PYRROLIZIDINES AND THE PULMONARY VASCULATURE

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INTRODUCTION

The lung is the Cinderella in the sorority of organs. This metaphor extends not only to the relative neglect of investigation it has suffered, but to the barrage of insults it sustains that reach it both via the airway – the lung being one of the select group of organs exposed to the extracorporeal environment – and from the circulation. All organs, of course are potentially exposed to circulatory insults in the form of toxins, pathogens or metabolic stress. The lung, however, unique among the organs, is situated between the systemic venous and arterial circulations; receives the full cardiac output; absorbs the brunt of digested dietary constituents which pass from the liver to the lung in relatively concentrated form, and is similarly exposed to relatively high concentrations of any substance – drug or nutrient – given intravenously. The lung must unwearyingly respond to every change in cardiac output by a reciprocal change in pulmonary resistance to allow the change in flow without alteration in blood pressure. It is not surprising to find the lung consequentially or causally involved in many pathological processes. Obstructive or fibrotic lung disease is a major cause of incapacitation in middle age /1, 2, 3/. Pulmonary impairment is an intimate part of both left- and right-sided heart failure. Dietary pulmonary hypertension may be an underreported disease because of diagnostic difficulties /4/.

Why has not the same attention been paid to this hard-working Cinderella as to her sister-organs? One reason has been the lack of appropriate technology. Furthermore, the low metabolic rate of the lung, it using about 1-2% of the total energy consumption of the body compared with, say, 16% for the heart, led to an early focus on non-metabolic, respiratory functions of the lung. Perhaps the biggest barrier to progress is the difficulty of studying an organ with over 40 cell types, most of them difficult to separate and characterize, and many of them present in rather low abundance /5/. Only the macrophage, obtainable by lavage, has been amenable to easy isolation and purification /6/.

Gross pathological changes are consequent upon biochemical changes at the cellular or subcellular level; i.e. microscopic events underlie and parallel macroscopic events. In this article, we review first; the relationship between the biochemistry and pharmacology of the pulmonary circulation and the physiological functioning of the lung, and secondly; models of pulmonary vascular disease that give the possibility of insight into the molecular mechanisms involved in the pathogenesis of such disease. In particular, we discuss the actions of pyrrolizidine

alkaloids and their analogs.

ANATOMY OF PULMONARY ARTERIAL SYSTEM

Pulmonary arteries can be divided into 3 types: elastic, transitional and muscular /7/. Elastic arteries have well-defined internal and external laminae with 7 or more elastic laminae between. Muscular arteries, however, have less than 4 elastic laminae between the internal and external laminae. Depending on the species, muscle arteries have a diameter of around 2000 microns at the origin, narrowing to 100 microns at the level of the terminal bronchiole, with the walls becoming progressively thinner. In dogs and monkeys, smooth muscle is present down to 30 microns and in oxen, pigs, and horses down to 20 microns.

The elastic arteries consist predominantly of the pulmonary trunk and the left and right large pulmonary arteries. The media is largely composed of elastic fibrils, but does contain some smooth muscle fibers /8/. Also present are collagen and acid mucopolysaccharides (ground substance). The conducting arteries gradually merge into muscular arteries, in which there are circularly oriented smooth muscle fibers bounded by the internal and external elastic laminae. There is some interspecies variation in smooth muscle. For example, the muscular pulmonary arteries of rabbits have consistently thick muscles compared to other species /9/. The walls of pulmonary arteries are much thinner than those of the systemic circulation, as the blood pressure is only about one sixth that of the systemic circulation.

PULMONARY HYPERTENSION

Pulmonary hypertension can exist as a primary disease entity, although it is not very frequent and rather hard to diagnose, as definite diagnosis requires right heart catheterization. Pulmonary hypertension can also occur following congenital defects, or acquired abnormalities of the heart, such as mitral stenosis. More frequently, pulmonary hypertension develops as a complication or correlate of such common illnesses as bronchiectasis, emphysema, pulmonary fibrosis and cor pulmonale. It may also result from recurrent pulmonary embolism. The disease is difficult to recognize in its early stages as minimal knowledge of the basic physiological and biochemical abnormalities is absent. Normally, pulmonary hypertension and the diseases with which it is asso-

ciated are not recognized until irreversible damage has been done. In general, treatments of these diseases are palliative rather than curative.

There are many factors that can lead to production of the disease, and these factors may interact and depend on each other in a complex way. Regardless of the cause, however, the resulting pathological alterations in the lung may be very similar. The pattern of alterations that occurs in the pulmonary arterial vasculature in hypertension is briefly described below.

CHANGES IN PULMONARY ARTERIAL SYSTEM IN HYPERTENSION

Pulmonary hypertension may be classed according to the clinical signs /10/ or by the mechanism of the elevation in the blood pressure /11/. Wood's classification was: passive – due to an increase in pulmonary venous pressure; hyperkinetic – an increase in the pulmonary blood flow; obstructive – thromboembolism; obliterative – due to partial destruction of the vascular bed; vasoconstrictive; polygenic. However the blood pressure is elevated, one result is the hypertrophy of smooth muscle in the media of pulmonary veins and arteries. Muscle also extends to cover small precapillary vessels which do not normally have muscle /12/. It does not appear to have been shown whether the hypertrophying response is neurogenic, humoral, or myogenic in origin. Indeed, it has not been unequivocally demonstrated that the increase in muscle mass is due to hypertrophy rather than hyperplasia. Analogy with other systems, such as cardiac enlargement in which there is clear demonstration from RNA/DNA ratios that the increase is due to hypertrophy /13/, strongly suggests, however, that the arterial muscle is hypertrophying.

The result of the increase in muscle mass is a constriction of the lumen of the vessel, which further increases blood pressure, thus setting up an autocatalytic process. Increased blood pressure induces hypertrophy, and hypertrophy increases blood pressure. Eventually, the increased transmural pressure causes breakdown of integrity of the vessels, resulting in fibrotic lesions, diapedesis, and hemosiderosis.

The changes that occur in the muscular pulmonary arteries during the progression of pulmonary hypertension may be conveniently summarized using the scheme put forward by Heath and Edwards /12/. In this scheme, six grades of hypertension are recognized, based on changes in the media and intima of the small pulmonary arterial walls (Figure 1).

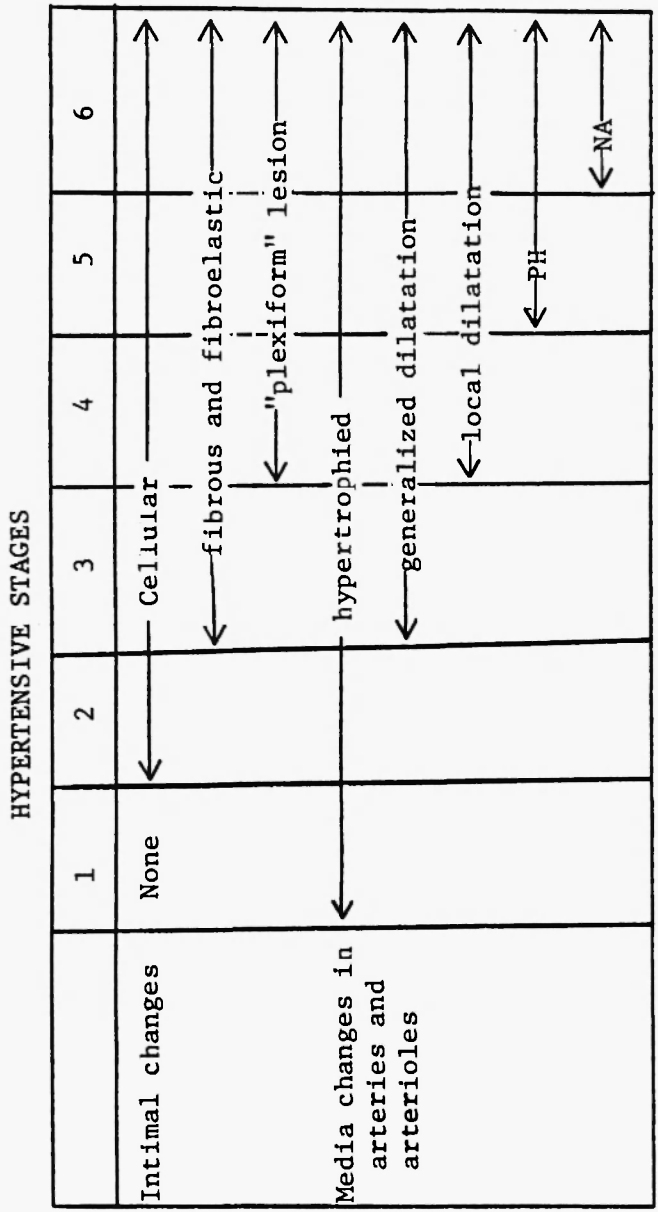


Fig. 1. Heath-Edwards Classification of vascular changes in pulmonary hypertension.
PH — haemosiderosis + distended thin walled arterial vessels through lung. NA — necrotizing arteritis.

