may be compared to the findings of Hsu et al. (26) following subcutaneous administration (Table 8). Discrepancies exist between these two studies for total organ activity 7 days following injection. At this time, we found the kidney to contain minimal activity, whereas Hsu *et al.* found kidney levels to be high. The converse is the case with the lung. If the premise of Mattocks (7) is accepted that the toxicity of pyrroles is due to covalent bond formation with cell macromolecules, the distribution of bound radioactivity is a more important parameter than total activity. The rapid fall-off in total tissue label found in both studies indicates that most dehydroretronecine is not covalently bound. The toxic effects are caused by the small percentage of dehydroretronecine that is covalently bound.

In view of the electrophilic nature of pyrroles, sulfhydryl groups on the endothelial cell surface are, quantitiatively, probably the most important substrates. At low exposure levels a specificity of interaction may be predicted for the less reactive pyrroles, such as dehydroretronecine. The relatively low reactivity of dehydroretronecine is indicated by the bound activities in Table 8. A proportion of injected pyrrole survives the passage to the lungs, and a proportion survives through the lung, ending up bound to the left heart and other structures.

The effects of dehydroretronecine pretreatment on endothelial function differed from those of monocrotaline. Thus, serotonin metabolism was increased 24 hours after dehydroretronecine injection (100 mg/kg s.c.), suggesting that cell permeability had been increased, causing faster release of metabolites from the cell. These differences may be due to the large doses used, chosen to be consonant with dose levels used by others (7, 26), and the different times examined. Under these conditions, specificity of interaction may have been lost. Or these differences may indicate that the pulmonary toxicity of monocrotaline is not due to conversion to dehydroretronecine but is caused by another pyrrole metabolite (25).

Perfusion of monocrotaline through isolated lungs caused no alteration in endothelial function. Dehydroretronecine was chemically modified on perfusion, in that a high percentage of the pyrrole chromophore was destroyed. Furthermore, dehydroretronecine bound to lung tissue at the rate of 0.3 μmole/min/g dry weight. A significant decrease in AMP and HHL hy-

drolysis occurred, the metabolism of each being decreased by 16%. However, the metabolism of serotonin was significantly increased by 34%. No alteration in retention of [^{3}H]AMP occurred, but pyrrole-perfused lungs showed a 44% decrease in retention of tritium from serotonin. Perfusion of lungs with high concentrations of a reactive pyrrole results, therefore, in relatively nonspecific damage to luminal enzymes and cell permeability.

Major conclusion. Of the four pulmonary endothelial functions we have examined, the slow release of pyrroles from the liver into the bloodstream results in the specific inhibition of serotonin transport.

Public health aspects. Rats in this study were given 3.6 mg/kg/day of monocrotaline. The LD_{50} for monocrotaline following a single injection is 210 mg/kg. The finding that low levels of a pyrrolizidine alkaloid can produce impairment of pulmonary nonrespiratory functions and gross cardiopulmonary damage has a public health relevance. It has been shown that certain ethnic groups in the United States have been chronically exposed to pyrrolizidine alkaloids as a result of drinking herbal teas (11, 12, 27). There is increasing concern that low levels of these alkaloids may be present in food supplies (13, 28). Exposure levels may be far below those at which clinical symptoms appear, yet cryptic damage to the lungs may occur.

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