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THE TOXIC ACTIONS OF PYRROLIZIDINE (SENECIO) ALKALOIDS

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I. INTRODUCTION

The pyrrolizidine alkaloids are esters of the amino-alcohols derived from the hetercyclic pyrrolizidine nucleus (see structures I–IV, section III A). There appears to be no evolutionary logic in their distribution among the botanical families. They occur in a number of unrelated families, from the grasses up, and are found in widely separated parts of the world. Their function in the plant is not known, though, having a bitter taste, they may serve as a protection from grazing animals. Many of the alkaloids are not toxic. The chemical criteria conferring toxicity have been well worked out (section III).

The common names of some of the plant species containing toxic pyrrolizidine alkaloids are listed in table 1. A series of tables listing the molecular structures of the alkaloids, plant sources of each alkaloid, and alkaloid content of the relevant plant species has been published by Bull *et al.* (30).

The toxic alkaloids are of importance as hazards to farm animals and to man. Among grazing animals, those which have access to plentiful pasture seem to learn not to eat the toxic plants. One can see ragwort (*Senecio jacoboea*) in many English meadows, but ragwort poisoning is not recognized as a major veterinary problem in England. Where pasture is scanty, however, the plants are eaten and the consequent loss of stock presents a serious economic problem in Australia. When contaminated hay or grain is fed, the animals cannot protect themselves by selecting what they eat.

Poisoning in man has occurred by contamination of cereals and by the use of poisonous plants in traditional medicines. In the West Indies it has been an important cause of cirrhosis of the liver in man.

The alkaloids are of theoretical interest because of the long-lasting effect of single doses, possibly related to cell division, in some tissues. This effect led to the study of their action as antitumour substances. It may also be the basis of their disputed action as carcinogens. There is, therefore, justification for the study of the basic mechanism responsible for this effect.

Opportunities for understanding the modes of action of the toxic alkaloids have recently opened out in two directions. Firstly Mattocks (143) has developed

TABLE 1

Common names of some plants containing pyrrolizidine alkaloids

Botanical Name	Common Names	Location of This Name	Ref.*
Amsinckia intermedia	Fiddleneck	U.S.A.	127
	Tarweed	U.S.A.	127
	Fireweed	U.S.A.	127
	Yellow forget-me-not	Australia	87
Crotalaria	Rattle box	U.S.A.	127
	Rattle pod	Australia	87
Crotalaria dura	Wild lucerne	South Africa	228
Crotalaria globifera	Jaagsiektebossie	South Africa	228
Crotalaria fulva	Whiteback	Jamaica	
Crotalaria mucronata	Streaked rattle pod	Australia	87
Crotalaria retusa	Wedge-leaved rattlepod	Australia	
	Earring plant	South Africa	228
Echium plantagineum	Viper's bugloss	California	127
	Salvation Jane	Australia	87
Heliotropium europaeum	Common heliotrope	Australia	87
	Caterpillar weed	Australia	87
	Potato weed	S.E. U.S.A.	127
Senecio jacoboea	Common ragwort	U.K.; New Zealand	87
	Stinking Willie	U.S.A.; Canada	127
Senecio latifolius	Dan's cabbage	South Africa	228
	Groundsel	South Africa	228
	Ragwort	South Africa	228
	Rhodesia ragwort	South Africa	228
Senecio retrorsus	Dan's cabbage	South Africa	228
	Ragwort	South Africa	228
Senecio ridelli	Woolly groundsel	U.S.A.	127
	Thread-leaf groundsel	U.S.A.	127
	Riddell's groundsel	U.S.A.	127
Senecio spartioides	Broom groundsel	U.S.A.	127
Senecio vulgaris	Common groundsel	U.K.; California	127

* (See also ref. 18a, 182a.)

a colourimetric assay for the alkaloids and their metabolites which has made their determination in blood, urine, and tissues relatively easy. He has also demonstrated and tested a toxic metabolite of the alkaloids. Study of the toxic effects of this and related metabolites opens up a new field of investigation. Secondly, studies of the cell cycle (109), which began in bacteria but are now applicable to mammalian tissues (18), have encouraged the development of techniques that could be used to look at the effect of the alkaloids upon cell division. It has long been known that liver cells suffering the long term effects of pyrrolizidine alkaloid poisoning accumulate an excess of nuclear chromatin. More recently (77) it has been shown that these cells fail to divide. The divorce of deoxyribonucleic acid (DNA) synthesis from cell division is an effect well worth study. The present wave of interest in nucleic acid metabolism is now providing us with the techniques necessary to dissect this phenomenon.

The reader should note two points about the design of this article.

1) An important monograph on the pyrrolizidine alkaloids has recently been published by Bull, Culvenor, and Dick (30). It will be assumed that everyone interested in these alkaloids has read this book. The present article will not attempt to cover the same ground. The veterinary problem and the clinical pathology of chronic poisoning will be dealt with very briefly, whereas the study of lung and vascular lesions, and more recent work relating to the production of a toxic metabolite and to effects on nucleic acid metabolism will be considered in greater detail.

2) In writing about the alkaloids one is faced with the dilemma whether to speak of them collectively (which would involve serious inaccuracy) or whether to define them one by one (which would make the article unreadable). Whenever possible, the author has chosen to speak of them collectively. Readers are therefore warned that, for the planning of experiments, differences between individual alkaloids are important, and reference must be made to original papers when selecting a particular point for further study.

II. TOXICITY IN THE WHOLE ANIMAL

A. Veterinary studies

The veterinary problem has recently been reviewed by Sippel (204) and by Bull *et al.* (30). Different species present different syndromes.

Cattle were among the earliest to be investigated (70, 169) when the farmers of Nova Scotia noted that their cows fell ill after eating the imported weed Stinking Willie (*S. jacoboea*). Their observations survived the denial of the government inspector, who was convinced the illness was an infection, and were vindicated by the experiments of Pethick (169), who reproduced the symptoms by feeding Senecio. Other experiments with Senecio on cattle were reported by Cushny (70), Markson (141) and Thorpe and Ford (220), with Heliotropium by Bull *et al.* (40) and Kinnaird *et al.* (128), with Amsinckia by Fowler (83), and with Crotalaria by Bras *et al.* (23). Cattle succumb rapidly, within about a month; the common signs are violent diarrhoea with tenesmus, wasting, a straddled gait, inability to stand, and death. Cattle fed Crotalaria develop ascites and veno-occlusive disease.

Sheep are resistant to Senecio (30). Field and experimental studies with Heliotropium and its alkaloids have been carried out by Bull *et al.* (38) and Jago *et al.* (114). Sheep are affected only slowly by heliotrope and most survive one season's grazing. During the second season there are three patterns of illness, all different from that in cattle. Some die within a few days of transfer to lush pastures. From evidence collected among horses and rats this death is thought to be due to a high level of blood ammonia arising from the inability of the damaged liver to deal with a high protein intake (38). A second group die less rapidly with haemoglobinuria and very high liver copper levels. The mechanism underlying this syndrome will be discussed in section V C 1. A third group die much later, at least 9 months after the second grazing of Heliotropium (which is a seasonal plant), of gradual liver failure. These animals have small livers containing very large parenchymal

cells. Sheep fed Crotalaria exhibit yet another syndrome. They die within 3 weeks with fluid in the thoracic cavity (134).

The signs in horses are predominantly neurological, though stomach and oesophageal lesions are also reported (200). Rose *et al.* (180, 181) and Gardiner *et al.* (86) investigated "walk-about" disease of horses and found it distributed over the same areas as *Crotalaria retusa*. The symptoms are compulsive walking in a straight line and butting the head against any obstruction encountered. Feeding with *C. retusa* reproduced the illness. The onset of neurological symptoms coincided with a steep rise of blood ammonia. Severe pathological changes in the liver were recorded at death. Two species of Trichodesma, *T. incanum* and *T. zeylanicum*, are known to contain pyrrolizidine alkaloids (30). Trichodesma poisoning of horses in Uzbekistan is referred to as Dzhalangarsk encephalitis (203). Senecio poisoning of horses in Czechoslovakia is associated with liver lesions (224).

Chickens and turkeys poisoned with *Crotalaria spectabilis* seed died with lesions in liver, lung, and muscle (2, 8, 9). Administration of Senecio alkaloids to chickens can produce carcinomas (47). This is the only instance (so far as I am aware) of carcinogenesis due to these alkaloids outside the species commonly used in laboratory investigation.

Senecio (94) and Crotalaria (79a) poisoning in pigs produces liver, lung, and kidney lesions. Heliotrope poisoning in pigs (41) produces liver lesions and gastric ulceration. In dogs, heliotrope alkaloids give rise to liver failure in which ascites is the most prominent feature (125).

In summary, one cannot say that the veterinary syndromes fall into a recognisable pattern. Acute liver damage is seen in poultry. Chronic liver damage is seen in sheep, horses, pigs, and dogs. Lung damage is seen in sheep, poultry, and pigs. In addition horses suffer neurological disturbances which may be secondary to chronic liver damage but a primary neurotoxic action of the alkaloids has not been ruled out. Sheep exhibit a haemolytic syndrome, and cattle suffer an unexplained but fatal gastrointestinal disorder.

The differences may depend, not only upon the species of animal affected but also on the plant consumed. The alkaloids of Crotalaria are noteably more likely to produce lung lesions, and less likely to produce tumours in laboratory animals (191). In field experiments there is little control over the dose administered.

B. Laboratory studies

In the laboratory, by contrast, dosing can be rigidly controlled. The pattern of toxicity in laboratory animals varies with the dose employed.

1. Acute deaths

The LD50 values in rats for the more common toxic alkaloids are listed in table 2. It is evident that toxicity varies considerably between the alkaloids. The figures are calculated from animals dying in the first 3 days after a single dose.