The changes that occur at each stage are (Figure 1) /8/:

Grade 1: The muscular pulmonary arteries increase in medial thickness.

Grade 2: Hypertrophy of the media continues, but cellular proliferation of the intima starts.

Grade 3: The smaller arteries (less than 500 microns) start to undergo vascular occlusion due to the development of fibers under the endothelial lining. The larger muscular arteries show atherosclerotic changes rather than intimal fibrosis.

Grade 4: There is progressive generalized arterial dilatation with concomitant thinning of the media. In addition, local dilatations and plexiform lesions – or sacs – form. As these lesions age, they act as foci for the formation of thrombi, which in turn convert into fibroelastic tissue.

Grade 5: By this stage, the arteries are thin walled and greatly dilated.

Grade 6: This is a stage of necrotizing arteritis associated with very high pulmonary arterial blood pressure.

Similar changes occur in the elastic pulmonary arteries – the pulmonary trunk and arteries with diameters in excess of 1 mm. Muscle fibers that are present increase in thickness, and the intima becomes atherosclerotic. Chronic high blood pressure leads to an increase in the amount of elastic tissue and this appears to be irreversible. The walls of the pulmonary trunk may become thicker than the walls of the aorta. In fact, the ratio of the thickness of the media of the pulmonary artery to the media of the aorta has been used as an index of the severity of pulmonary hypertension (e.g. Ref. 14).

THE LUNG ENDOTHELIUM

We have considered above the normal structure of the pulmonary vasculature and its alteration in disease. Now we wish to consider normal function at the cellular and biochemical level, and how this may be altered in disease.

There has been a slow realization over the last 20-30 years that the lungs have important non-respiratory functions in addition to their function as a gas-exchange organ. One of the most dramatic of these non-respiratory functions is the ability to modify drastically on one

pass through the lungs the concentrations of certain vasoactive substances in the circulation.

Teleologically, there are a number of good reaons why the lung should have the capability to remove or release vasoactive substances from or into the circulation. The lungs are placed between the arterial and venous sides of the circulation, and thus are ideally located for modifying the composition of the blood entering the systemic circulation (Figure 2). Furthermore, the lungs are the only organ receiving the entire cardiac output (94% of the blood traversing the heart passes through the chambers rather than the collateral circulation). In addition, the lungs are in line with the blood flow from the liver, and so absorb the shock of digested substances. The ability to remove or inactivate sudden loads of vasoactive substances present in food (e.g. serotonin in bananas) may be an important physiological function of the lungs. Numerous reviews of this area have appeared within the last decade /15-22/.

The vasculature of the lung is lined with endothelial cells. These cells serve a number of apparent functions. They provide a non-thrombogenic lining for blood vessels, clear emboli and thrombi, serve as a barrier to prevent the diffusion of serum and proteins into tissue, and form a physical barrier between the vasculature and the alveoli. An intact endothelium is necessary for normal vascular relaxation /23, 24/. The endothelium has an enormous surface area. It has been pointed out that in the human, one ml of blood may fill 10 miles of capillaries. It is clear, therefore, that any substance present in the circulation must come into intimate contact with the endothelium. The metabolic functions of the cell serve to release a number of substances into the blood or to destroy certain substances already present in the circulation (Table 1). Vasoactive substances may be handled in the following ways:

(I)Cleared from the circulation;
(II)Released into the circulation;
(Ill)Precursors present in the blood may be activated;
(IV)Unaffected by the lungs.

The first mechanism can be broken down into the two subclassifications of substances metabolized at the luminal endothelial surface, and substances transported within the cell to be metabolized intracellularly. Examples of each are listed in Table 1.

Substances metabolized at the cell surface traverse the lungs at the same speed as the circulation. That is to say, the metabolites appear in

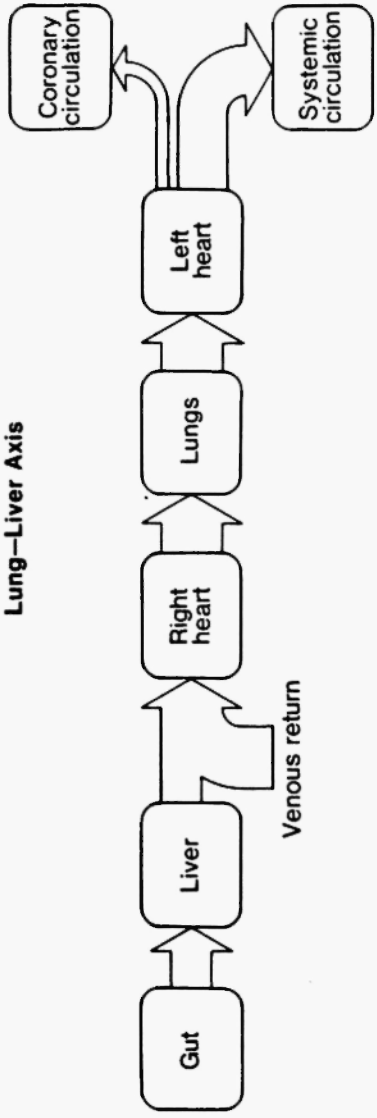


Fig. 2 The lung-liver axis, illustrating the dose circulatory relationship of the lung to the liver. Substances absorbed from the gut pass via the liver to the lungs in relatively concentrated form.

TABLE 1. *Handling of vasoactive substances by the lung.*

Metabolized at endothelial surface	*Unaffected by lungs (cont.)*
Bradykinin	Prostaglandin A
Adenine nucleotides (to adenosine)	Histamine
Angiotensin I (to angiotensin II)	Vasopressin
Metabolized intracellularly	Oxytocin
Serotonin	*Released from lungs*
Norepinephrine	Prostaglandins
Prostaglandin E	Slow reacting substance of anaphylaxis
Prostaglandin F	Kallikreins
Unaffected by lungs	
Angiotensin II	
Dopamine	
Epinephrine	

Substances classed as "metabolized" undergo in excess of 50% destruction on one pass through the lungs at physiological concentrations.

the "void volume" of the lung if substrate is pulsed into the organ. There is a finite delay for substances transported within the cell for metabolism before metabolites are released and appear in the pulmonary outflow.

Vane /25/ has made a useful distinction between "local" and "circulating" hormones. Substances handled by mechanism I can be considered to be local hormones, in that they do not enter the arterial circulation. The function of the lung can be considered to be that of inactivating them, so that they do not exhibit systemic effects. Substances released into the circulation by mechanisms II and III can be considered to be circulating hormones, exhibiting systemic effects.

The metabolic functions of the lung have to be highly active, in view of the short contact period. The capillary bed in the human holds about 60 ml of blood, and the passage time of blood through the lungs is less than a second.

The morphology of the endothelial cell is well suited to this metabolic role. A number of important enzymes are present on the luminal surface of the cell, and the cell is long and thin, presenting a high surface to volume ratio. Furthermore, these cells are highly vesiculated. The vesiculations of the endothelial cell surface may serve a number of functions. They probably act as pinocytotic vesicles, engulfing large par-

ticles into the cell. They appreciably increase the surface area of the cell, and we suggest that one effect they have is to cause turbulent flow of the blood across the endothelial surface. This would serve the function of ensuring that all substances in the blood come into close contact with the cell surface. From this point of view, it is interesting to note that the vesiculations are particularly rich in enzyme activities, containing a large proportion of the 5′-nucleotidase and angiotensin converting enzyme activity of the cell.

Endothelial cells are also a prime source of thromboplastin and plasminogen activator. The latter substance initiates fibrinolysis by converting plasminogen to plasmin.

