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# DIFFERENTIATION OF THE CARDIAC AND PULMONARY TOXICITY OF MONOCROTALINE, A PYRROLIZIDINE ALKALOID

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**Abstract**—Monocrotaline, given to rats as a 20 mg/l solution in drinking water for 3 weeks, doubled the mass of the right heart and lung. The rise in lung mass preceded that of the heart. These increases were accompanied by increases in the absolute protein content of the two organs, together with increases in the rates of both protein and RNA syntheses. The increase in lung mass was not accompanied by a change in total collagen content, as measured by two independent methods: 4-hydroxyproline content and detergent fractionation. In contrast, the right ventricle showed more than a 4-fold increase in total collagen content. Total pulmonary lipids increased by 86%, but the lipid:protein ratio was unchanged. Right ventricular lipids were unchanged in amount but the lipid:protein ratio fell by 29%. Lung DNA:RNA ratio decreased 49% and right ventricle DNA:RNA ratio decreased 69%, indicating that both of these organs were responding to monocrotaline with hypertrophy. These results suggest that the processes of hypertrophy differ in the two organs: in the lung, there was no fibrosis despite a marked increase in dry weight, while right ventricular hypertrophy was characterized by increased collagen deposition. There was no alteration in the left ventricle in any of the parameters investigated.

Pyrrolizidine alkaloids are of toxicological concern due to their ubiquity of distribution and their public health and economic impact. Monocrotaline is a pyrrolizidine alkaloid with effects in mammals that include various types of tumors, portal hypertension, veno-occlusive disease of the liver, pulmonary hypertension and right heart hypertrophy [1, 2]. Despite the increased use of monocrotaline as a model of pulmonary hypertension, little work has been done to characterize the biochemical changes in the lung caused by monocrotaline [3].

Monocrotaline produces a marked increase in dry lung weight when given to rats [4, 5]. This action of monocrotaline is poorly understood. Previous studies have described histological changes accompanying the increased mass [6–10]. In this paper, we report quantitative evaluation of changes in structural and cellular components of the lungs and right and left ventricles of the heart. These data further delineate the changes that occur in these organs during the period of rapid weight increase caused by monocrotaline.

## MATERIALS AND METHODS

*Radiochemicals*. [4,5-$^3$H]Leucine (sp. act. 60 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and [5-$^3$H]orotic acid from the Amersham-Searle Corp., Arlington Heights, IL. Monocrotaline was isolated from the seeds of *Crotalaria spectabilis* collected in Alabama [11].

*Statistics*. Comparisons were made between groups based on Student's unpaired *t*-test. Comparisons in Fig. 1 were made by analysis of variance and Dunnett's *t*-test.

*Rats*. Female Sprague–Dawley rats, initially weighing 45–55 g, obtained from the University of Arizona Division of Animal Resources, were housed six to a cage in a laminar flow hood.

*Monocrotaline administration*. Monocrotaline was dissolved in the drinking water of the animals at a concentration of 20 mg/l. Animals were placed on the water at various intervals such that, regardless of the length of exposure to monocrotaline, all animals were the same age at sacrifice.

*Organ dissection*. Animals were killed by decapitation. The lungs, right ventricle and left ventricle, plus septum, were surgically removed, washed in ice-cold 0.9% saline, patted dry, and weighed.

*Leucine incorporation*. Four hours after an intraperitoneal injection of [4,5-$^3$H]leucine (100 $\mu$Ci/kg), rats were decapitated, and the lungs and right heart were removed and weighed as described in "Organ dissection". Tissue proteins were precipitated with 4 vol. of ice-cold 10% trichloroacetic acid (TCA) and centrifuged at 10,000 *g*, and the pellet was washed two times in 5% TCA. The precipitated proteins were dissolved in 2 ml of Protosol, and radioactivity was assayed by liquid scintillation spectroscopy.

*Orotic acid incorporation*. Rats were injected i.p. with [5-$^3$H]orotic acid (1 mCi/kg; sp. act. 0.5 Ci/mmole) and killed 4 hr later by decapitation. Tissues were homogenized in 10% ice-cold TCA and analyzed as described above.