These animals die with severe haemorrhagic liver necrosis. All the common lab-

TABLE 2

LD50 in male rats, at 3 days after dose, for some toxic pyrrolizidine alkaloids

Alkaloid	LD50 mg/kg Body Weight	Empirical Formula	Molecular Weight	Ref.
Fulvine	40	$C_{16}H_{23}O_5N$	309	190*
Heliotrine	300	$C_{16}H_{27}O_5N$	313	32*
Lasiocarpine	72	$C_{21}H_{33}O_7N$	411	32
Monocrotaline	175	$C_{16}H_{23}O_6N$	325	32
Retrorsine	35	$C_{18}H_{25}O_6N$	351	190
Senecionine	85	$C_{18}H_{25}O_5N$	335	32
Seneciphylline	77	$C_{18}H_{23}O_5N$	333	32

* Alkaloids administered intraperitoneally in ref. 32. Route of administration not stated in ref. 190.

oratory species, except the guinea pig, are susceptible. In some species, survivors of doses of this magnitude develop vascular lesions in the liver (72, 156).

2. Hyperacute deaths

Hyperacute deaths are those which occur much more rapidly than the acute deaths. They are frequently associated with convulsions. They precede, and are therefore not dependent on, liver necrosis and they follow doses larger or much larger than the LD50. They are usually associated with intravenous administration. Chen *et al.* (51) reported such deaths in mice, rats, guinea pigs, frogs, and monkeys after large intravenous doses (85 to 200 mg/kg) of seneciphylline and platyphilline. Cushny reported (70) convulsions in cats but not rats after large doses (100 to 200 mg/kg) of senecifoline and senecifolidine (retronecine). Schoental and Mattocks (199) saw them in rats receiving semisynthetic diesters of retronecine. Some of these had unbranched ester side chains and were not hepatotoxic. Since this effect is produced by platyphilline, which has no 1—2 double bond in the pyrrolizidine ring, and since it appears in guinea pigs, which do not suffer liver necrosis from these alkaloids, it seems likely that the type of hyperacute death associated with convulsions is not related to "acute" death or to megalocytosis (See section II B 3).

Another type of hyperacute death was described by Bull *et al.* (39). Among female rats receiving large doses (400 to 600 mg/kg = 1.25 to 2.0 acute LD50's) all the deaths (26/50) occurred in the first hour. Only slight pulmonary congestion was found in these animals. Butler *et al.* (46) recorded death of all young male rats after monocrotaline pyrrole or retrorsine pyrrole in doses of 10 to 20 mg/kg. These animals died in the first 24 hr with severe, clear pleural effusions. In both these cases, death could be due to a rapidly developing capillary defect in the lung, or to neuromuscular block at the phrenic nerve-diaphragm junction, as described by Gallagher and Koch (85) (see section V B 2).

3. Chronic lesions—liver

Repeated administration of very small doses (0.1 LD50 3 times weekly) of alkaloid to rats produces a chronic liver damage characterised by the appearance of

grossly enlarged liver cells. This syndrome has been termed megalocytosis (37). A similar picture may be seen in rats surviving for 2 years after a single, nearly lethal, dose of alkaloid (196). The lesion may lead to fatal liver failure or may be compensated by hypertrophy of normal liver tissue. It is histologically very similar to one of the syndromes following heliotrope poisoning in sheep.

Liver tumours have arisen in rats and chickens after single or infrequently repeated doses of the alkaloids. These findings are disputed and will be discussed in section V A 2 d.

4. Chronic lesions—lung

Rats fed *C. spectabilis* seed 0.1 % w/w in the diet develop a severe pulmonary lesion and die with pulmonary oedema and cor pulmonale in the second month of feeding (103, 121, 122, 133). A similar fatal pulmonary lesion follows single doses of monocrotaline or fulvine (15) and monocrotaline and retrorsine pyrrole (46) (see section V B).

5. Summary—the clinical picture in laboratory animals.

Laboratory animals present a fairly simple clinical picture.

1) After a single administration of a suitable dose an acute liver lesion kills the animals within 3 days.

2) Smaller doses give rise to a chronic liver lesion, which is not always fatal but which progresses long after the alkaloid dosing is stopped.

3) Concurrent with the liver lesion, smaller doses also cause a progressive lung lesion which kills animals at about 5 weeks.

4) Very large doses kill the animals immediately. These deaths are associated with convulsions and are probably not related to the liver or lung lesions.

I believe this simple picture to be seriously misleading. Had this account been written 10 years ago it would have included no mention of lung lesions as a major item in the clinical picture. At that time the pathological descriptions in the literature already contained numerous passing references to lesions in the lungs, but the matter had not been taken up and investigated in the laboratory, and the alkaloids were regarded as liver poisons. A survey of the literature indicates that there are other areas in the body where the same story could be repeated, in particular in the brain, the kidney, and haematopoietic tissue. The clinical picture in laboratory animals is a useful simplification, but as a microcosm of the overall toxicity of the alkaloids I think it is seriously unbalanced.

C. The foetus and young animals

It has often been reported (83, 99, 115, 187) that young animals are more susceptible than adults to the toxic actions of pyrrolizidine alkaloids. However, the published evidence (table 3) is ambiguous.

Studies in the foetus. The alkaloids, or their proximate toxins, can cross the placenta in rats (92, 163, 213) and cattle (83). Acute (163, 213) and chronic liver damage is reported in foetuses whose mothers received doses of the alkaloids. Sundareson (213) did the interesting opposite experiment of injecting senecionine, at hysterotomy, into 19-day rat foetuses. Larger doses killed the foetus, smaller

TABLE 3

Toxic effects of pyrrolizidine alkaloids in young animals

Line No.	Alkaloid	Dose	Dose (as Fraction of Adult Acute LD50)	Dosed at Age	Result		Ref.
					Early	Late	
1	Lasiocarpine	100 mg/kg i.g.	1.3 to mother	13th day gestation	Liver necrosis and death of foetus		163
2	Lasiocarpine	75 mg/kg i.g.	1 to mother	14th day gestation	Liver necrosis in foetus		163
3	Lasiocarpine	35 mg/kg i.g. twice	0.5 twice to mother	15th and 17th day gestation	Liver necrosis in foetus	Megalocytosis at 7 weeks in foetal survivors	163
4	Senecionine	1 cc i.v. or 5 cc s.c. of 1% w/v solution twice	0.5 twice to mother	15th–19th day gestation	Liver necrosis in foetus		213
5	Senecionine	0.125 ml of 1% w/v	3 to foetus	19th day gestation	Survived 2 days		213
6	Senecionine	0.125 ml of 1% w/v	3	Newborn (within 1 day)	Died within 24 hr		213
7	Monocrotaline	140 mg/kg	1	Newborn (within 1 day)	9/14 liver necrosis, half died within 3 days (*i.e.*, same as adult)		155
8	Retrorsine	27 mg/kg followed	0.8 followed by	5 days	All survived 9 days		187
	Retrorsine	by 16 mg/kg	0.5	14 days	9/6 died within 10 days		187
9	Heliotrine	250 mg/kg i.p.	0.8	12 days	Mean survival 44 hr		99
10	Heliotrine	250 mg/kg i.p.	0.8	20 days	Mean survival 55 hr		99
11	Heliotrine	250 mg/kg i.p.	0.8	26 days	Mean survival 70 hr		99
12	Heliotrine	250 mg/kg i.p.	0.8	30 days	Mean survival 89 hr		99
13	Heliotrine	250 mg/kg i.p.	0.8	100 days	Mean survival 85 hr		99
14	Retrorsine	33 mg/kg oral	0.8	35–50 g body wt. (21–28 days)	All died within 30 days		187
15	Retrorsine	23–30 mg/kg oral	0.6–0.75	40–65 g body wt. (24–30 days)	3/6 died within 30 days		187
16	Retrorsine	36–48 mg/kg oral	0.9–1.2	230–260 g body wt. (adult)	All survived 30 days	1/6 died before 6 months	187
17	Monocrotaline	30 mg/kg	0.2	21 days	5/4 survived 4 weeks	5/4 survived 9 weeks	102
18	Monocrotaline	60 mg/kg	0.35	21 days	11/12 survived 4 weeks	9/12 survived 9 weeks	102
19	Monocrotaline	30 mg/kg	0.2	28 days	7/25 survived 4 weeks	5/25 survived 9 weeks	102
20	Monocrotaline	60 mg/kg	0.35	28 days	3/24 survived 4 weeks	9/24 survived 9 weeks	102
21	Heliotrine	260 mg/kg	1	30 days	Half died at 3 days (*i.e.*, same as adult)		32
22	Lasiocarpine	7–30 mg/kg	0.1–0.4	14 days		Severe megalocytosis at 6 weeks	115
23	Lasiocarpine	7–30 mg/kg	0.1–0.4	10 weeks		No effect	115

doses (0.125 ml of 1% w/v solution = 3 times LD50) allowed the foetus to survive until birth 2 days later. The maternal livers were damaged. This implies that alkaloid crossed the placenta in the opposite direction. In all these experiments, the administered alkaloid had access to the maternal liver and could have been activated there (see section IV C), the metabolites returning to damage the foetus. There are no studies on isolated foetuses.

Green and Christie (92) report foetal malformations, including dwarfing, deformed ribs, and hypoplasia of the lower jaw, after injection of alkaloids into the mothers between the 9th and 16th days of gestation; 12th-day injections yielded the highest number of abnormalities.

Transmission of the harmful effects of retrorsine through the milk was reported by Schoental (187). When lactating mother rats were given lasiocarpine or retrorsine, the suckling young suffered liver damage including necrosis, vascular lesions, and megalocytosis.

Newborn and young animals. There are conflicting reports of the relative susceptibility of the young, compared with adult, animals. The reported experiments have been set out in table 3. For the purposes of this table the results have been divided into liver necrosis with "early" death and megalocytosis with "late" death, but this division is highly arbitrary. The "early" group includes deaths between 24 hr and 4 weeks after dose. On this basis, if we look at necrosis and early death, it seems that newborn animals (155, 213) (table 3, lines 6 and 7) and 4-week-old animals (32) (line 21) have about the same susceptibility as adults. In the intervening period, the experiments of Harris *et al.* (99) and one series of Schoental's experiments (187) (lines 9 to 16) show that susceptibility decreases with age, but Hayashi and Lalich's (102) (lines 17 to 20) and another series of Schoental's experiments (187) (line 8) show that it increases. There is no satisfactory way of interpreting these results.

Looking at megalocytosis, Jago's experiments (115) show clearly that young animals are more susceptible. This is doubtless the basis for veterinary experience that younger animals are at risk.