VASOACTIVE SUBSTANCES AT THE ENDOTHELIUM

(a) Serotonin

Serotonin is an example of a substance whose passage is retarded through the lungs. Between 65-98% of a dose of serotonin injected into the right atrium disappears on one pass through the lungs /26/. Disappearance is due to endothelial uptake, retention within the endothelial cell, metabolism within the cell followed by release of metabolites. If radioactive serotonin is perfused, the released radioactivity is mainly metabolic degradation products. Efficient removal of serotonin from the pulmonary circulation has been found in many species, including man, dog, cat, rabbit, guinea pig and rat. Almost complete removal occurs over the concentration range 0.005-17 μM. In rats, the Michaelis constant (Km) for removal is 5.2 μM, and the Vmax is 12.8 nmole/min/g lung /27, 28/.

Serotonin is not liposoluble, and does not diffuse across the endothelial cell membrane. It is carried by a transport system dependent on Na, K-ATPase. Uptake is inhibited by ouabain, cooling, hypoxia, and metabolic inhibitors. Uptake is sodium dependent.

Evidence for Cellular Location: A number of techniques agree in locating the site of serotonin transport on the endothelial cell. Electron microscopy radioautography of lungs perfused with (^{3}H) serotonin and monoamine oxidase inhibitor revealed that over 50% of the radio label was in endothelial cells /29/. This finding has been confirmed by further radioautography /30/ and fluorescence spectroscopy /28/. Mast cells are not involved in the removal process /31/. Transport sites for serotonin appear to be uniformly distributed along the endothelium of both capillaries and larger vessels.

Intracellular Metabolism: Serotonin is not stored in the lung. There is an efficient intracellular metabolic pathway, whereby serotonin is initially metabolized by monoamine oxidase to the corresponding aldehyde, and further oxidized via aldehyde dehydrogenase to hydroxyindolyl acetic acid (Figure 3). The rate limiting step in serotonin removal, however, is not metabolism but transport into the cell. Monoamine oxidase inhibitors block metabolism almost completely, but do not affect removal of serotonin from the perfusate or circulation of the lung.

The physiological significance of this active removal system for serotonin is uncertain. It may be important in states such as the carcinoid syndrome in which large amounts of serotonin are released into the bloodstream. Normally, it may have a role in maintaining low serum levels of serotonin, in order to prevent platelet aggregation and thrombi formation. It may also function to remove large loads of serotonin entering the circulation from the diet.

(b) Norepinephrine

Norepinephrine is handled in a similar way to serotonin. However, the transport sites are different, and do not exhibit overlapping affinities. The removal rate for norepinephrine is rather less efficient than that for serotonin, and the degree of metabolism is less. Thus, after an i.v. bolus, 20-35% of the norepinephrine is removed on one passage through the lungs. The retained norepinephrine and its metabolites are released over the next 2 hours, with the metabolites appearing sooner. As with serotonin, the rate limiting step is transport.

Norepinephrine is metabolized intracellularly by monoamine oxidase, and catechol-O-methyl transferase (Figure 4). The metabolic route differs from that present in the adrenals, for example, in which the major metabolite is epinephrine. If the metabolism of norepinephrine is inhibited by tropolone, iproniazid or pargyline, the removal rate is not affected /32, 33/.

The Michaelis constant (Km) for removal in the rat lies between 1.1 to 1.4 μM, and the Vmax lies between 2.2 to 2.8 nmole/g lung /28, 32, 33/. The transport site shows no stereospecificity for D or L norepinephrine. Transport is inhibited by iodoacetate, but not by anoxia /28/. Uptake of norepinephrine from the circulation occurs extraneuronally, and has been shown to take place in the endothelial cell. This has been demonstrated by radioautography /32, 33/ and fluorescence spectro-

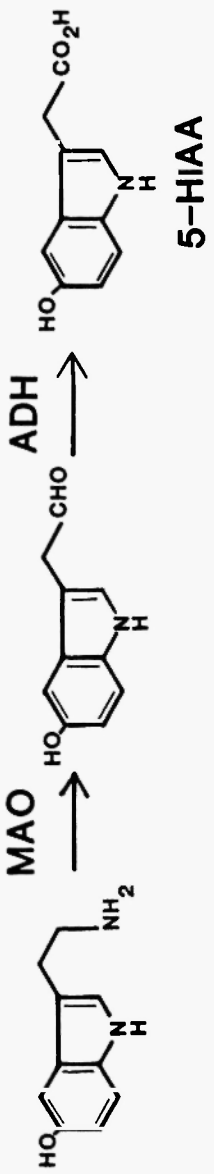


Fig. 3. Pulmonary metabolism of serotonin by monoamine oxidase and alcohol dehydrogenase to give 5-hydroxyindolylacetic acid.

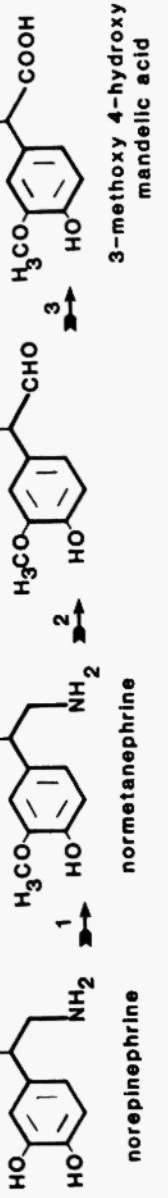


Fig. 4. Pulmonary metabolism of norepinephrine. The enzymes are: (1) catechol-O-methyl transferase; (2) monoamine oxidase; (3) aldehyde dehydrogenase.

scopy /28/. The lung can distinguish between the three pressor amines, dopamine, norepinephrine and epinephrine. Norepinephrine is retained whereas the other two substances traverse the lung unaffected. This is in contrast to systemic vascular beds, in which norepinephrine and epinephrine are removed to the same extent.

There is a possibility that removal sites for norepinephrine are not distributed evenly along the endothelium. It has been suggested that there is little uptake into the large arteries or the capillaries, and that the most active uptake occurs in the pre-capillary and post-capillary vessels /33/.

Further evidence that the pulmonary uptake of norepinephrine and serotonin occurs in the endothelium and is not related to adrenergic innervation is that pretreatment with 6-hydroxydopamine (an agent that destroys adrenergic nerve terminals) does not affect the uptake /34/.

(c) Angiotensin Converting Enzyme

The angiotensins are components of the renal pressor system. Angiotensin I is a decapeptide generated in blood by the action of renin upon an α 2 globulin, angiotensinogen. Angiotensin I has little pressor activity, but is converted to angiotensin II, an octapeptide, which has 50 times more pressor activity. The enzyme converting angiotensin I to angiotensin II is a dipeptide hydrolase, known as angiotensin converting enzyme. The lung is the major physiological site for this conversion in dog, man, rat, guinea pig, sheep, hog, cat and rabbit. Converting enzyme activity exists in plasma, but the activity is too low to account for the rapidity of metabolism.

Angiotensin converting enzyme is present on the luminal aspect of the endothelial cell. Evidence for this is provided by a number of lines of research. If (^{14}C) angiotensin I is perfused through isolated lungs no radioactivity is retained within the organ /35/. Converting activity has been shown to be restricted to endothelial cells by techniques such as immunocytochemical and immunofluorescence localization /36/, electron microscopy /37/, and antibody labelling /38/. Sandler and Huggins /39/ showed that enzyme activity was limited to the plasma membrane fraction from lung, and Ryan and Smith /40/ further demonstrated that isolated endothelial cells had high activity. As a result of the localization of the enzyme on the luminal aspect of the endothelium, the degree of conversion of angiotensin I to angiotensin II depends on the pulmonary vascular area, and the transit time /41/.

Another peptide, bradykinin, is efficiently inactivated by the lungs. It has been shown that the enzyme inactivating bradykinin, kininase, is identical to angiotensin converting enzyme.

(d) Adenine Nucleotides

Adenine nucleotides, which are vasoconstrictors, are efficiently metabolized on one passage through the lungs to the non-vasoactive substance adenosine. Pulse perfusion of rat lungs with (^{3}H) AMP leads to 60% of the activity emerging as adenosine on one pass. Similarly, ATP is largely converted to adenosine on one pass. Both ATP and AMP and their metabolites clear the lung simultaneously with dextran blue injected at the same time. No radioactivity is retained by the lungs. This indicates that, as with angiotensin I, metabolism occurs while the substrate is in the blood /42/.

Metabolic activity has been localized to the luminal surface of endothelial cells. In particular, ATPase and 5′-nucleotidase activities are high on pinocytotic vesicles. That these are the enzymes responsible for the metabolism of adenine nucleotides was shown by co-perfusion with AMP and lead nitrate. Lead deposits were restricted to the pinocytotic vesicles /43/.