*Determination of 4-hydroxyproline*. Tissue proteins were obtained by TCA precipitation of homogenized organs as indicated above. The pellet was further washed four times with absolute ethanol to

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remove lipids and dried at 60° for 48 hr. Approximately 20 mg of the dried pellet was hydrolyzed in 10 ml of 6 N HCl for 18 hr at 100–110°. The acid was removed by evaporation, and 4-hydroxyproline (4-HP) was determined in the residue by the method of Stegmann [12]. Protein was estimated fluorometrically in hydrolyzed samples by comparison to similarly hydrolyzed albumin standards [13].

*Detergent fractionation of proteins.* Proteins were differentially solubilized according to the methods of Duhamel *et al.* [14]. Tissues were homogenized in 4 vol. of distilled water containing 0.9 mM phenylmethylsulfonyl fluoride (PMSF), 30 mM sodium azide, 20 mM *N*-ethylmaleimide (NEM) and 20 mM ethylenediamine tetraacetic acid (EDTA). The homogenate was then extracted two times (24 hr each) with 3% Triton X-100* (octyl phenoxy polyethoxyethanol) at 20° to remove soluble cytoplasmic proteins.

Proteins remaining after the Triton treatment were extracted two times with 1% lithium dodecyl sulfate (LDS) in distilled water at 20° to remove cellular structural protein as well as some low molecular weight extracellular matrix proteins. This was repeated. Non-collagen extracellular proteins were obtained from the Triton and 1% LDS insoluble proteins by extracting twice with 1% LDS and 5% β-mercaptoethanol (BME) at 20°. The material remaining insoluble after this treatment was considered collagen protein. None of the fractions obtained by the above extractions contained detectable 4-HP (data not shown).

The collagen proteins were fractioned into newly synthesized collagens and highly crosslinked collagens. New collagens were obtained by extracting the protein pellet remaining after the Triton, LDS and LDS–BME washes with hot (100°) 1% LDS–5% BME. The material remaining insoluble after this extraction consisted of highly crosslinked collagen protein and some structural sugars. Proteins in all extracts were precipitated with ice-cold TCA, washed two times in 5% TCA and three times in absolute ethanol. The resulting protein pellet was hydrolyzed in 2 N NaOH for 48 hr at 100° and the protein was estimated by fluorimetry [13].

*Total lipid assay.* Total lipids were determined gravimetrically according to the methods of Sperry [15].

*Nucleic acid determination.* Colorimetric assays of RNA and DNA were conducted according to the method of Schneider [16] with *p*-diphenylamine to determine DNA. RNA was detected colorimetrically after reaction with orcinol.

*Electrophoresis.* Detergent fractions from organs were analyzed for qualitative differences in protein distribution by Laemmli electrophoresis on 6% polyacrylaminde gels and stained with Coomassie blue R-250 [17].

## RESULTS

*Time course of changes in the lung and right heart.* Monocrotaline, administered to male Sprague–Dawley rats at concentrations of 20 mg/l in their drinking water, caused a gradual increase in lung mass that became statistically significant by day 9 and persisted for the remainder of the experiment (Fig. 1A). Preceding this event was an increase in RNA synthesis which was maximal on day 9 as well as an elevation of the rate of synthesis of protein. The rate of protein synthesis was highest when the rate of weight gain was greatest.

Five days following initiation of the above sequence of changes in the lung, similar events occurred in the right ventricle. Right ventricular mass was elevated significantly from day 14 onwards, and this was accompanied by an increase in the rate of synthesis of protein (Fig. 1B). There were no changes in left ventricular RNA synthesis, protein synthesis, or tissue to body weight ratio.

*Effect of treatment with monocrotaline on 4-hydroxyproline levels.* Monocrotaline in the drinking water of rats (20 mg/l) caused a 25% increase in wet lung weight and a 55% increase in lung protein content after 21 days (Table 1). However, monocrotaline did not alter the total lung content of 4-HP, a marker of collagen content [18]. In the right ventricle of animals treated with monocrotaline, there was a significant (132%) increase in wet organ weight and a 31% increase in protein content (Table 1). In contrast to the lungs, this elevation in weight