Implications of toxic reactions in newborn animals. A clear study of toxic effects in infancy could help our understanding in two directions. It could relate toxicity to the activity of one of the several enzyme systems known to undergo sharp variations in activity in the perinatal period; and it could give us a guide to human risks.

Evidence to be considered in section IV C suggests that pyrrolizidine alkaloids are metabolised to a proximate toxin. The site of this reaction is uncertain, but the liver microsomal hydroxylating system is one possibility. It is almost impossible to compare the results in table 3 usefully with what is known about microsomal hydroxylating enzymes in young animals. A number of species including rat, rabbit (165), and, by implication, man (62) have very low levels of microsomal hydroxylating enzymes in their livers at birth.

Table 3 shows that the newborn rat is apparently susceptible to the necrogenic effects of monocrotaline and senecionine at a time when liver microsomal hydroxylating activity is very low. The activity of these enzymes rises to adult levels

over a time course which is variable in rats (91, 118). In general the enzyme activities rise slowly in the first 3 weeks of life and then jump to adult levels at weaning, between 3 and 4 weeks. But for individual enzymes the achievement of adult levels is uneven (sometimes humped) and one would need to know exactly which enzymes were activating the alkaloids before one could interpret the data in table 3 in these terms. The data themselves are simply conflicting, and further experiments are badly needed. The experiments must take into account the difference between acute death and later deaths from chronic lesions and should employ a recognised method for the determination of LD50. The implications for man are debatable. Liver cell megalocytosis is not reported in man, so that the clearly demonstrated stimulating effect of youth on this lesion may not be applicable. The fact that the poisons are transmitted through the placenta and in the milk is more ominous. Congenital malformations have not yet been connected with pyrrolizidine poisoning. Green and Christie's experiments (92) suggest that a search might be made in this direction.

D. Human intoxication

Reports of poisoning from pyrrolizidine alkaloids in man all fall into the category of acute poisoning. Cirrhosis is certainly one of the late effects of the acute poisoning seen in Jamaica. Chronic pyrrolizidine poisoning, as seen in grazing animals with megalocytosis and gradual liver failure, is not recorded in man. Neither are lung or kidney lesions reported. The possible role of pyrrolizidine poisoning in the genesis of primary carcinoma of the liver in man is debated.

1. Acute intoxication. Veno-occlusive disease

Outbreaks of acute intoxication have been reported by Willmot and Robertson (231), Selzer and Parker (201), Savvina (184), and Braginskii and Bobokhadzaev (21). It is endemic in the West Indies (25). It has occurred sporadically in widely scattered parts of the world (105).

Willmot and Robertson (231) and Selzer and Parker (201) (in South Africa) both saw patients poisoned by Senecio contamination of bread. They commented on the clinical similarity to the Budd-Chiari syndrome. A majority of these patients died. Their reports emphasise centrilobular haemorrhage in the liver. Selzer described "intimal swelling and occlusion of the central and sublobular veins." Selzer also gave the opinion that the low protein diet eaten by these families enhanced the effects of Senecio. Another interpretation can be put on the results. There was a strong correlation, both in Willmot's and in Selzer's patients, between low economic status and severity of the attack but, as Willmot pointed out, the poorer the family the higher the proportion of the diet eaten as bread, and the bigger the dose of Senecio. In both outbreaks it was "poor whites," whose staple is bread, who suffered. Negro families, whose staple was maize (corn), did not succumb.

Heliotrope poisoning is endemic among farm animals in Central Asia where seeds of *Heliotropium lasiocarpium* contaminate the barley (41). From time to time the seeds also get into human food. Twenty-eight patients were described in

a paper by Savvina (184). Braginskii and Bobokhadzaev (22) described an outbreak due to contaminated wheat flour and linseed oil. Sixty-one patients suffered acute intoxication. Accounts of the acute phase are not available in English. A 4-year follow-up on these patients is available (21). Ascites appeared in the majority 10 to 12 months after poisoning. Thirty had hepatosplenomegaly, two had cirrhosis. None are reported as having died. Among the group with hepatosplenomegaly, liver function tests with respect to protein synthesis were abnormal with high globulins and low albumen. Other liver function tests were normal. The authors equate this with "a sluggish chronic hepatitis." All the patients were capable of work. These good results were attributed to energetic treatment with methionine and vitamins B_6 and B_{12}.

In Jamaica in the 1950's, Hill *et al.* (107) described an illness, common among children. The clinical picture was very like Budd-Chiari syndrome (obstruction of the trunk or large branches of the hepatic vein): severe abdominal enlargement of acute onset with hepatosplenomegaly, ascites, and the development of collateral venous channels over the abdomen. Liver biopsy revealed a lesion peculiar to this disease, collagenous occlusion of the small branches of the hepatic venous tree. In the vicinity of these occlusions, dilated sinusoids form by-pass channels in the substance of the parenchyma (fig. 1). Liver biopsy in the acute stage of the disease showed massive centrilobular congestion and centrilobular necrosis. This syndrome was named veno-occlusive disease (26, 25).

Stuart and Bras (210) made a careful follow-up of a group of these patients. Half of them recovered completely from acute attack, 20% died of liver failure in the acute attack, and 30% went on to a subacute form of veno-occlusive disease. Of these, two-thirds made a complete recovery and one-third (10% of the original group) progressed to cirrhosis and died with haematemesis. The overall mortality was thus 30%. The cirrhosis arising in this way is recognisable to the pathologist because it largely spares the portal triads (nonportal cirrhosis). Cirrhosis of this type accounted in 1961 for one-third of the cirrhosis seen at autopsy in Jamaica (24). In this area, pyrrolizidine alkaloids are thought to get into the human diet in "bush tea" (207). Bush tea is drunk regularly by all the poorer sections (*i.e.*, 70%) of the population. It consists of a hot water infusion of leaves picked from the garden of the hut, or from "the bush." "The bush" is what we would call "the wilderness," a thick and intensely prickly growth of scrub covering every untilled acre of the island. The great majority of leaves so picked are harmless. It is doubtful whether pyrrolizidine infusions are drunk as part of the normal diet because they are bitter. However, when a child has a cold (and "cold" covers everything from mild teething trouble to fatal gastroenteritis) he may be given a herbal medicine, and these medicines are also called bush tea. *Crotalaria fulva* (which contains fulvine) is used as a medicine for children. A number of plants containing pyrrolizidine alkaloids were from time to time on sale as material for medicines in Kingston market. The use of pyrrolizidine infusions in medicinal, but not in the daily, bush teas means that alkaloids are taken in single large doses and are frequently taken by patients already ill from some other cause. The danger from

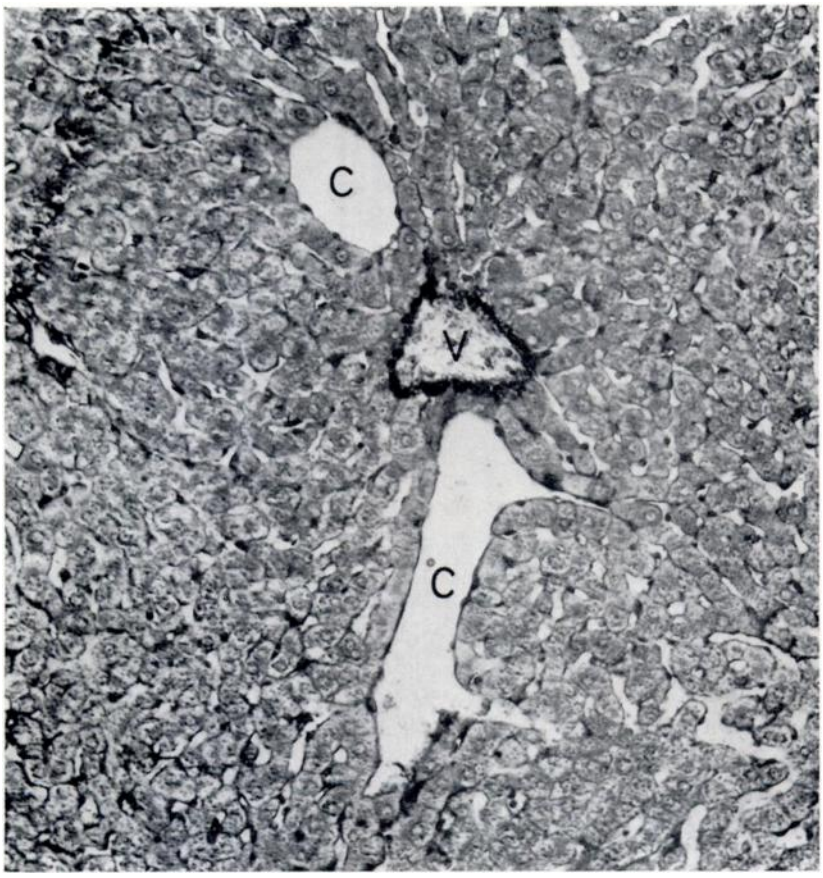


FIG. 1. Subacute veno-occlusive disease in rat liver. V = venous occlusion; C = by-pass channels. At this stage of the disease the centrilobular necrosis and haemorrhage has been repaired and the centrilobular area contains normal liver tissue. (Photograph by courtesy of Professor G. Bras.)

these plants is now recognised by the government and pictorial campaigns have been waged against their use. These have had considerable success and the incidence of new cases is falling.

Veno-occlusive disease of the liver in man has also been described after therapeutic doses of radiation to the abdomen (178). The pathogensis of this lesion has not been investigated.

2. Chronic intoxication, and late effects of single doses

Braginskii's patients and the Jamaican survivors of veno-occlusive disease (proven at biopsy) constitute a group known to have suffered exposure to at least one dose of pyrrolizidine alkaloids. Liver cell megalocytosis has not occurred among them. Veno-occlusive disease is a major source of cirrhosis in Jamaica (24). Primary liver cancer is not more common among them than among other cirrhotic groups. This may be because veno-occlusive disease is largely due to Crota-

laria alkaloids, which are among the least carcinogenic of the pyrrolizidine alkaloids.