PHARMACOLOGY OF THE ENDOTHELIUM

A large number of drugs are removed on passage through the lungs (Table 2). Many of these drugs are transported either by the serotonin or norepinephrine sites, and competitively inhibit the removal of these substances (Table 3). As a result, tricyclic antidepressants such as imipramine or desmethylimipramine potentiate the pressor action of norepinephrine, and these and antihistamines such as tripelennamine inhibit the pulmonary clearance of serotonin and norepinephrine. Patients with high serum levels of these drugs may have norepinephrine and serotonin in the arterial circulation.

BIOCHEMICAL STIMULI TO PULMONARY HYPERTENSION

The mechanism whereby the initiating event in hypertension is translated into the pathological alterations that are seen is complex and not well understood. Many physiological adaptations appear to predispose to pulmonary hypertension. One of the most intriguing is the influence of hypoxia. A decrease in the oxygen saturation of pulmonary arterial

TABLE 2. *Drugs inactivated by the lung.*

Chlorpromazine	Propranolol
Lysergic acid diethylamide	Sulfanilamide
Δ-9-Tetrahydrocannabinol	Cyclizine
Imipramine	Chlorcyclizine
Desmethylimipramine	Tripelannamine
Nortriphyline	Diphenhydramine
Mepacrine	

(From Ref. 16).

TABLE 3. *Drugs that affect serotonin and norepinephrine removal.*

Serotonin	*Norepinephrine*
Inhibitors:	
imipramine	imipramine
amitryptiline	nitrous oxide
trancylpromine	halothane
chlorpromazine	estradiol
cocaine	cocaine
phenoxybenzamine	phenoxybenzamine
normetanephrine	normethanephrine
	aminophylline
	phentolamine
No effect:	
aminophylline	propranolol
phentolamine	metaraminol
reserpine	
norepinephrine	

blood causes immediate vasoconstriction /44/. Two types of mechanism might be responsible: (i) the vasoconstriction is an indirect action mediated through a hypoxia-stimulated release of a local vasoconstrictor metabolite; or (ii) hypoxia has a direct vasoconstrictor action on vascular smooth muscle. The balance of evidence at the moment appears to favor mechanism (i), as alveolar hypoxia is more effective than arterial in causing vasoconstriction, suggesting that the alveoli release a substance that then acts upon the arteries. Furthermore, vascular strips exposed to hypoxic conditions contract if lung parenchymal tissue is

still attached, but not if the strip is free of such tissue. The nature of the substance (or substances) released is unproven. However, up to 58% of the response can be blocked with indomethacin or aspirin, indicating that prostaglandins are at least partially involved /45/. Antihistamines inhibit the hypoxic pressor response in rats, which might suggest the released substance is histamine. This is refuted, however, by the rather low sensitivity the rat pulmonary vasculature exhibits towards histamine.

Neural mechanisms are not important in the response. Atropine or hexamethonium do not alter the pressor response to acute hypoxia /46/. Methylsergide, pyrilamine maleate, propranolol and phenoxybenzamine also are without effect on hypoxic vasoconstriction /45, 47/. Robin et al. /48/ isolated from hypoxic calf lung a substance producing pulmonary hypertension in calves and dogs without affecting the peripheral circulation.

Prolonged exposure to hypoxic conditions can lead to a permanent elevation in pulmonary blood pressure /46, 49, 50/. Weidman et al. /49/, for example, found that cats kept in 10% oxygen developed pulmonary hypertension, while Naeye /46/ kept mice in 10% oxygen and found they developed medial hyperplasia and right ventricular enlargement. Naeye further found that chronic administration of α-methyldopa during the period of hypoxia blocked the development of pathological alterations. Chronic alterations in environmental oxygen tension lead to changes in lung size. In rats, hypoxia causes an increase in lung volume, and hyperoxia a decrease /51/. The lung to body weight ratio also increases in hypoxia /52/. This indicates the importance of environment in the regulation of lung growth processes. The fetal and neonatal lung are even more responsive than the adult lung to hypoxic stimulation, due to the greater content of medial smooth muscle in immature lung.

People living at high altitudes have permanently higher pulmonary blood pressures, and this is associated with more prominent medial musculature.

Acidosis sensitizes the pulmonary circulation to the effects of low oxygen pressure. At a given oxygen pressure, an increase in carbon dioxide content of inspired air will cause an increase in pulmonary blood pressure.

Another agent with a strong vasoconstrictor action on pulmonary vessels is serotonin /53/. The effect is inhibited by serotonin antagonists. α-Adrenergic agents such as phentolamine also antagonize the vasoconstrictor effects of serotonin, even though these agents have vasocon-

stricting actions of their own. However, serotonin receptors are not identical to α-receptors, because small pulmonary arteries are not responsive to norepinephrine but are highly sensitive to serotonin. The vasoconstrictor effect of serotonin is a direct one, as it persists after denervation or reserpine treatment /54/.

Mast cells in the lung of some species contain serotonin /55/, and other vasoactive substances, such as histamine. Mast cells are probably involved in the response of lung to a number of stimuli, but their function in such responses is unclear.

The polypeptide, bradykinin, causes an increase in pulmonary arterial pressure in dog /56/ and man /57/, mainly due to its positive inotropic action leading to increased cardiac output.

MODEL STATES OF PULMONARY HYPERTENSION IN ANIMALS

(1) Hypoxia. The pulmonary hypertension produced on keeping animals under chronic low oxygen tension has been the most widely used experimental model on which to study cor pulmonale and pulmonary hypertension /46, 49-52/. Much of the research has been performed on animals maintained at high altitudes. However, there is an important difference between the hypoxic patient with chronic obstructive lung disease and a person living at high altitude. Chronic obstructive lung disease usually leads to a decrease in the pulmonary capillary bed, but the opposite may be true of the high altitude resident.

(2) Papain emphysema leading to pulmonary hypertension. Patients with emphysema develop chronic hypoxia with pulmonary hypertension and right ventricular hypertrophy /58/. Many enzymes have been administered into the lungs of animals in order to mimic the destructive lesions seen in emphysema. Will and Kay /59/ investigated whether papain-induced emphysema would proceed to pulmonary arterial hypertension and right ventricular hypertrophy. They found that 25% of rats that survived exposure to a papain aerosol developed emphysema, and a small percentage developed hyperplasia of the media of muscularized pulmonary arteries. No animals with right ventricular hypertrophy were found in their study, probably because the animals were not kept long enough for it to develop. It appears on the basis of this study and others that the hypertension is a result of the chronic hypoxia rather than the destruction of the vascular bed, as increases in blood pressure occur before the bed is damaged enough for vascular resistance to increase.

Although papain emphysema induced hypertension appears to be a good model of the human disease, it is not a very convenient one. In the study quoted /59/, only 2 animals out of 40 rats developed pulmonary hypertension.

(3) Increase in flow resistance of left heart. All stimuli to pulmonary hypertension act via an increase in resistance of some part of the pulmonary circulation. Hypoxic vasoconstriction occurs in the arterioles, and to a lesser extent in the pulmonary arteries. Obstructive lung diseases can lead to an increase in resistance due to destruction of the capillary bed. Increased resistance in the left heart, such as occurs in mitral stenosis, may also lead to pulmonary hypertension. In the normal lung, the bulk of flow resistance occurs in the capillary bed, and little resistance is found between the capillaries and left atrium. If the left atrial pressure increases, the pressure is transmitted back to the capillaries, and pulmonary arterial pressure and right ventricular work must increase to meet the increased load. In such a situation, although the right heart has compensated, it is at the expense of an elevation in the pulmonary capillary pressure. If this is raised enough, pulmonary edema develops. This situation arises in mitral stenosis. The state can be produced experimentally by banding of the pulmonary vein.

The earliest changes occur in capillaries /8/. These rupture, leading to hemosiderosis, followed by siderofibrosis (impregnation with elastica and reticulin). Medial hypertrophy and severe intimal fibrosis occur in the veins as a result of the elevated blood pressure. Hypertension which primarily affects the veins – as in mitral stenosis – causes characteristic alterations. Muscle fibers in pulmonary veins are normally arranged irregularly and are intermingled with collagen and elastic fibrils. With severe chronic hypertension, a distinct muscular media develops, with no collagen or elastic fibrils – in other words, the vein takes on the appearance of arterial media. The hypertrophy of smooth muscle is pathognomonic of pulmonary venous hypertension.