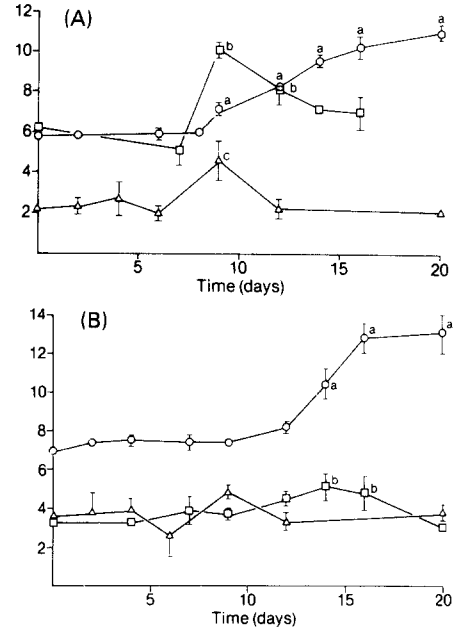


Fig. 1.(A) RNA synthesis, protein synthesis, and weight changes in the lungs during exposure to monocrotaline. Key: (△—△) RNA synthesis ([$^3$H]orotic acid incorporation, mean dpm/g wet weight × $10^{-4}$) ± S.D., a = $P < 0.05$; (□—□) protein synthesis ([$^3$H]leucine incorporation, mean dpm/g dry weight × $10^{-5}$) ± S.D., b = $P < 0.05$; and (○—○) mean organ to body weight ratio × $10^2$ ± S.D., a = $P < 0.05$. Significances are compared to zero days of exposure. Error bars are omitted where they are too narrow to be clearly shown. (B) RNA synthesis, protein synthesis, and weight changes in right ventricle during exposure to monocrotaline. Symbols are the same as in Fig. 1A except protein synthesis is dpm/g × $10^{-6}$ and right ventricle to body weight ratio is × $10^4$.

* "Triton" is a registered trademark of the Rohm & Haas Co.

Table 1. 4-Hydroxyproline content, organ wet weights and protein content in monocrotaline-treated animals*

| Tissue | (μg 4-HP/organ) | | Organ wet weight (mg) | | Protein/Organ (mg) | |
|---|---|---|---|---|---|---|
| | Control | Monocrotaline | Control | Monocrotaline | Control | Monocrotaline |
| Lung | 2906 ± 517 | 2861 ± 779 | 1474 ± 286 | 1837 ± 128‡ | 143 ± 11 | 222 ± 23† |
| Right ventricle | 25 ± 4 | 133 ± 16† | 151 ± 45 | 350 ± 55† | 16 ± 3 | 21 ± 3† |
| Left ventricle plus septum | 80 ± 32 | 50 ± 11‡ | 566 ± 47 | 473 ± 72‡ | 57 ± 11 | 38 ± 9 |

* Male Sprague–Dawley rats were treated with monocrotaline in their drinking water (20 mg/l) for 21 days. Animals were killed by decapitation, and organs were removed, washed, weighed, and analyzed for 4-hydroxyproline [12] and total TCA precipitable protein [13]. Values are means ± S.D. for twelve animals per group.

†‡ Significance of difference from control values: (†)$P < 0.001$, and (‡)$P < 0.01$.

was accompanied by a greater than 4-fold increase in 4-HP levels. In the left ventricle, wet weights and 4-HP levels were not elevated in the treated group but were slightly depressed because of the smaller weight gain of the animals treated with monocrotaline [final weights (mean ± S.D.); 223 ± 19 g control, 173 ± 11 g monocrotaline-treated, $N = 72$]. The differences in left ventricle wet weights and 4-HP levels between control and treated animals disappear if the wet weights and 4-HP levels are expressed as a fraction of body weight.

Figure 2 (A–C) depicts the relationship between the fraction of protein which is collagen and the protein content of the organ. We have used the ordinate (4-HP/mg protein) as an indicator of the proportion of collagen-containing protein. There was no increase in the fraction of protein that contained collagen in lungs from treated animals, although there was a significant increase in total protein (Fig. 2A). Indeed, there was a slight decrease in the proportion of collagen protein.

In the right ventricle of treated animals there was more protein, consonant with right ventricular hypertrophy, and a higher proportion of the protein was collagenous (Fig. 2B). The left ventricle, however, was unchanged in both total protein content and proportion of 4-HP (Fig. 2C).

*Detergent separation into non-collagen and collagen proteins.* To investigate further the relationship between increased lung mass caused by monocrotaline and the synthesis of collagenous proteins, we separated non-collagenous from collagenous proteins by detergent fractionation. The results of these experiments were consonant with the results of the experiments measuring 4-HP, and they served to support the interpretation that there was no change in lung collagen content but that there was a substantial increase in collagen in the right ventricle (Table 2).