Williams *et al.* (229) is recording histories of the use of herbal medicines in cases of hepatocellular carcinoma in infancy and childhood in Western Nigeria, so far without positive correlations. Dunham (78) is investigating the relationship between oral cancer and plants. Where betel and nass are chewed, the incidence of nasopharyngeal cancer is high, and higher in men (who do more chewing) than in women. The quid made up for chewing is a mixture of plants with lime ($Ca(OH)_2$), and nothing is yet known of the possible co-carcinogenic action of the epitehlial damage due to lime. A number of plant extracts, including *Heliotropium ternatum*, are being tested on lime-damaged hamster cheek pouch.

Chronic intoxication in the sense of repeated exposure to small doses is not reported in man, but this could be an artifact attributable to the world distribution of medical services. For a report to reach the literature, at least two criteria must be fulfilled. First, the illness must occur in an area that has a medical service. Since the use of herbal remedies will vary roughly inversely with the availability of the medical services, we probably know least about the populations most at risk. Second, the patient must connect the symptoms with some unusual incident sufficiently strongly to tell the doctor about it. He is not likely to connect the symptoms with something which is utterly routine to him. The link between smoking and lung cancer came out of prospective surveys on a statistical basis. It could never have come out of patient histories. Gaps in our knowledge of human reactions to the alkaloids cannot, therefore, be used as negative evidence. They are simply gaps.

The International Agency for Research in Cancer Working Conference on Liver Cancer (112) has made the sensible suggestion that environmental studies should be made in areas of known *low* liver cancer incidence as well as the numerous studies already under way in areas of high incidence, in an effort to pick up relevant differences.

E. Summary

1) In laboratory animals a fairly simple picture of toxicity of the alkaloids emerges. Large doses cause an acute necrotic lesion in the liver, and vascular lesions in the liver. Smaller doses cause a progressive lesion in the liver, characterised by megalocytosis, and a progressive lesion in the lungs. There is a disputed carcinogenic effect in liver.

2) In veterinary practice the acute and chronic liver lesions and the lung lesions are frequently seen. Carcinogenesis is not reported. Other syndromes are common causes of death, *viz.*, diarrhoea in cattle, neurological signs in horses, and a haemolytic syndrome in sheep.

3) Young animals are much more susceptible to liver cell megalocytosis than adults. The view that young animals are particularly susceptible to other effects of the alkaloids is not supported by the published evidence.

4) All the reported instances of human intoxication fall into the category of acute liver damage and its sequelae. There is no evidence on the often-discussed role of the alkaloids as liver carcinogens in man.

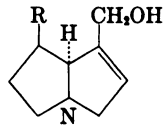
III. CHEMISTRY OF THE ALKALOIDS

The chemistry of the pyrrolizidine alkaloids has recently been reviewed (30, 190, 227). Only those aspects of their chemistry necessary to an understanding of current work on their toxic actions will be dealt with here.

A. Chemical structure

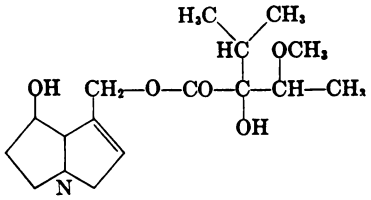
The structure and ring numbering of pyrrolizidine is shown in I. Two five-membered rings share a common nitrogen at position 4. All the toxic alkaloids are derivatives of II (1-hydroxymethyl-1:2 dehydropyrrolizidine) (190). The two rings are inclined towards each other along the C (position 8)-N axis like the wings of a butterfly. Side chains that project from one face, say the upper side of the "wing," are now in a different relation to the rest of the molecule from side chains projecting down from the other face. These two positions are distinguished in the chemical nomenclature by α (which stands for a downward projection when the ring nucleus is viewed as a V) and β.

I

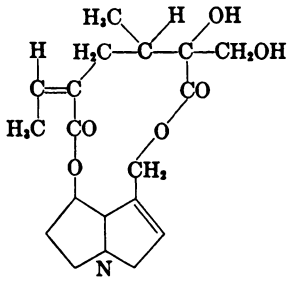


R = H or OH

II



III



IV

The toxic alkaloids are esters of II. Ester linkages are possible at positions 1 and 7. Some alkaloids, *e.g.*, heliotrine (III), are open esters. In others, *e.g.*, retrorsine (IV), the esters join to form a ring. Only esters of branched chain acids confer toxicity (199). The unsaturated pyrrolizidine nucleus itself (II) is not toxic. The nitrogen atom of the nucleus readily undergoes oxidation and N oxides of the alkaloids are commonly found together with the alkaloid in plants. They have the same type of toxicity as the alkaloids.

It is thus well established that there are three essentials for toxicity: 1) that the ring nucleus contain a double bond at 1:2. 2) that the nucleus carry esterified hydroxyl groups; and 3) that at least one of the ester side chains contain a branched carbon chain.

The double bond between positions 1 and 2 is not essential for actions of the

alkaloids unrelated to cell damage (section VI). McKenzie (149) found platyphylline, which has a saturated ring base, to be the most active in some of these respects. The alkaloids are chemically very stable. Oxidation of the ring nitrogen is the reaction most readily available. They are only slightly soluble at neutral pH, the open-ester alkaloids being considerably more soluble than the cyclic esters. For biological experiments they are usually dissolved in 0.1 N HCl and the solution then neutralised before administration.

B. Methods for determination

Until 1967, the methods available were labelling the alkaloids by growing plants in labelled media and chemical determination of the pyrrolizidine ring. Neither of these were convenient, and little progress was made. Recently Mattocks (143) has developed a colourimetric assay which is much more convenient. The colour reaction demonstrates the presence of substances of structure XI (see section IV C) named "pyrroles" because of the position of the double bonds in the ring. These are obtained by dehydration of the N-oxides of the alkaloids. The colour reaction is quantitative. When used with appropriate controls it is specific for the pyrrolizidine alkaloids.

IV. METABOLISM

A. Distribution in the body

The fate in the body of ingested alkaloids was first worked out with alkaloids laboriously labelled by growing plants in labelled media. Schoental (189) used ^{15}N, Hayashi (100) tritium, and Bull *et al.* (36) ^{14}C. The results agree with those of the simpler, but less direct, pyrrole methods now available. Up to 80% of the pyrrolizidine ring comes out unchanged in the urine (36, 100), 30% of the tritium label (but no unchanged alkaloid) appears in bile in the first 3 hr after dose (100). Ten percent of ^{14}C appears in expired CO_2 within 4 hr (36). Liver, kidney, and stomach (36, 100) show the highest labelling at 3 and 4 hr. By 72 hr only liver shows a significant level. Other organs (including lung) show very low levels at the times tested.

The figures of Jago *et al.* (114) for the sheep, based on chemical identification of the intact pyrrolizidine ring, differ from the above, but may not be irreconcilable. Heliotrine was administered to conscious sheep by slow or rapid intravenous infusion, or by duodenal infusion. Determinations were made on blood, bile, and urine over 48 hr. Blood levels (of intact pyrrolizidine ring) at the end of infusion were high (175 μg/ml) after rapid intravenous infusion and low (7 μg/ml) after slow infusion, but in all cases they fell very rapidly, reaching 1 to 4 μg/ml at the end of the first hour after infusion. Between 10 and 15% of total dose appeared in the urine in 48 hr. Up to 0.3% of the dose appeared in bile. About 80% of the dose was unaccounted for. No tissue levels were estimated. In attempting to compare these figures one must remember that the alkaloids differ in their toxicity. Hayashi used monocrotaline (LD50 175 mg/kg), Bull used lasiocarpine (LD50 72 mg/kg), and Bull and Jago used heliotrine (LD50 300 mg/kg). A difference in toxicity must mean a difference in the proportion of an administered dose that

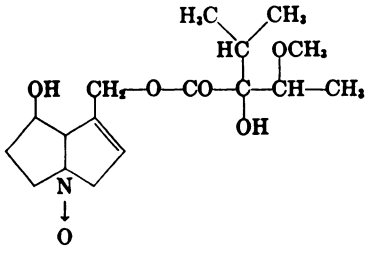
becomes available to the relevant binding site, leaving a different proportion to be wasted along other metabolic paths.

I think these results are best summarised as follows. Regardless of the route of entry, administered alkaloid disappears very rapidly from the blood and is mostly gone within 1 hr. More than half of the dose is eliminated in the urine with its pyrrolizidine nucleus intact, or in a form in which it has been chemically mistaken for an intact pyrrolizidine nucleus. A further large fraction of the dose is broken down sufficiently to appear as tritium label without an intact pyrrolizidine ring in the bile, or as $^{14}CO_2$. Again regardless of the route of administration, label from the alkaloid adheres to liver, intestines, and kidney and not to other tissues.

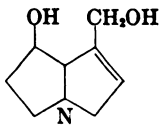
B. Metabolic reactions available

The toxic alkaloids are chemically fairly stable. Oxidation of the ring nitrogen is the only reaction readily available.

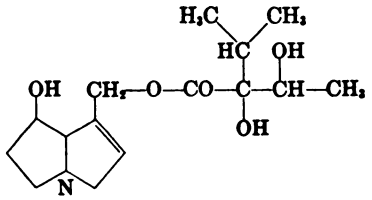
Bull, Culvenor, and Dick (36) and Jago *et al.* (114) have demonstrated heliotrine N-oxide (V) and heliotridine (VI) in rat and sheep urine after dosing with heliotrine (III). They also found heliotridine glucuronide and heliotridine trachelanthate (VII), in which the ester side chain has lost a methyl group. Rat liver microsomes can carry out this reaction *in vitro* (116). All of these metabolites are less toxic than the parent alkaloid.



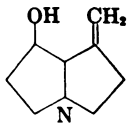
V



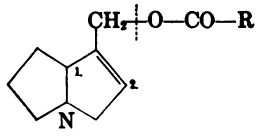
VI



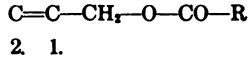
VII



VIII



IX



X

A further metabolic route is peculiar to the sheep, whose rumen detoxicates heliotrine and other alkaloids to the 1-methylene derivative (VIII) (68, 76). Deprived of its allylic ester group the pyrrolizidine nucleus loses its toxicity.

Culvenor *et al.* (67) suggested that the alkaloids might alkylate tissue constituents though alkyl-oxygen fission (IX) in the allylic ester group. The term "allylic ester group" refers to the ester on position 1 which, because of the 1—2 double bond, can be viewed as an ester of allyl alcohol (X). Culvenor showed such a reaction *in vitro* under the influence of strong alkali and benzyl mercaptan. The idea that the alkaloids could exert their action by alkylation was an important one, but it now seems unlikely that they do so by exactly this reaction (see section IV C 2).