(4) Monocrotaline-induced pulmonary hypertension. A well documented animal model of pulmonary hypertension is that produced by ingestion of the seeds of *Crotalaria spectabilis*, or of the pyrrolizidine alkaloid, monocrotaline (Figure 5), which is isolatable from the seeds. *Crotalaria spectabilis* is a large plant, growing to a height of 1 meter. Although indigenous to India, it was introduced into Florida as a cover crop in 1921, and now grows wild throughout the south-eastern states. Poisoning after feeding on the plant has been reported for many animals /60/.

Fig. 5. Structures of some acyclic diester and macrocyclic diester pyrrolizidines.

The actions of monocrotaline were first reported in 1942 /61/. This agent produces veno-occlusive lesions of the liver /62/. All the pathological changes caused by *Crotalaria spectabilis* seeds are also produced by monocrotaline /63/.

Even though the changes produced by monocrotaline have been quoted by many to be an excellent model of pulmonary hypertension, the bulk of the work reported on the system has been morphological. Until recently, little work had been done on the biochemical changes induced by monocrotaline.

The most detailed studies on *Crotalaria* poisoning have been carried out on rats. Rats fed ground up *Crotalaria* seeds mixed in with their normal diet show dilatation and hypertrophy of the right ventricle, with no change in the atria, left ventricle or valves. Right ventricular hypertrophy can occur following a single subcutaneous injection of monocrotaline /64, 65/. Occasionally, myocarditis of the right ventricle also occurs /65-67/.

Administration of *Crotalaria* leads to increases in medial thickness of the muscular pulmonary arteries. Occasionally, arteritis develops, followed by destruction of the external elastic lamina. The increase in medial thickness strongly correlates with increase in the ratio of right ventricular to body weight /60/. Increase in right ventricular weight, therefore, is a good index of the progressive damage caused by pulmonary hypertension. Increase in medial thickness is the earliest structural change found in pulmonary arterial hypertension. Pulmonary arteritis appears to be more of a response to the rate of increase of pulmonary blood pressure rather than to the absolute blood pressure /8/. This is indicated because of the lack of correlation between the presence of arteritis and right ventricular weight.

The electron microscopic changes occurring in arterial vessels in monocrotaline-induced disease have been well documented /68/. This study showed an increased number of subcellular organelles, indicating an increase in certain metabolic activities. However, no biochemical studies were performed.

Associated with the above changes in the muscular arteries was medial hypertrophy of the pulmonary trunk, due to an increased amount of muscle. A linear relationship also exists between the ratio of medial thickness of pulmonary trunk to aorta and right ventricle/body weight ratio – a further demonstration of the validity of taking right ventricle weight as an index of hypertensive disease progression. This relationship between right ventricular and arterial medial hypertrophy is also seen in

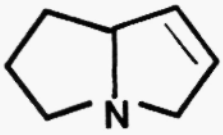
human essential pulmonary hypertension, but is not seen in hypertension due to hypoxia /69/. In the latter case right ventricular hypertension occurs in the absence of medial hypertrophy. This is relevant in that chronic hypoxia is frequently used as an experimental model of hypertension.

The other correlations of importance are first, a linear relationship between right ventricular systolic pressure and right ventricular weight and secondly, linear relationships between medial thicknesses of both muscular pulmonary arteries and pulmonary trunk and right ventricular systolic pressure. So there is a constant relationship between right ventricular weight, pulmonary arterial systolic blood pressure, and arterial hypertrophy,

The detailed mechanism whereby monocrotaline induces an increase in the resistance of the pulmonary circulation is unknown. It is not a direct effect, however, as injection of monocrotaline has no immediate action on right ventricular blood pressure /70/. Effects are not apparent until days following injection /71/. The metabolism of monocrotaline and other pyrrolizidines is discussed below. In general, however, these agents are converted to pyrroles such as dehydromonocrotaline in the liver which appear to be the proximate toxins (Figure 6).

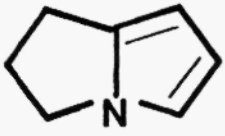
In sum, monocrotaline disease mimics closely the pathological changes associated with essential pulmonary arterial hypertension in humans. However, monocrotaline is not unique among the pyrrolizidines in producing lung damage. Many other pyrrolizidine alkaloids, capable of being metabolized to toxic pyrroles in the liver, are also pulmonary toxins.

As is the case with monocrotaline, much of the work reported on the other pneumotoxic pyrrolizidines has been histological. Despite the varied routes of administration and despite the various pyrrolizidines used in these studies most reports present a similar histological picture. Many of the effects of pneumotoxic pyrrolizidines have been summarized by McLean /72/. Early changes are dilated lymphatic vessels, alveolar edema, and hemorrhage, all as a result of increased capillary permeability. Intermediate changes are proliferation of cells and megalocytosis and late changes are pulmonary hypertension leading to right heart hypertrophy and right heart failure. These changes are seen with *Crotalaria spectabilis* seeds, or monocrotaline in the diet. They are also seen following single intraperitoneal injections of fulvine, fulvine N-oxide and monocrotaline (Figure 5) or single intravenous injections of monocrotaline pyrrole or retronecine pyrrole (Figure 7). Since McLean's



pyrrolizidine
(pyrrolizine)

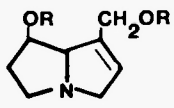
(a)



pyrrole
(dehydropyrrolizidine)

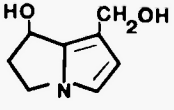
(b)

Fig. 6. The hepatic metabolism of pyrrolizidines (a) to pyrroles (b) as proposed by Mattocks /74/, which can serve as biological alkylating agents and account for the toxicity.



I R=H retronecine

II R= C_2H_5NHCO· dl-N-ethylcarbamyl retronecine



retronecine pyrrole

(dehydroretronecine)

Fig. 7. Structure of retronecine, its carbamate ester and its dehydro form.

review there have been more detailed investigations of pneumotoxic pyrrolizidines and additional ones have been reported.

Early changes in pyrrolizidine-treated lungs are edema, hemorrhaging and pleural effusions. Since these occur as a result of increased vascular permeability, Kay et al. /73/ termed this damage exudative lesions. The exudative phase of pulmonary pyrrolizidine toxicity occurs 1-3 days after administration of the toxin. As a single dose of alkaloid is cleared within 24 hours /74/ it is puzzling that the first effects are not observed immediately. The pyrrole metabolites postulated to be responsible for the toxicity of the alkaloids /74/ are chemically reactive. Presumably, chemical reactions with tissues are fast following exposure to pyrroles, but a period of days elapses before transformations at the molecular level translate into histological and ultrastructural changes.

The exudate is likely due to direct damage to the pulmonary endothelium causing a loss of integrity of the endothelial barrier and resulting in pulmonary edema. Edema could then cause swelling of the lung parenchyma, decreasing vascular lumen diameter and resulting in pulmonary hypertension and the lesions described. The major histological lesion in the lung from pyrrolizidines appears to be due to severe and sustained vasoconstriction, although other lesions have been reported and doubtless contribute to the overall course of response (see Ref. 72 for review).

In 1971 Kay et al. /75/ reported that fulvine (Figure 5) administered to female rats (50 mg/kg i.p. or 80 mg/kg gastric intubation) resulted in hypertensive pulmonary vascular disease leading to right heart hypertrophy and failure. Although 11 of the 30 test animals died within 23 days of extensive centrilobular necrosis of the liver, the animals that survived from 24-37 days developed a thickened medial layer in the pulmonary trunk characterized by hypertrophied smooth muscle fibers and fragmented laminae. In addition, there was medial hypertrophy of both muscular arteries and pulmonary arterioles. A similar description of the pulmonary toxicity of seneciphylline (Figure 5) was given by Ohtsubo et al. /76/. Animals treated with seneciphylline at 80 mg/kg i.p. showed medial thickening of arterioles 3 weeks post injection. In 1976 Dingemans and Wagenvoort /77/ described similar lesions and suggested that fulvine had a direct vasoconstrictor action. One week after injection of fulvine (50 mg/kg), veins and venioles showed an increased number of small smooth muscle cell protrusions which occasionally increased in size and extended through the basement membrane into the endothelial layer causing some protrusion of endothelial nuclei into the

vascular lumen. Histamine-contracted veins and venioles showed similar changes which led the authors to believe fulvine was causing contraction of vascular smooth muscle. In contrast, arteries from fulvine-treated rats had no smooth muscle excrescences into the endothelial layers but protrusions into the internal elastic laminae. In addition the lumen was so narrowed from apparent smooth muscle constriction that it was nearly occluded by endothelial cells. Smith and Heath /78/ described hypertrophied smooth musculature and newly formed immature muscle cells internal to elastic laminae in lungs from rats fed *Crotalaria spectabilis* seeds. The endothelial cells from pulmonary veins were uneven and appeared distended by cysts which were, in fact, smooth muscle cells protruding from the medial layer. The subtle distinctions made in this paper regarding the electron micrographic appearance of the contracted smooth muscles are important and should be helpful to other investigators attempting to decipher the histological appearance of contracted smooth muscle. Vascular smooth muscle may contract from cations contained in the fixative. Contracted vasculature has an undulating appearance and smooth muscle protuberances similar to those seen with pyrrolizidine-contracted smooth muscle. However, the pyrrolizidine-contracted smooth muscle has protuberances which are electron-lucent and which, compared to the medial smooth muscle layer, contained pale flocculent material. Normal contracted smooth muscle has protuberances which have similar density and appearance to that of the parent smooth muscle in the medial layer. Therefore distinctions can be made between the appearance of normal and pyrrolizidine-contracted vascular smooth muscle.