*Electrophoresis.* There were no observable differences in electrophoretic patterns of corresponding protein fractions after detergent treatment of tissues. There were the same number of bands in both the monocrotaline and control gels and the relative positions of all bands were similar.

*Total lipid determination.* We measured total lipids to determine if the increased lung mass caused by monocrotaline was partially due to lipids. Monocrotaline caused an 86% elevation in total lung lipids (Table 3). However, the increased lipids were

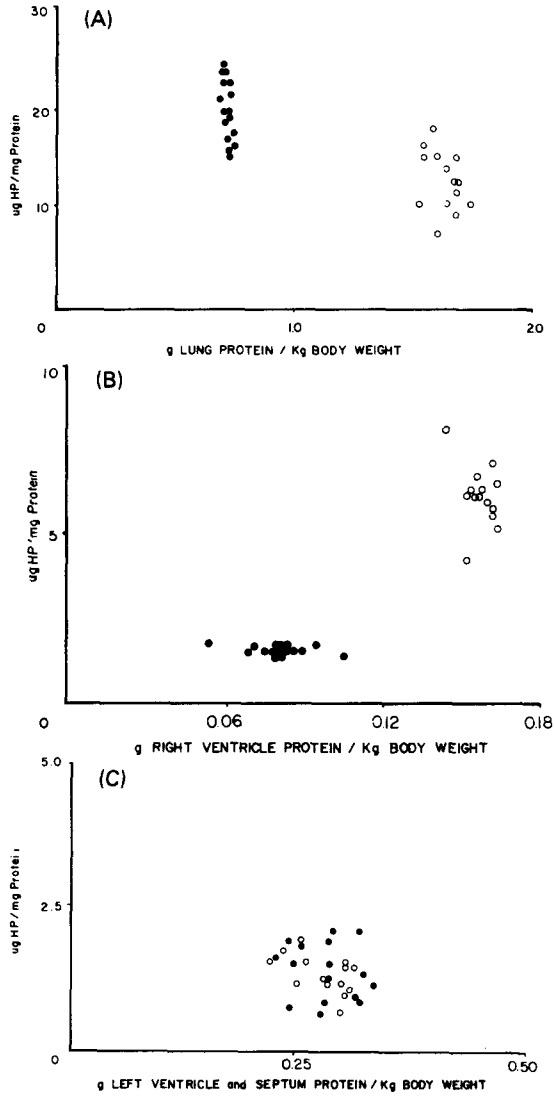


Fig. 2. Protein and collagen in the hearts and lungs of monocrotaline-exposed animals. Animals exposed to monocrotaline for 21 days were killed, and lung, right ventricle and left ventricle plus septum were removed and analyzed for total protein and for 4-HP. The y-axis indicates the proportion of total protein that is collagenous. The x-axis represents the total TCA precipitated protein per organ in protein per kg body weight. Key: (A) data obtained from analyses of lungs, (B) right ventricle, and (C) left ventricle plus septum. Closed circles are control animals: $N = 16$. Open circles are monocrotaline-treated animals: $N = 14$.

Table 2. Detergent separation of organ proteins into collagen and non-collagen fractions*

| Detergent | Protein (mg/organ) | | | | | |
|---|---|---|---|---|---|---|
| | Lung | | Right ventricle | | Left ventricle and septum | |
| | Control | Monocrotaline | Control | Monocrotaline | Control | Monocrotaline |
| Non-collagen | | | | | | |
| Triton | 118 ± 7 | 197 ± 8† | 14.5 ± 1.7 | 16.1 ± 2.2 | 50.8 ± 7.2 | 34.4 ± 8.01† |
| Cold LDS | 1.72 ± 0.16 | 1.93 ± 0.17‡ | 1.22 ± 0.16 | 3.64 ± 0.39† | 4.27 ± 0.96 | 3.37 ± 1.06 |
| 20° LDS–5% BME | 0.39 ± 0.14 | 0.72 ± 0.16† | 0.09 ± 0.02 | 0.51 ± 0.11† | 0.34 ± 0.11 | 0.22 ± 0.19 |
| Collagen | | | | | | |
| 100° LDS–5% BME | 1.62 ± 0.21 | 1.25 ± 0.44‡ | 0.09 ± 0.03 | 0.59 ± 0.12† | 0.28 ± 0.17 | 0.18 ± 0.10 |
| 2 N NaOH | 21.3 ± 3.1 | 21.0 ± 5.0 | 0.09 ± 0.02 | 0.41 ± 0.19† | 0.39 ± 0.10 | 0.26 ± 0.08§ |

* Values are means ± S.D. for twelve tissues per group.
†–§ Significance of difference from control value for same organ: (†)$P < 0.001$, (‡)$P < 0.02$, and (§)$P < 0.01$.

accompanied by elevated protein levels which left the lipid:protein ratio unchanged.