None of these reactions, however, (with the possible exception of IX) accounts for the toxic capacity of the alkaloids. It has always been a puzzle that the very stable alkaloids were so highly toxic. In the case of a number of other toxins (139, 151, 162) it has been shown that enzyme reactions in the body may convert a substance, harmless in itself, into an intensely harmful metabolite. This mechanism could account for the toxicity of these alkaloids and evidence in this direction has recently been published by Mattocks (143, 144) and by Culvenor *et al.* (68).

C. Metabolism to pyrrole derivatives

Mattocks found that urine and tissue extracts from animals dosed *in vivo*, and liver homogenates and slices treated *in vitro* with retrorsine gave a positive colour reaction for pyrrole derivatives. This suggested that the whole animal does, and the liver can, make substances of structure (XI) from retrorsine (IV) (144). Pyrroles identified and isolated in this fashion are referred to by Mattocks as "metabolic" pyrroles. The chemical background to this work is dealt with in detail by Mattocks (143, 145) and by Culvenor *et al.* (68, 69). The biological evidence was presented by Mattocks (143, 144), Butler *et al.* (46), and Culvenor *et al.* (68) and can be summarised as follows:

Pyrrole derivatives produced by the body. Rats dosed with retrorsine (60 mg/kg) put out 14% of the dose in the urine as pyrroles within 48 hr. Tissues from rats given the same dose and killed 4 hr later showed pyrroles in liver, lung, heart, spleen, and kidney (not stomach and small intestine). Of these, liver showed much the highest level. The time-course for pyrrole levels in the liver showed a maximum between 2 and 5 hr (144).

Cell fractions. Estimation of pyrroles in liver cell fractions showed greater activity associated with the microsomal and solid debris fractions and less associated with the mitochondrial and soluble fractions.

Pyrrole derivatives produced in vitro. Rat liver slices can form pyrroles *in vitro* from added alkaloid (144), and so can rat liver microsomes (146). Lung slices and lung and kidney microsomes (147) cannot. These "metabolic" pyrroles are different in their optical absorption spectra from their chemically formed analogues.

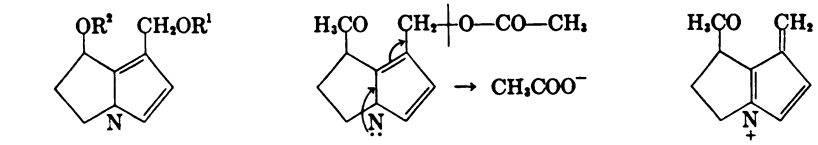
1. The role of liver microsomes in producing pyrrole derivatives

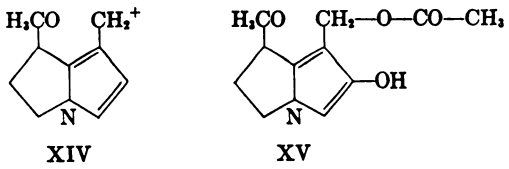
The role of liver microsomal enzymes and of enzyme induction, in the metabolism of foreign compounds has recently been reviewed by Conney (62).

Mattocks has shown that prior administration of phenobarbitone to the rat increases the quantity of pyrrole formed by that rat's microsomes *in vitro*. This is as one would expect if the pyrroles were being manufactured in the microsomes.

Results from animals *in vivo* are less straightforward. A sucrose (low protein) diet raises the LD50 of retrorsine in male rats, and phenobarbitone treatment lowers the LD50 in female rats (147). This is as one would expect. But phenobarbitone raises the LD50 of retrorsine in male rats (147) and DDT has no effect on the LD50 of retrorsine in male rats (150a). These two results do not fit. In the case of these experiments, either it is not the pyrrole which is responsible for death, or the microsomes have a further action on the pyrrole which detoxicates it without altering its capacity to take part in the colour reaction by which it is detected. Mattocks favoured the latter, and he offered the following explanation.

The alkylating capacity of structure XI depends on the transfer of electrons from the nitrogen as in XII, allowing the ester to ionise and leaving structure XIII. This resonates with XIV, which is an alkylating agent. Microsomal hydroxylating enzymes could add an OH to the ring in position 2 (XV) which would prevent the necessary electron transfer, but not interfere with the colour reaction.





If the microsomes both produce and detoxicate the pyrrole derivative, there must be some provision for escape of the active pyrrole derivative between these two steps, otherwise there would be no toxic effects.

2. Alkylating capacity of pyrrole derivatives

Pyrroles are chemically highly reactive and, unlike the parent alkaloid, are themselves capable of alkylating, The alkylating ability of pyrroles of retrorsine, monocrotaline, lasiocarpine, and heliotrine have been shown (145) to be high in circumstances where the parent alkaloids and N-oxides do not react at all (as measured with 4-*p*-nitrobenzyl pyridine). Pyrroles are capable of producing alkylating groups at the site of both the ester linkages (68, 145). Bifunctional alkylating agents of this type can be compared to nitrogen mustards and mitomycin C, which are believed to inhibit mitosis by linking themselves across the two strands of DNA. Since the alkaloids have a strong inhibitory effect on mitosis (See section V A 2 C) this chemical characteristic is of great interest.

3. Toxic reactions of pyrroles

Mattocks (145) investigated the toxic capacity of a number of chemical pyrroles of retrorsine and found that maximal toxicity was limited to structure XI. They gave this substance to rats by a number of different routes. By mouth it had no effect. Given intravenously it produced lesions in the lungs 4 or 5 weeks later very similar to those seen after monocrotaline and fulvine (15). Given intraperitoneally it caused death with haemorrhage and sloughing of the peritoneum. Culvenor used a pyrrole of heliotrine and also found it fatal when given intraperitoneally. In neither case was any acute or chronic effect observed on the liver. This may be due to the high reactivity of the pyrrole, which could all be mopped up by sites available in the peritoneum without ever reaching the liver.

In later experiments, Butler, Mattocks, and Barnes (46) showed that slow injection of retrorsine pyrrole into the inferior mesenteric vein produced necrosis of the walls of the small branches of the portal vein with infarction of the part of the liver served by these branches. It did not produce liver cell necrosis in a lobular distribution. These results will be discussed in sections V A 4 d and IV C 4.

Culvenor has tested heliotrine and lasiocarpine pyrroles for nucleotoxic effects and found them mildly mutagenic in Drosophila and Aspergillus and capable of inhibiting the replication of a DNA virus (68).

Chemical pyrroles prepared from retrorsine, monocrotaline, heliotrine, and lasiocarpine have identical absorption spectra. It is not yet known whether this is also true of the corresponding metabolic pyrroles. There are considerable differences in the toxic reactions that commonly follow administration of these different alkaloids. Lung lesions, for instance, frequently follow monocrotaline, but not the others. Lung lesions were produced by chemical retrorsine pyrrole. If the corresponding metabolic pyrroles are identical, some other mechanism must be found to account for the observed differences in toxic pattern (145a).

4. Pyrrole derivatives as the proximate toxin

We have conclusive evidence that pyrroles are formed in the body and that chemical pyrroles can mimic some of the toxic actions of the alkaloids. There is strong circumstantial evidence, but no conclusive evidence, that metabolic pyrroles are the metabolic products responsible for the toxicity of the alkaloids. It is asking a great deal to demand conclusive evidence on this point. Substances that stay around in the tissues long enough to be measured do so because they are in excess of the tissue binding sites. This argues a large quantity of the substance or a low chemical reactivity. In many toxic situations the converse is true. Very small quantities of a highly reactive proximate toxin do severe damage, but the proximate toxin itself possesses only a fleeting existence.

Nevertheless this is a dilemma which the toxicologist must face. One way of doing so is to pinpoint a reaction and show that a toxic effect is augmented or diminished as the rate of that reaction is raised or lowered by manipulation of

the enzymic machinery of the cell. This was done successfully for carbon tetrachloride (151). Even such evidence, however, does not conclusively incriminate one chemical substance but produces a black-box type of answer (albeit quite a small box) indicating "The toxic capacity arises here."

Pyrroles microsomes and species specificity. Looking for such a black box among the evidence at present available, one's eyes turn hopefully towards the liver microsomes.

A number of species are resistant to pyrrolizidine poisoning, and while this resistance need not reside in the microsomes (sheep, *vide supra*), it is worth looking at their microsomal status for the light it casts on the story so far.

Guinea pigs are resistant to the acute hepatotoxic effects of senecionine. There is disagreement about the microsomal activities of guinea pigs. The Millers (161) found low levels of excretion of N-OH acetyl amino fluorine, after feeding this compound to guinea pigs and concluded that the N-hydroxylating systems were weak or absent in this species. Subsequently, Hlavica and Kiese (108) and Kiese and Wiedemann (126) have shown that guinea pigs break down nitroso compounds faster than rats or rabbits; this suggests a high N-hydroxylating ability. Debackere and Uehleke (75) looked at C and N hydroxylation of aromatic amines in microsomes from a number of species and found it high in guinea pigs and chickens and lowest in rabbits. Kiese has now found (126) that N-hydroxarylamides have an extremely short half-life in guinea pig blood, being removed and broken down very rapidly by the red cells. Mattocks and White (147) have shown that microsomes from male rats produce pyrroles 30 to 80 times faster than microsomes from male guinea pigs.

Groundsel (*Senecio vulgaris*) is gathered as food for canaries. Their resistance to its poisonous effect is not, however, common to all birds, since chickens and turkeys get typical acute liver damage when fed *C. spectabilis* seed (monocrotaline) (2, 8, 9). The susceptibility of chickens is a serious flaw in the argument for microsomal production of pyrroles since this species is resistant to carbon tetrachloride (93) which is activated in the microsomes (151). Either the alkaloids are activated by some other system in chickens, or the functions of the microsomal mixed-function oxidase system are separable, chickens possessing the fraction responsible for pyrrole production and lacking that responsible for carbon tetrachloride metabolism. The question of the unity or otherwise of the microsomal mixed-function oxidases is a highly controversial one (71), and has been discussed by Kunzmann (ref. 89, p. 349).