Meyrich and Reid /79/ studied the time course of the pulmonary vasoconstriction caused by pyrrolizidines. Male rats weighing 230-276 g were fed diets containing 0.19% ground *Crotalaria spectabilis* seeds. At day 3 of the experiment, muscle appeared in normally non-muscularized arteries. By day 7, medial layers of muscular arteries were hypertrophied and by day 21, the number of small arteries was reduced which caused an increase in the alveolar relative to arterial volume. It has been suggested that the pulmonary vasoconstriction observed with pyrrolizidines is due to hypoxia /77, 78/. Sustained hypoxia (below 10% O_2) will cause vasoconstriction leading to medial hypertrophy of the muscular and non-muscular veins and arteries. This hypertrophy produces lesions similar to pyrrolizidine induced lesions. When Meyrich and Reid /79/ compared lesions from hypoxia-induced pulmonary hypertension to pyrrolizidine-induced hypertension they demonstrated that the

hypoxic lesions appeared at a different time. Later, through more direct measurements using indwelling catheters /80/ they showed that blood gases were unchanged in pyrrolizidine treated animals thereby providing further evidence that hypoxia was not the primary mechanism of pyrrolizidine induced vasoconstriction.

Another lesion commonly described is capillary thrombi. Whether this is a cause or a result of pulmonary hypertension is uncertain, but this lesion is reported in studies using *Crotalaria spectabilis* seeds /78/, fulvine /75, 81/, and monocrotaline /82/.

Although pyrrolizidines are well demonstrated hepatocarcinogens we are aware of only one report which demonstrates pneumocarcinogenic potential. Chronic treatment of rats with lasiocarpine (0.005% in diet) resulted in angiosarcomas in 35% of the animals and of these 57% developed lung tumors of an undescribed nature /83/.

PYRROLES

The above discussion reviews histological sequelae to pyrrolizidine alkaloid exposure. Pyrrole derivatives produce similar changes.

Mattocks /74/ first proposed that the toxicity of pyrrolizidines is due to the pyrrole or dehydro metabolites of these alkaloids (Figure 6). The histology of lungs from animals treated with pyrroles is similar to that of alkaloid treated animals, supporting Mattock's proposal that the pyrrole metabolites are responsible for the lesions that follow exposure to pyrrolizidines. As with pyrrolizidine alkaloids, pyrroles cause edema and hemorrhaging, hypertension, vascular smooth muscle hypertrophy and hyperplasia, endothelial proliferation and eventually right heart hypertrophy and failure.

In 1970 Butler and coworkers /84, 85/ described pleural effusion in rats administered 5-10 mg/kg dehydromonocrotaline. Work by Plestina and Stoner /86/ verified this observation using female rats. In rats weighing 65-80 g given 30 mg/kg dehydromonocrotaline respiratory difficulty appeared after 15 hr. The relative wet lung weights of the animals increased significantly after 6 hr but fell back to normal weights within 9 hr. Pleural effusion increased at a time when the relative lung weights were decreasing. The protein concentration of these effusions was similar to that of lymph fluid. The hematocrit increased significantly after 12 hr. Albumin labeled with ^{125}I was used as a marker of pulmonary vascular leakage. Eighteen hours after injection of dehydromonocrotaline, 50% more albumin remained in the lungs, indicating

vascular leakage. (^{125}I) albumin was also found in pleural fluid, implying a vascular source for this fluid. Using colloidal carbon labelling and histological scoring, Plestina and Stoner /86/ were unable to locate the precise site of vascular lesion since the entire pulmonary capillary bed stained uniformly with carbon 9 hr after administration of the pyrrole. From this work and with data from other reports /84, 87-89/ the authors postulated the following series of events: there was interstitial edema and an increase in residual blood content of the lung within 3 hr. When this edema could no longer be drained away by the lymphatic system an effusion appeared in the pleural cavity. Further changes, intermediate and late, were a result of the edema and vascular leakage. Using dehydromonocrotaline Lalich et al. /82/ showed arteriolar and capillary fibrin thrombi and alveolar edema prior to right heart hypertrophy in rats treated with monocrotaline pyrrole (2 mg/kg i.v.). They concluded the pyrrole injures the endothelium which then releases factors causing fibrin thrombi. These authors suggest thrombi are a frequent occurrence and that this may be the mechanism of pulmonary hypertension.

The use of pyrroles have also led to speculation that a third mechanism of pulmonary hypertension may produce the lesions observed in pyrrolizidine toxicity. Raczniak et al. /90/ reported that interstitial and alveolar fibrosis corresponded to changes in pulmonary pressures. In these experiments, beagles were injected with dehydromonocrotaline (3 mg/kg) in the pulmonary artery. Pulmonary pressures and blood gases were monitored with indwelling catheters. By day 14 histological techniques revealed intraalveolar fibroblast proliferation: By day 21 post-injection, there was elevated vascular resistance, and severely proliferated connective tissue. Direct measurement of blood gases revealed no changes in pO_2 even at 28 days post-injection when the histological changes were most severe. The authors concluded that the pulmonary hypertension observed was due not to hypoxia but to changes in pulmonary structure caused by intraalveolar fibrosis which increased vascular resistance. This paper makes no mention of the medial hypertrophy observed by others in the rat. It is possible that there is more than one response to the pyrrolizidine insult and that in the beagle the fibrotic response is predominant.

PYRROLIZIDINES AND LUNG PHARMACOLOGY

Since the lung is the primary site of modulation for many vasoactive compounds there is a possibility that pyrrolizidines exert their effects –

most importantly vasoconstriction – by altering normal lung pharmacology.

Gillis, Huxtable and Roth /70/ showed that monocrotaline changes the pulmonary removal of 5-HT and norepinephrine. Isolated perfused lungs from rats treated with monocrotaline (22 mg/l) in their drinking water showed a 50% decrease in ability to remove 5-HT. This change was due to impaired delivery as monoamine oxidase activity in lung homogenates was unchanged. At slightly lower levels of exposure, Huxtable et al. /71/ found a specific inhibition of serotonin transport by the endothelium, with no effect on norepinephrine removal.

A similar result was seen on perfusing the pyrrole, dehydroretronecine (Figure 7), through isolated lungs of rats not previously exposed to pyrrolizidines /71/. It was further shown both *in vivo* and *in vitro* that covalent binding of radioactivity to lung tissues occurred after exposure to ^{3}H-dehydroretronecine. A selectivity in binding was observed, as norepinephrine uptake was unimpaired, and the activities of endothelial surface enzymes 5′-nucleotidase and angiotensin converting enzyme were unaltered. In isolated, perfused lungs, dehydroretronecine caused a 16% decrease in 5′-nucleotidase activity and angiotensin converting activity. Similar events occurred when dehydroretronecine was used to pretreat the animal. Twenty four hours after dehydroretronecine injection (100 mg/kg) serotonin metabolism in the isolated perfused lung increased by 80%. It is surprising that chronic treatment with the parent alkaloid causes one effect while a pyrrole produces quite different results. Histological changes produced by the alkaloid and by the pyrrole were similar. It is possible that the pyrrole caused increased endothelial permeability to serotonin permitting enhanced intracellular delivery of serotonin for oxidation by monoamine oxidase. That this was not observed in longer feeding studies may simply reflect a different stage in the course of toxicity. By 21 days of exposure to monocrotaline, cell repair may have occurred to prevent leakage while the active transport system for serotonin has been inhibited by the toxin.