There was no change in the lipid content of the right ventricle, but the amount of lipid per mg protein was decreased significantly.

The left ventricular data reflect the unresponsiveness of the organ to monocrotaline. The total lipid content of the left ventricle in treated animals was 21% lower. When the lipid content of the organ was expressed as a fraction of the body weight, the levels were similar for control and monocrotaline-treated animals. The lipid concentration per mg protein was likewise unchanged. Therefore, both lipid and protein analyses suggest that the left ventricle is unaffected by monocrotaline treatment.

*Nucleic acid determination.* DNA levels in lungs from animals treated with monocrotaline were unchanged, whereas RNA levels were elevated significantly (Table 4). This resulted in a significant decrease in the DNA:RNA ratio which indicates that the lung responds to monocrotaline by a process of hypertrophy. Right ventricles also showed a decrease in DNA:RNA ratios following monocrotaline, while left ventricle ratios were unchanged.

## DISCUSSION

*Monocrotaline-induced changes in the lung.* It is indubitable that wet and dry lung weights increase in rats exposed to monocrotaline. It has been reported that rats treated with monocrotaline in their drinking water develop increases in lung dry weight, pulmonary hypertension, and right heart hypertrophy [4, 5]. The increased lung mass is a significant event in the course of monocrotaline toxicity yet little work has been reported on elucidating the biochemical nature of the increased lung weight (for a recent review, see Ref. 3). Other workers have histologically evaluated lungs from animals treated with monocrotaline in order to determine what caused the increase, but reports have been conflicting [6–10]. In some instances [6, 8–10], investigators report a marked medial hypertrophy of small arterioles in rats. Others [7] have reported that, in rats, fibrin occlusion of pulmonary vasculature may lead to pulmonary hypertension. Raczniak *et al.* [19] reported that beagles treated with dehydromonocrotaline, a proposed active metabolite of monocrotaline [20, 21], developed pulmonary hypertension and pulmonary fibrosis.

We found that lungs from rats treated with monocrotaline contain 55% more protein than do controls but have no significant fibrosis, as shown by 4-HP levels and detergent separation. We report that pulmonary RNA synthesis and protein synthesis increased in response to monocrotaline administration. This does not exclude the possibility that some of the protein may derive from an extra-pulmonary source.

Kameji *et al.* [22] have reported that rat pulmonary arteries stressed by various stimuli, including monocrotaline-induced hypertension, respond by increasing collagen synthesis. They suggested that increased pressure on the vascular wall is ample stimulus for this increase. Their findings are not inconsistent with ours as arteries comprise only a

Table 3. Lipid distribution*

| | Total lipids (mg) | | (mg Lipid/mg Protein) × 100 | |
|---|---|---|---|---|
| | Control | Monocrotaline | Control | Monocrotaline |
| Lung | 28 ± 7 | 52 ± 9† | 20 ± 5 | 23 ± 6 |
| Right ventricle | 8 ± 2 | 8 ± 3 | 52 ± 8 | 37 ± 12‡ |
| Left ventricle and septum | 29 ± 5 | 23 ± 7§ | 51 ± 11 | 59 ± 14 |

* Values are means ± S.D. for twelve tissues per group.
†–§ Significance of difference from control value for same organ: (†)$P < 0.001$, (‡)$P < 0.01$, and (§)$P < 0.05$.

Table 4. Nucleic acids*

| | DNA (μg) | | RNA (μg) | | DNA:RNA | |
|---|---|---|---|---|---|---|
| | Control | Monocrotaline | Control | Monocrotaline | Control | Monocrotaline |
| Lung | 9342 ± 870 | 9006 ± 2268 | 2224 ± 603 | 3550 ± 453† | 4.381 ± 0.865 | 2.235 ± 0.220† |
| Right heart | 989 ± 268 | 862 ± 310 | 299 ± 93 | 817 ± 75† | 3.236 ± 0.912 | 1.032 ± 0.219† |
| Left heart | 1106 ± 175 | 972 ± 214 | 667 ± 70 | 647 ± 136 | 1.663 ± 0.230 | 1.485 ± 0.324 |

* Values are means ± S.D. for twelve tissues per group.
† Significance of difference from control value for same organ, $P < 0.001$.

small percentage of total lung weight and this change may be masked by the whole organ preparation we report.