Many foetal and newborn animals lack microsomal hydroxylating enzymes and develop them during the first few weeks of life. The general pattern is that of a slow rise in activity from 1 to 3 weeks, followed by a jump to adult levels at weaning, between 3 and 4 weeks (74, 91). Activities at birth are nil. Newborn man follows this pattern, being unable to glucuronidate bilirubin. Human foetal liver is thought not to possess mixed-function oxidase activity.

Mattocks (146) found that liver microsomes from rat foetus at 18 days gestation produce pyrrole derivatives from retrorsine and monocrotaline at about half the rate at which they are produced by adult female rat liver. McLean found

monocrotaline hepatotoxic to newborn rats. The LD50 was about the same as for adult rats. Adult Gunn rats (which lack the capacity to glucoronidate bilirubin) were also susceptible (155). Human foetal liver cells in tissue culture do not produce pyrroles from heliotrine (146). They do not suffer necrosis after exposure to heliotrine but they do undergo cell enlargement (211). It is difficult to assess the microsomal status of these cells. Microsomal hydroxylating activity has not been measured in them. If they are equivalent to human foetal liver, there should be none, but some cultured cells are known to differ from those of their organ of origin and this particular enzyme system is known to react very sensitively to the environment.

Cinnabar moths not only succeed in feeding on Ragwort (Senecio) but actually concentrate the alkaloid in their tissues and put it out, after metabolic alteration of the ester side chain, as a defensive secretion (10). Mattocks found no pyrrole derivatives in these caterpillars though larval insects do possess microsomal hydroxylating enzymes in their mid-gut (130).

Taken together, this evidence argues against microsomal mixed-function oxidases as the sole source of the proximate toxin of these alkaloids. It seems worth investigating the guinea pig, the canary, and the chicken since these species apparently offer variations from the normal response. An understanding of these variations might allow us to pinpoint the mechanism of susceptibility in other species.

The case for identifying the pyrrole derivatives with the proximate toxin gains spurious weight from the fact that these are the first chemically active derivatives to have been detected and tested. Among other possible active derivatives, Schoental (190a) favoured the epoxides as the proximate toxin. There is only one report of the biological testing of epoxides (68). Culvenor found them inactive when injected intraperitoneally. This is not conclusive, as adequate testing of such highly reactive compounds would require intravenous administration. Experiments in this direction may alter the emphasis on the role of the pyrrole derivatives.

D. Conclusions

1) We have conclusive evidence that pyrrole derivatives of the alkaloids are formed in the body.

2) The pyrrole derivatives are strong alkylating agents.

3) Administered by intravenous injection, the pyrrole derivatives produce toxic reactions in the lung and in the endothelium of small blood vessels, which are comparable with the toxic actions of the alkaloids.

4) There is contradictory evidence on the role of liver microsomes in the production of these pyrroles.

V. THE PATHOLOGICAL BASIS OF TOXICITY

The bulk of the pathological data available refers to the toxic syndromes exhibited by laboratory animals. In this section I will consider it under the headings: Liver (acute, chronic, and vascular), Lung, and Other tissues.

A. Liver damage

1. The acute lesion in the liver

a. Liver parenchymal cell necrosis. The acute hepatotoxic actions of the alkaloids have been reviewed by Chen (50), Schoental (188, 189), Magee (140), and Bull *et al.* (31, 32). The typical reaction of small laboratory animals to doses close to the LD50 is a confluent, haemorrhagic, centrilobular necrosis in the liver reaching its peak 24 to 48 hr after dose and first demonstrable with the light microscope about 12 hr after dose. In general, the same pattern follows dosing by mouth, subcutaneously, or intraperitoneally. Reactions following this pattern have been described for cats and rabbits (70), hamsters (99), mice (51, 96–98), rats (15, 39, 51, 53, 72, 156, 202), and chickens (8, 47). Guinea pigs are notably resistant to this syndrome (50). In some species, some, but not all, of the alkaloids produce a periportal necrosis. This has been reported for mice (97, 98, 183), monkeys (182, 226), and hamsters (182). Occasionally central, midzonal, or periportal lesions were variously seen in different animals of the same experiment (97, 98). Haemorrhage into the liver lesion was common, regardless of the lobular site of the necrosis. (72, 97, 98, 156, 182, 226). It seems, therefore, that the exact site of attack in the liver lobule does not depend solely on the individual alkaloid structure or animal species. The observed differences are probably best ascribed to individual variations in the enzymic geography of the liver lobule.

Attempts to account for the necrosis by lesions in separate subcellular particles have progressed according to the biochemical fashion of the moment. A number of such lesions have been demonstrated. But though we can now draw up a timetable of these, we still do not know which of them causes necrosis and which are only incidental, or fully reversible.

The timetable runs roughly as follows. After a single dose ($\simeq$LD50) by mouth or intraperitoneally, cytoplasmic protein synthesis falls extremely rapidly (95), reaching 30% of control levels at 15 min and 6% at 1 hr.

A number of changes in the nuclei, which will be discussed below (section V A 1 b), run a closely similar time course. The time course observed does not seem to allow for the depression of cytoplasmic protein synthesis to be secondary to the nuclear events. To explain the observations of Harris *et al.* (95) one must postulate a direct action of the alkaloid on the ribosomes.

Polyribosome disaggregation (95, 225) is severe at 1 hr and progresses to 6 hr. Pyruvate oxidation by liver homogenates and by mitochondrial preparations falls off progressively from 1 hr (54). At 3 hr the centrilobular cells begin to lose their glycogen (208). At 9 hr mitochondria in the centrilobular area are swollen and fat droplets appear. At 12 hr and later there is increased lysosomal activity as observed by light microscopy (29, 208), and by lysosomal enzyme techniques (124). At 16 hr (but not earlier) the nicotine adenine dinucleotide (NAD)-dependent enzyme systems of isolated mitochondrial preparations begin to fail (54). At 18 hr NAD synthesis by nuclear preparations begins to fall (58). Later than this the cells are frankly necrotic. To make any sense of these events one

must consider first the observations which have been made on the biochemical activities of the nucleus.

b. The acute lesion in liver cell nuclei. At the present moment, the major alteration in liver cell nuclear metabolism known to occur on exposure to pyrrolizidine alkaloids is a rapid and profound drop in DNA-mediated ribonucleic acid (RNA) synthesis (176). A slower fall in DNA synthesis is also reported (84, 211). Work in this field is going so fast that quite a different state of play will probably obtain by the time this article appears in print.

Svoboda and Soga (217) have demonstrated changes in the ultrastructure of the nucleolus of rat liver cells after lasiocarpine. The nucleolus, normally a random mixture of fibrillar and granular components, draws apart into globules in which these components are separated, within 1 hr after lasiocarpine dose. Nuclear RNA synthesis falls sharply, reaching 30% of control values 15 min after dose and 6% in 1 hr. This fall in RNA synthesis is concurrent with the fall in cytoplasmic protein synthesis (95) (see section V A 1 a). RNA polymerase activity is inhibited, showing 40% of normal activity at 2 hr. The liver loses its capacity to respond to an enzyme-inducing stimulus; tryptophan pyrrolase induction by hydrocortisone is reduced to 20% of normal values when first sampled 6 hr after lasiocarpine administration (176). All these changes revert to normal over 72 hr.

Fayssinet and Moulé (84) reported a drop in DNA synthesis of 70% 1 hr after an LD50 of lasiocarpine, and of 92% at 3 hr. They also showed that the fall in RNA polymerase activity is due to an alteration of the DNA, not to an effect on the polymerase itself. A system containing DNA from a lasiocarpine-treated rat, labelled cytosine, and RNA polymerase from a micrococcus, showed 50% inhibition of polymerase activity over control systems. Alkaloid added *in vitro* to such a system containing DNA from a normal animal, had no effect. It seems that the active principle in this system was a metabolite of the alkaloid.

Sullman and Zukerman (211) have studied the response to heliotrine of human foetal liver cells in tissue culture (see section V A 2 b). These cells do not undergo necrosis and do not show nucleolar abnormalities. It seems probable, therefore, that they are not in the same state as the other acutely poisoned calls under discussion. They found a dose-dependent drop in DNA synthesis after 3 days exposure to the alkaloid. The number of cells labelled with ^{3}H-thymidine dropped progressively after exposure to the alkaloid and continued to drop after the alkaloid was removed from the incubating medium.

Of these changes, RNA synthesis, protein synthesis, and nucleolar segregation are much the earliest.

The functions of the nucleolus include the production of RNA for making ribosomes (166, 167). This is probably "packaged" within the nucleus to protect it from nuclear RNAases and then transported to the cytoplasm where it must join other ribosome units, linked together by messenger RNA to make a polyribosome, before it is capable of protein synthesis. Messenger RNA is thought to be manufactured on the nuclear chromatin, and is therefore not directly affected by the nucleolar changes described.

Autoradiographic studies (176) show that lasiocarpine does not prevent the migration of label from the nucleolus (*i.e.*, the migration of RNA already formed) but does prevent the incorporation of label, that is, synthesis in the nucleolus of RNA. Chiga *et al.* (52) and Svoboda and Higginson (215) showed that thioacetamide stops migration of RNA from the nucleolus. When nucleolar RNA synthesis was stopped by actinomycin D, nucleolar segregation could be prevented by thioacetamide. It seems, therefore, that what is happening after lasiocarpine is a cessation of RNA synthesis followed by disintegration of the nucleolus as the last of the RNA moves away.

Can these events be responsible for the very rapid drop in cytoplasmic protein synthesis? (95) (see section V A 1 a). Evidence from actinomycin-D studies suggests they cannot. Reich et al. (178a) using mammalian cell cultures, found protein synthesis unchanged at 4 hr after a dose of actinomycin-D sufficient to reduce RNA synthesis to 40% of control values. If lasiocarpine inhibits protein synthesis via inhibition of RNA synthesis, the mechanism of action must be different from that of actinomycin-D.