Structure and Activity in Pyrrolizidine Toxicity

The structural simplicity of the nitrogen-containing ring system in pyrrolizidine alkaloids is belied by the difficulties of synthesizing it. Formal syntheses in low yield have been performed to verify structure, and there is much recent interest in pyrrolizidine syntheses (for review

see 91, 92). Practical routes for the synthesis of substituted pyrrolizidines in quantities sufficient for biological studies may be achieved in the near future. However, to date, synthesized pyrrolizidines have not been available for study. Instead, biological research has concentrated on three groups of compounds: plant alkaloids, semisynthetic derivatives of naturally occurring alkaloids, and studies on chemically synthesized monocyclic pyrrole and pyrroline derivatives.

The field of endeavors with synthetic analogs is dominated by the studies of Mattocks. One of his major contributions derived from his hypothesis that pyrrolizidines were toxic due to hepatic conversion to pyrroles, which then serve as alkylating agents /74/. He isolated from the pyrrolizine nucleus the structural elements required for alkylating ability, and deduced that relatively simple pyrroles, and the corresponding pyrrolines that on oxidation would yield pyrroles, should exhibit the same toxicity as is found in the natural alkaloids (Figure 8).

Studies with monocyclic synthetic analogs such as those shown in Figure 9 have enabled the absolute structural requirements for toxicity to be verified, and have given a degree of insight into how modification of structure modifies toxicity. Perhaps most importantly, these studies have given some indication as to the different structural elements responsible for the expression of the different toxic effects of pyrrolizidines. That is to say, the various toxicities of pyrrolizidines, such as megalocytosis, pulmonary edema, and arthritis do not inevitably co-occur but each has its own structure activity relationships.

Semisynthetic Pyrrolizidine Derivatives

Ester hydrolysis of naturally occurring alkaloids is readily achieved. Necine bases, such as retronecine (Figure 7), have thus been useful intermediates from which to synthesize other esters and carbamates /93-100/. Studies with semisynthetic esters have shown that structural requirements for toxicity include, in addition to a 1:2-double bond, esterification at C9 /98/. The degree of toxicity is strongly dependent on the nature of the esterifying acid, and toxicity increases with degree of steric hindrance at the carbon atom /98/. This is presumably because the more hindered this position, the lower the stability of the ester relative to its hydrolytic products.

Toxicity is enhanced by esterification at C7. Cyclic diesters are more toxic than the acyclic esters, and Senecio-type alkaloids are more hepa-

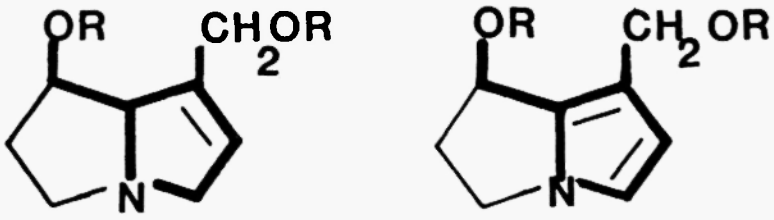
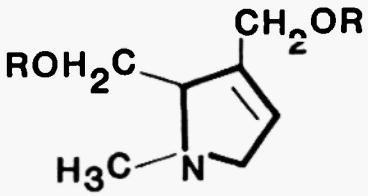


Fig. 8. Structural relationships between naturally occurring bicyclic pyrrolizidines (left) and synthetic monocyclic pyrrolines (superimposed in heavy lines), and between pyrroles formed from pyrrolizines (right) and from pyrrolines (superimposed in heavy lines).



I R=H synthanecine A

II R=C_2H_5NHCO· synthanecine A bis-N-ethyl carbamate

II R=CH_3 synthanecine A dimethyl ester

Fig. 9. Synthetic pyrrolines: synthanecine A and its esters.

totoxic than the Crotalaria-type having a macrocyclic diester ring one carbon smaller.

Another factor regulating the toxicity of diesters is the presence of branching in the ester moiety. Di-N-butyryl and di-N-valeryl retronecine were not toxic even at doses as high as 1.3 g/kg and 0.7 g/kg respectively. However, the branched esters ditiglyl and disenecionoyl retronecine were toxic at doses of 250-500 mg/kg /94/.

Another indication that toxicity is inversely related to ease of hydrolysis of the ester grouping is the much higher toxicity of the carbamate analogs /101/. Di-N-ethylcarbamyl retronecine (Figure 7) is as toxic as monocrotaline /99, 101/, producing both lung and liver damage.

One advantage of the semisynthetic alkaloids is that they may be radiolabelled with tritium. Retronecine can be tritiated relatively readily before esterification on the 9 position, whereas radiolabelled alkaloids are unavailable. The distribution of (^{3}H) di-N-ethylcarbamyl retronecine has been studied, and this agent has been found to concentrate in liver, lung, kidney, spleen and blood /99/.

Dehydroretronecine

Dehydroretronecine is a secondary metabolite of monocrotaline. That is to say, monocrotaline is metabolized to the pyrrole, dehydromonocrotaline, which in turn is metabolized to dehydroretronecine. It is readily prepared from monocrotaline and may be readily radiolabeled. Its properties have therefore received a degree of study even though owing to its relative inactivity it is probably not the metabolite responsible for the toxicity of monocrotaline. Despite this, on intravenous injection dehydroretronecine reproduces all the toxic effects of monocrotaline: right ventricular hypertrophy, pulmonary hyperplasia and pulmonary arterial hypertension /71/. It also has the same actions on pulmonary endothelial functions. Furthermore, as discussed above, on perfusion through rat lung, it is bound covalently, and inhibits serotonin removal: i.e. perfusion of this substance produces the same changes in 10 minutes as are seen after 3 weeks treatment *in vivo* with monocrotaline /71/. The distribution of ^{3}H-dehydroretronecine in rats has been studied by two groups /71, 102/ and radioactivity found to concentrate in the liver and stomach. The high levels found in the stomach may be a reflection of the acid lability of pyrroles. Radioactivity is concentrated in the mucosal layer. Dehydroretronecine produces gastric hemorrhaging and ulceration.

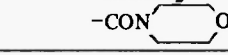
In rats, the major route of elimination is in the urine, 75% of a 65 mg/kg dose being excreted within 24 hr by this route /102/. In rhesus monkeys, however, biliary excretion is also a major route /103/. Rhesus monkeys showed high levels of radioactivity along the endothelium of pulmonary capillaries, consistent with the proposal that pulmonary endothelium is a major site of interaction of pyrrolizidine metabolites /71, 104/.

3-Pyrrolines

The first report on duplication of pyrrolizidine toxicity with a synthetic compound appeared in 1971 /101/. Other reports have followed /99, 105-109/. A series of diesters of 2,3-dihydroxymethyl-1-methyl-3-pyrroline were tested in rats /101/. Ethyl carbamate, methyl, and isovaleryl esters (Figure 9) were employed by analogy with a similar series of semisynthetic retronecine derivatives that had been previously tested. The methyl and isovaleryl esters were not toxic, and were not converted to pyrroles to any significant degree by rats. On the other hand, the carbamate ester proved to be even more toxic than monocrotaline, doses of 60 mg/kg killing most rats within 2-6 days. Survivors had megalohepatocytes and other toxic changes associated with pyrrolizidine poisoning. Within two hours of intraperitoneal administration, large quantities of pyrroles were detectable in the liver, lungs, and in the urine. This difference in toxicity between pure esters and the carbamates is also observed with the semisynthetic esters derived from retronecine, and is probably due to the speed with which esters are hydrolyzed. Carbamates, however, are resistant to the action of serum esterases. That the instability of the esters is responsible for the lack of toxicity, rather than the absence of the requisite structural features, is shown by the extreme toxicity of the pyrrole chemically synthesized from the dimethyl ester /101/.