We found in the lung a large and significant increase in non-collagen proteins, primarily from the Triton-soluble fraction which consisted of cytoplasmic proteins. The treated lungs also contained more lipid and RNA which suggests that a component of the increased lung weight was cellular. The decreased DNA:RNA ratios in monocrotaline-treated animals also indicate that cellular hypertrophy was occurring. However, as we did not measure DNA synthesis, hyperplasia, as reported by others [23], cannot be ruled out.

The cellular source of the increased lung weight is still a major question. One of the proposed metabolites of monocrotaline, dehydromonocrotaline, is electrophilic, and it has been postulated that it binds to the nucleophilic sulfhydryl groups on the pulmonary endothelium as the initial toxic event [2, 4, 24, 25]. What happens to the lung after this binding is unknown. Ultimately, pulmonary hypertension and medial hypertrophy of arterioles develop. The latter is much like that observed in hypoxia-induced pulmonary hypertension [9]. However, the medial layer of vascular smooth muscle comprises little of the mass of the lung, so it is unlikely that hypertrophy of this material could account for the 80% increase in dry lung weight. On the other hand, endothelial cells comprise about 40% of the mass of normal lung [26] and proliferation of these cells following exposure to monocrotaline has been reported previously [2]. Therefore, it is probable that a proportion of the increased lung mass is due to hypertrophy of endothelial cells. By what mechanism this increase occurs is unknown.

*Changes in the right ventricle.* The right ventricle from animals treated with monocrotaline developed fibrosis, as demonstrated by elevated levels of 4-HP and detergent solubilized collagen proteins. Both the lung and right ventricle hypertrophied but, unlike the lung, a significant fraction of the increase in mass of the right ventricle was attributable to collagen.

In normal cardiac tissue, a fine balance is obtained between the necessary structural support provided by collagen proteins and compliance of the heart. Ideally, the percentage of protein which is collagen would remain unchanged as the heart progresses from the normal to the hypertrophied state. However, in pathological hypertrophy, the collagen concentration of the heart increases, thereby decreasing the compliance of the heart and predisposing the animal to heart failure [27]. Collagen levels in pathologically hypertrophied right ventricles have been shown to increase in rodents [28–31] and felines [32]. Studies with pressure overload induced right heart hypertrophy have shown that the rate of [$^3$H]proline incorporation increased at the end stage of hypertrophy [30, 31].

In monocrotaline-treated animals, the differing biochemical changes in the right ventricle and lungs suggest that the stimuli causing hypertrophy differed. It appears that the lung was responding to direct chemical damage, whereas the right ventricle was responding to a physiological stimulus: the increased workload caused by pulmonary hypertension.

Right ventricular hypertrophy caused by monocrotaline is characterized by an increase in membranous proteins. Detergent fractionation of lung tissue revealed that the largest increase in mass occurred in the Triton-soluble fraction. This fraction contains primarily cytoplasmic proteins. But the greatest increase in mass in the right heart was the LDS fraction which is comprised of cytoplasmic matrix proteins. This difference in protein fractionation between lung and right ventricle further reflects the divergent response of the two organs.

Our findings underline the conclusion that pyrrolizidines have marked actions on tissues apart from the liver. Acute dosing with relatively large amounts of alkaloids produces primarily liver damage, the course of which masks delayed damage to the lungs. Rats consuming low levels of the pyrrolizidine alkaloid monocrotaline in their drinking water do not develop liver damage to any degree but do develop pulmonary arterial hypertension and right ventricular hypertrophy [4]. This damage is the result of an activation of the pyrrolizidine alkaloid occurring in the liver. Furthermore, damage to the lung cannot be viewed in isolation but must be seen in terms of the relationship of the lung to regulatory processes and the functioning of other organs. One such relationship is probably involved in the production of right ventricular hypertrophy. The sequence of increased RNA synthesis, increased protein synthesis, and increased mass in the right ventricle occurs later than the parallel changes in the lungs, and is probably in some way consequential on the lung damage.

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