Alternatively, could the nuclear events be secondary to the failure of cytoplasmic protein synthesis? The drop in DNA synthesis could, since protein synthesis is required for DNA synthesis (17). The drop in RNA synthesis could also be explained as follows. If newly-synthesised nucleolar RNA requires a protein package (167) to protect it during transport, then delivery of RNA to the cytoplasm would be dependent on cytoplasmic protein synthesis, since the nucleus is thought not to synthesise protein (81). A failure of RNA metabolism at the point at which it leaves the nucleolus could cause a drop in nuclear and cytoplasmic RNA levels. It ought not, however, to cause nucleolar segregation, since RNA synthesis in the nucleolus is proceeding normally. Cycloheximide, which stops cytoplasmic protein synthesis, does cause nucleolar fragmentation, but this is different in electronmicroscopic morphology from the nucleolar segregation of lasiocarpine, aflatoxin, and actinomycin D poisoning.

Cells without nuclei (red blood cells) do not appear to suffer directly from exposure to the alkaloids or their metabolites.

c. Summary. 1) All the toxic alkaloids tested cause acute zonal necrosis of liver cells, which is usually centrilobular.

2) Among a large number of biochemical lesions, the earliest yet described are a fall in cytoplasmic protein synthesis and, concurrently, a fall in DNA-mediated RNA synthesis.

2. The chronic lesion in the liver

The chronic lesion in the liver was recognised by Cushny (70) and Davidson (72). The first systematic studies were made in sheep (29). It has now been reported in many other animals, and Bull has christened the condition "pyrrolizidine alkaloidosis." In the interests of the English language I shall refer to it throughout this article as "pyrrolizidine poisoning."

The histological hallmark of this disease is megalocytosis of the parenchymal cells of the liver, associated with failure of liver function. This is by no means

the only change to be seen histologically in the liver. Bile duct proliferation (198), fatty change, fibrosis (32), cirrhosis (9), and vascular lesions (197) are also seen. But the grossly enlarged liver cell is the constant feature.

Laboratory investigation of liver cell megalocytosis has shown that the alkaloids have a pronounced antimitotic action, and that they are carcinogenic. Accordingly, I shall consider the chronic lesion in the liver under the headings: Megalocytosis; Tissue culture experiments; Antimitotic action; and Carcinogenesis.

a. Megalocytosis. The literature dealing with this phenomenon has recently been reviewed in an excellent article by Jago (115). The term was coined by Bull (29) to describe the large parenchymal cells in the livers of sheep which had grazed on *Heliotropium europaeum* for 2 seasons. These cells have 10 to 30 times the volume of normal cells, with nuclei enlarged proportionately (29). To the light microscope the only gross abnormality of structure is an increase in nuclear chromatin. They are capable of entering mitosis, but it seems highly doubtful that they are capable of completing it. Many abnormal mitoses appear among them (198). Livers abundant in such cells have been found after single doses of the alkaloids to rats (196–198), and after multiple doses of the alkaloids or the relevant plants, to rats (137), mice (183), sheep (29, 32, 38), cattle (40, 83, 128, 141, 220), horses (106, 180, 181), and pigs (94). Similar cells are also seen in cultures of human foetal liver cells after exposure to the alkaloid (211). There are occasional reports of comparable large cells in the kidney (33, 94) and the lung (15, 46, 223). In the intact animal the lesion is typically slow to appear; a number of workers report it at 3 to 4 weeks after a single dose (115, 164, 197).

The electronmicroscopic morphology of these cells after single doses of the alkaloids in rats has been described by Svoboda and Soga (217) and by Afzelius and Schoental (1), whose findings differ sharply. After lasiocarpine, Svoboda and Soga found no abnormality of the nucleolus in megalocytes, and no constant feature to distinguish them in electronmicroscopic appearance from other liver cells. After retrorsine, Afzelius and Schoental found a large number of impressive changes, which were a constant feature of these cells. The changes are too numerous to list here in full. The ones that appear to me to tie up with other available information are: a striking proliferation of the rough endoplasmic reticulum; loss of the normal organisation of cytoplasmic organelles; the presence in the cytoplasm of several centrioles (normal cells in interphase contain two); an increase in the number of nuclear pores; and granular aggregations within the nucleus similar to the nucleolar segregation seen in acute poisoning. The authors concluded that many of these changes point to increased metabolic activity with increased exchange of material between the nucleus and the cytoplasm. One could view such increased activity as a response to deficiencies in liver function. Since pyrrolizidine poisoning is progressive, we know that this response is not successful.

Reliable indices of the effect of megalocytosis on liver function are difficult to come by, since livers always contain a greater or smaller proportion of apparently normal cells as well as the megalocytes. Scheuer (185) and Thorpe and

Ford (220) have made histochemical studies in rats and calves, respectively, which are in broad agreement. Megalocytes have no or little glycogen. Staining for succinic dehydrogenase is weak but for nonspecific esterase remains normal in these livers. Estimations in blood and serum show anaemia, a raised bilirubin, and low serum proteins in rats (186). In calves Ford *et al.* (82) found a fall in the albumen/globulin ratio of serum proteins as a late feature only; serum levels of glutamic oxalic transaminase, glutamate dehydrogenase, and ornithine carbamyl transferase were raised, but glutamic pyruvic transaminase was normal. These high serum levels were independent of the degree of liver damage assessed histologically. It seems likely (150) that raised levels of serum transaminases may occur as part of the process of repair of liver damage and should not be regarded only as indices of current liver cell necrosis. The capacity of the liver to handle ammonia is also severely impaired. High blood ammonia levels have been found in horses (181) and rats (35) with chronic pyrrolizidine poisoning and are thought to occur in sheep. These observations do not lend themselves to a single interpretation. It is clear that livers containing a high proportion of megalocytes are strained beyond their powers of adaptation and eventually fail to meet the demands of the body. It is not clear in which of the liver systems this failure starts.

A number of other toxins including thioacetamide (174), bentonite (232), dimethylnitrosamine (55), aflatoxin (43), and vitamin B_6 deficiency (90) are capable of producing megalocytes in the liver. Of these, the lesion produced by acute doses of aflatoxin seems closest to that seen in pyrrolizidine poisoning, but none has the same persistence. The problem has recently been discussed by Jago (115).

b. Tissue culture. Two studies of the alkaloids have been made with tissue culture techniques. Hirchinson and Hill (104) put monocrotaline in the medium of Hu Li foetal liver cells. The cultures died more rapidly than controls. There was no inhibition of mitosis, but a large number of abnormal mitoses. Many cells with large or multiple nuclei were seen and nucleoli were abnormal.

Sullman and Zuckerman (211) used two types of foetal liver cells, a primary human embryo liver cell culture, and a continuous cell line derived from human foetal liver. Heliotrine, in doses 1 to 250 μM/ml (0.7 to 160 $\times$ LD50) in the medium did not cause necrosis in these cells. No pyrrole derivative was detected in these cells (146), but the parent organ did give a positive reaction for pyrroles (212). The alkaloid caused enlargement of the cells, to 50% above normal levels, within 3 days. Cell enlargement progressed even after alkaloid was removed from the medium. At doses above 50 μM/ml there was severe inhibition of mitosis. No abnormalities of nucleoli were seen.

Among biochemical observations made, the most striking was a profound and dose-dependent drop in DNA synthesis after 3 days exposure to heliotrine. RNA synthesis fell off more quickly but much less profoundly, and was not dose-dependent. Protein synthesis fell progressively from 18 to 72 hr exposure, and was reduced by 90% after 3 days exposure to the highest dose.

In assessing these results, one should bear in mind that rat Novikoff hepatoma

cells in tissue culture go through startling changes in the synthesis of DNA, RNA, and protein according to their phase of growth. When changing from the exponential to the stationary phase of growth they show a marked drop in the synthesis of DNA, RNA, and protein with loss of RNA transferase activity and complete disaggregation of polysomes (170). It is not recorded that they show nucleolar abnormalities at this time. It is difficult to know what relation these observations bear to the behaviour of liver cultures grown from normal foetal liver. Perhaps the alkaloid has changed the foetal culture in one aspect which is seen in neoplasia, or perhaps the foetal culture itself is reacting more as one would expect neoplastic cells to react and less as one would expect normal cells. At the very simplest these observations underline the fact that cells in culture are not always in a steady state, and that, when they are, it is unlikely to be identical with the steady state of cells in the parent organ in the body.

These are, so far, the only measurements of DNA synthesis during pyrrolizidine poisoning. Further observations at a later stage after dose are urgently needed. The drop in DNA synthesis presumably coincides with the early severe inhibition of mitosis described in the next section (V A 2 C). At a later stage, as cell and nucleus begin to enlarge, one would expect a return to levels of DNA synthesis at least as high as normal. Sullman's cell cultures (211) can survive 16 days or longer. Observations relating DNA synthesis to mitotic index at about that interval after dose would be very helpful.

Heliotrine causes chromosome breaks in cultures of leucocytes from the Tasmanian kangaroo (19a).

c. Antimitotic action. It was noted in the early studies on the experimental production of liver cell megalocytosis that the liver cells showed too few mitoses, and that the majority of mitoses seen in megalocytes were abnormal (197, 198). Schoental (188) suggested that the alkaloids had a specific inhibiting action on mitosis. The evidence has recently been reviewed by Bull *et al.* (34).

Peterson (168) and Downing and Peterson (77) have shown that the wave of mitosis in liver parenchymal cells after partial hepatectomy is cut to 50% or less of normal levels by prior treatment with heliotrine, lasiocarpine, or lasiocarpine-N-oxide. The effect is dose-dependent. For a standard dose, capable of reducing the cumulative mitotic index, at 16 hr after partial hepatectomy, to 50% of control levels, the alkaloid reaches its full effect one week after dosing.