The 7 day LD_{50} of the carbamate ester in rats is 44 mg/kg, which compares to, say, monocrotaline at 95 mg/kg. Unlike some pyrrolizidine alkaloids, this ester, and other carbamates, does not produce cancers in rats. Comparison of a small series of carbamate esters is shown in Table 4 /105/. For a compound to be toxic, there must be a 3:4 double bond, and the compound must be capable of dialkylation. Toxicity, as evidenced by the LD_{50} or the presence of megalocytes in the liver, directly correlates with the levels of pyrrole produced *in vivo* by the compound, a further indication that these compounds are toxic because of

TABLE 4. *Toxicity of pyrroline diesters and diamides.*

Compound	R	Liver pyrrole[a]	LD_{50} (mg/kg)	Megalo-hepatocytes[b]
I	-COCMe(Et)OAc;Ac[c]	0.2	164	+
II	$-PO(OEt)_2$	0.8	Not V. Toxic	+
III	-CONHMe	0.7	65	+
IV	-CONHEt	1.3	44	+
V	$-CONMe_2$	0.9	73	+
VI	$-CONEt_2$	0.6	75	+
VII	$-CONBu_2$	0.1	Not V. Toxic	0
VIII		0.7	91	+

(Data from Ref. 105) Substituents refer to Fig. 9.

[a]Pyrroles formed in liver 24 hr after 100 mg/kg of pyrroline.

[b]Within 3 weeks of dosing.

[c]Esterifying groups at C2 and C3 hydroxymethyl groups, respectively.

the formation of metabolic pyrroles from them. The substituents in compound I (Table 4) were chosen so as to be analogous to the naturally occurring alkaloids. Compound II (Table 4) is interesting in that although large amounts of pyrroles are produced, the compound is not very toxic and thus contradicts the general rule of a correlation between toxicity and pyrrole production. In addition, although it produces hepatomegaly, it does not cause necrosis. It appears that the pyrrole produced from this compound does not alkylate very strongly. Compound VII is only partially metabolized, and this may be because of marked steric hindrance. Compound IV is the most toxic, and pyrroles are also found in the lungs, at approximately one third the level in the liver. In compound VI, lung pyrrole levels are extremely high, being approximately 75% of that found in the liver. Both compound IV and VI produce lung damage in keeping with the occurrence of pyrroles in this organ. Compound VI, in addition, is cytotoxic to a number of other organs including gut, spleen, and bone marrow.

The ability of a pyrroline to be metabolized, and the stability of the pyrrole metabolite formed, are strongly affected by the nature of the ester moiety. Pyrroles formed from these pyrrolizidines are bifunc-

tional alkylating agents and are highly toxic and chemically unstable, rapidly hydrolyzing to pyrrole diols.

In rats, the distribution of radio-labeled synthanecine A bis-N-ethylcarbamate (II, Figure 9), has been followed for up to 340 days /109/. Within a few hours of intravenous dosing at a level of 40 mg/kg, two thirds of the radioactivity in the liver is already in the form of pyrroles, and a high proportion of this is not extractable, indicating some type of binding has already occurred. High levels of activity are also found in erythrocytes, but only low levels occur in the stomach. When the bis-hydroxy compound, synthanecine A, is similarly administered, extremely high levels are found in the stomach. This indicates that synthanecine A is not a major metabolite of synthanecine A bis-N-ethylcarbamate. Other organs accumulating radioactivity were lungs, kidney and spleen. The lungs and spleen lose activity very slowly over a period of months. Activity also remains bound to erythrocytes for the life of the cell.

A comparison of the bis-N-ethylcarbamates of synthanecine A and retronecine (II, Figure 7) is interesting. The retronecine ester is less toxic, and is probably not so well metabolized. However, it produces greater lung damage than does the synthanecine ester. It is possible that the less toxic alkaloids may produce greater lung damage than the more toxic ones because of the greater stability of pyrrole metabolites formed from them. These are able to survive the passage from the liver to the lung more effectively.

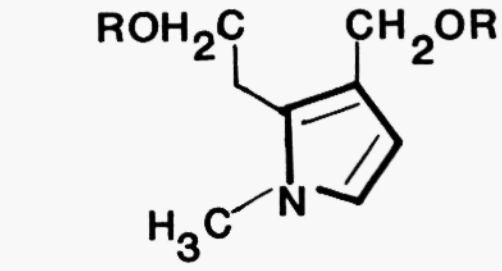
Esters of synthanecine D and synthanecine E have been synthesized. The interesting aspect of these pyrrolizidine analogs is that they may be metabolized to pyrroles which are monofunctional and not difunctional alkylating agents (Figure 10) /107/. *In vivo*, these agents cause acute cytotoxic effects such as tissue necrosis, but do not produce delayed antimitotic effects in liver, or progressive lung damage. It would appear, therefore, that bidentate alkylation is an integral step in the sequence of events leading to pulmonary hypertension and right ventricular hypertrophy.

Monocyclic Pyrroles

The monocyclic pyrroles are more amenable to synthesis than the alkaloid pyrroles /101, 105-107, 109/. A further demonstration that synthetic pyrroline derivatives have the same mechanism of toxicity as do pyrrolizidine alkaloids is that 1-methyl-2, 3-diacetoxy methyl pyrrole

Fig. 10. Synthanecine A can form carbonium ions at two centers that are stabilized by delocalization of charge. It is therefore, a bidentate alkylating agent. Synthanecine D can form only one stabilized carbonium ion and is therefore, a monodentate alkylating agent.

(1, Figure 11) produces the same toxic effects as pyrrole esters such as dehydromonocrotaline prepared from alkaloids, or the semisynthetic esters such as diacetylretronecine pyrrole. The diacetoxy methyl pyrrole, given intravenous at 5-12 mg/kg, causes death to rats within 20-35 days. The lung changes produced are the same as for dehydromonocrotaline. Studies with these two compounds showed that reactive pyrrole metabolites cause the same cytotoxic effects that pyrrolizidine alkaloids do, and that such effects can be caused by compounds with only one five membered ring /101/.



I R= $CH_3CO\cdot$

II R= $C_2H_5NHCO\cdot$

III R= H

Fig. 11. Synthetic pyrrole esters.

The metabolism of pyrrolizines and pyrrolines is complex, and usually both primary and secondary pyrroles are formed. Thus, from synthanecine A bis-ethylcarbamate is formed a primary pyrrole (II, Figure 11), which has been chemically synthesized and found to be a highly reactive alkylating agent /106/, and also the secondary pyrrole 2,3-dihydroxymethyl-1-methyl pyrrole (III, Figure 7). This has been found in the urine of rats given the synthetic carbamate. Both classes of pyrroles (primary and secondary) have toxic properties.

As is the case with the bicyclic pyrroles, the alcohol is less reactive than the esters (which decompose rapidly in water). The alcohol accumulates in the liver follow oral administration or injection, but very little is bound to the lungs. Furthermore, no specific lung toxicity is seen. Esters of this pyrrole, however, are highly pneumotoxic. The distributions of radioactivity following administration of the pyrrole alcohol and synthanecine A esters or carbamates differ considerably. It may be safely concluded, therefore, that although compound III is a metabolite of synthanecine A esters, it is not primarily responsible for the toxicity of these esters. The esters and pyrrole alcohol stand in the same metabolic relationship as monocrotaline and dehydroretronecine.

In summary, pyrrole esters are bifunctional alkylating agents, that are highly toxic. They are rapidly hydrolyzed to pyrrole diols, which are mild alkylating agents. At high doses, pyrrole diols are cytotoxic. When given at concentrations at which pyrrolizidine alkaloids and synthetic esters are hepatotoxic, pyrrole diols have no apparent toxicity. Therefore, they are not the metabolites responsible for pyrrolizidine poisoning.

Some general conclusions have been reached regarding pyrrole alkylating ability based on reactivity towards 4-p-nitrobenzyl pyridine (i.e. reaction to form a C-N bond by analogy with C-S bond formation) /107/. This confirmed the greater electrophilic reactivity of esters and carbamates relative to the corresponding diols, and revealed that the reactivity of the 2-position is greater than that of the 3 position. Other substituents on the pyrrole ring enhanced reactivity, whereas increasing size of the ester group decreased reactivity.

CONCLUSION

The toxicology of pyrrolizidine alkaloids is a subject of enormous and ubiquitous economic and public health interest because of the wide distribution of plants containing these substances and the damage they inflict on man and beast (for reviews see 104, 110, 111). Their mechanism of toxicity makes these substances of interest to the biomedical scientist because they permit detailed study of the molecular toxicology of the lung, and provide a ready tool for studies of pulmonary vascular disease and right heart hypertrophy. Different disciplines ranging from synthetic chemistry to veterinary science have come together in the study of this unique class of bidentate alkylating agents. The availability of a range of synthetic and natural analogs will be a spur to research in an area where many fascinating problems remain unresolved.

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