These experiments lent elegant support to the view that megalocytes are liver cells that fail to carry out mitosis and continue to grow without dividing. Further support for this view comes from the experiments of Jago (115), who argued that any stimulus to liver cell growth and division ought to speed up the appearance of megalocytes after small doses of alkaloids. She showed that the growth stimulus represented by youth had exactly such an effect. Single doses in the range 0.1 to 0.4 LD50 of lasiocarpine given to 2-week-old rats gave severe megalocytosis 4 weeks later. The same fraction of LD50 to 10-week-old rats had no effect. Repeated liver injury with carbon tetrachloride (with consequent repeated waves of liver cell regeneration) after single small dose of lasiocarpine, also increased the speed of appearance and the severity of megalocytosis. In

TABLE 4

Liver tumours following administration of pyrrolizidine alkaloids

Line No.	Dose	Alkaloid	Number Surviving	Survival Time	All Tumors	Hepatoma	Metastasizing Liver Tumour	Other Tumours	Diet	Other Treatment	Ref.
1	Oral, 30 mg/kg once	Retrorsine	25	1 year +	19	5	1	13	MRC 41B	Whole body irradiation, 400 r	192
2	Oral, 30 mg/kg once	Retrorsine	29	1 year +	11	5		6	MRC 41B		192
3	Oral, 30 mg/kg once	Retrorsine	9	1 year +	3	2		1	MRC 41B	Partial hepatectomy	192
4	Nil		6	1 year +	4			4	MRC 41B	Whole body irradiation 400 r	192
5	Oral and i.p., 0.02 mg/ml in drinking water, 2 days in drinking water, 2 days weekly for 6 months; 4 i.p. weekly for 6 months; 4 i.p. doses 25–30 mg/kg up to 1 year	Ridelliine	16	1 year +					Shearer's pig weaner nuts + MRC 41B	3 had liver biopsy	195
6	I.p. 25–30 mg/kg, 6 injections over 5 months	Retrorsine	4	18 months +	1	1			Shearer's pig weaner nuts + MRC 41B	2 had betaine supplement to diet	195
7	Oral or i.p., 0.03 mg/ml in drinking water, 2 days weekly for 1 year or 25–30 mg/kg 5 injections over 2 months	Isatidine (= retrorsine-N-oxide)	20	1 year +	6	6			Shearer's pig weaner nuts + MRC 41B	6 high casein diet; 3 betaine supplement to normal diet	195
8	Nil		15	1 year +	2			2	Shearer's pig weaner nuts + MRC 41B		195
9	Oral and i.p., 0.03 mg/ml in drinkwater 2 days weekly for 6 months; up to 3 injections, 4–12 mg/rat until 1 year	Monocrotaline	6	1 year +	3	2		1	Shearer's pig weaner nuts		194
10	Oral, 0.05 mg/ml in drinking water 3 days weekly over an interrupted course until death	Alkaloids of *Senecio jacoboea*	10	1 year +	2	2			Aberdeen rat cake and Shearer's pig weaner nuts		193
11	Oral, 0.03 mg/ml in drinking water 3 days weekly until death	Retrorsine	4	10 months +	5	4		1	Aberdeen rat cake and Shearer's pig weaner nuts		193

12	Oral, 0.03–0.05 mg/ml in drinking water 3 days weekly throughout	Isatidine	22	11 months +	10	9	1		Aberdeen rat cake and Shearer's pig weaner nuts		193
13	Oral, 0.03–0.05 mg/ml in drinking water 3 days weekly throughout	Isatidine	7	14 months +	4	4			Aberdeen rat cake and Shearer's pig weaner nuts	Choline supplement to diet	193
14	I.p. and skin painting, 2 mg i.p. once; 0.5% w/v solution to skin 3 days weekly for 15 months	Isatidine	5	11 months +	1	1			Aberdeen rat cake and Shearer's pig weaner nuts	Choline supplement to diet	193
15	Oral, 0.05–0.1 mg/ml in drinking water, intermittently throughout	Alkaloids of *Senecio jacoboea*	2	10 months +	3	2		1	Aberdeen rat cake		63
16	I.v., 0.5 LD50 weekly for 8 weeks 8 weeks (chicken)	Alkaloids of *Senecio jacoboea*	12	3–12 weeks	3	3			Poultry research centre, balanced ration		47
17	I.v., 0.5 LD50 weekly for 8 weeks (chicken)	Alkaloids of *Senecio jacoboea*	8	3½ weeks–8 months	3	2		1	9% protein and low choline		47
18	Oral, milled plant in diet, 3.5–7.5% w/w for 14 weeks (chicken)	*Senecio jacoboea*	40	7 weeks–5 months	4	4			Poultry research centre balanced ration		47
19	Oral, 1% w/w in feed throughout	*S. aquaticus*	7	7 months +	6	6					79
Totals excluding control experiments (lines 1, 4, 8)			201			53		11			

further observations of great interest she showed that, in the very young animals, the mitotic index in the liver cells of normal size fell to 2% of the normal level 1 day after a single dose of alkaloid, 10% at 2 days and reached 200% at 28 days. The mitotic index in megalocytic cells was the same as for normal cells in an untreated liver throughout.

A recent experiment of Rogers' (129a) showed that the antimitotic section of lasiocarpine can be overridden. Rats dosed with lasiocarpine (40 mg/kg, 3 doses) and subjected to ⅔ hepatectomy after intervals of 2 weeks or 3 months, showed a dramatic reduction of mitotic index and of DNA synthesis at 24 and 48 hr after hepatectomy as compared with controls. Rats dosed with lasiocarpine and subjected, after a 2-week interval, to 11 weeks feeding with 2-acetylaminofluorene (AAF) had mitotic indices and DNA synthesis levels no different from lasiocarpine-free controls 1 week after the end of feeding. In both AAf groups, the incidence of carcinoma was 50% at 15 weeks.

In unpublished experiments, Bull *et al.* (32) showed that large (0.5 to 1.0 LD50) intraperitoneal doses of heliotrine had a severe inhibitory effect on mitosis in rat duodenal mucosa. He did not quote figures from the mitotic index, but mitoses were rare or absent in the tissue examined between 2 and 48 hr after dose. At lower doses (0.05 to 0.2 LD50) the mitotic index was reduced at 4 and 7 hr after dose, but had returned to normal at 48 hr. These results are particularly interesting because of the light they cast on the role of a proximate toxin of the alkaloids. A highly reactive proximate toxin produced by the liver ought not to survive in the bloodstream long enough to reach the duodenum. Three solutions of this problem suggest themselves, *i.e.*, the substance responsible for inhibition of mitosis in duodenal mucosa is 1) the aklaloid itself, known to persist in the bloodstream for an hour after dose (IV A) (114), 2) a metabolite from the liver which is not a very highly reactive substance, or 3) a metabolite from some other tissue. This tissue would have to be along the route taken by the alkaloid from the peritoneal cavity to the duodenum. Much the most likely route is by lymph drainage of the peritoneum to the blood stream. The supposed metabolite would then pass the lungs (but not the liver) before reaching the duodenum.

The literature contains two other instances of the alkaloids exerting biological effects in tissues where one would not expect them to have undergone metabolism. They suppress mitosis and DNA synthesis and cause large cells in human foetal liver cell cultures (section V A 2 b) (211), and they cause chromosome breakage when added *in vitro* to cultures of leucocytes from the Tasmanian kangaroo *Potorous tridactylus* (Section V C 2) (19a, b).

d. Carcinogenesis. There is disagreement about the carcinogenic capacity of the pyrrolizidine alkaloids. The evidence has been reviewed by Barnes and Schoental (16), Schoental (190), and Bull *et al.* (36). The positive data available in the literature are summarised in table 4. The crude totals, excluding control experiments and excluding the experiment incorporating the use of whole body radiation (lines 1, 4, and 8) show a 25% incidence of hepatoma and a 5% incidence of other tumours. Two metastasizing liver tumours have been reported. Neither whole-body radiation nor partial hepatectomy increased the

incidence of tumours; 9% protein, high choline, high casein, and betaine supplements to the diet did not alter tumour incidences.

Tumours of the pancreas have recently been reported (192a) after administration of alkaloids of *Amsinckia intermedia* and *Heliotropium supinum.*

All reported tumours occurred in rats and chickens. In 10 of these 19 experiments (table 4, lines 5 to 14) (193–195) rats were maintained for all or part of the time on a commercial diet (Shearer's pig weaner nuts) containing 8.5% groundnut and soya meal (193). All the alkaloids here shown to be carcinogenic are cyclic esters.

The case against the carcinogenic capacity of the alkaloids cannot be presented in any such satisfactory fashion, since it is the custom not to publish negative results. It rests upon three considerations. Firstly, Bull and his collaborators in Australia have carried out a large number of small long-term experiments in which rats survived 3 to 11 months. Most of the alkaloid used derived from Heliotropium and Crotalaria. Retrorsine and isatidine (retrorsine-N-oxide) were not used. Small doses, (0.02 to 0.1 LD50) were given at frequent intervals throughout the experiments. The total number of late survivors is not quoted in Bull's book, but the figures quoted amount to at least 65 animals. They reported (33, 36) two cholangiocarcinomas. They do not report any hepatomas.

Secondly, there exist numerous groups of farm animals, and two groups of human subjects, known from descriptions of acute outbreaks of pyrrolizidine alkaloid poisoning, to have been exposed to at least one dose of the alkaloids. Neither liver nor other tumours are reported among sheep (39), cows (40), horses (180, 181), pigs (41), or fowl (2, 8, 9) which had suffered acute pyrrolizidine intoxication.

Veno-occlusive disease is endemic in Jamaica and there is strong circumstantial evidence that it is due to pyrrolizidine intoxication. The incidence of primary liver cancer in Jamaica is lower than that in England (110). Heliotrope poisoning is endemic in South Central Asia (21, 41). It has not been connected with primary liver cancer.

Thirdly, there is disagreement over the morphological definition of hepatoma. Chronic pyrrolizidine poisoning gives rise to extremely knobbly livers. Some of the knobs are cystic and appear to be of biliary origin (198) whereas other are hyperplastic nodules consisting mainly of parenchymal cells. The criteria for distinguishing a hyperplastic nodule from a hepatoma are hotly debated among pathologists. The recent International Agency for Research in Cancer Working Conference on Liver Cancer (113) concluded that, in the mouse, this distinction could not be made. Their recommendations include the abandonment of the term "hepatoma" in favour of "liver cell carcinoma and liver cell adenoma." As far as pyrrolizidine alkaloid carcinogenesis is concerned then, an important part of the argument rests upon the disputed identity of the lesions reported as hepatomata. The authors themselves appear to have had misgivings on this point. Dybing and Erichsen (79) wrote "It is possible to dispute to what extent these areas of new growth are to be interpreted as hyperplasia or true tumours. Their ill-defined borders and destructive effect on the preformed liver tissue may justify

the diagnosis hepatoma." Schoental and Head (195) wrote of the six hepatomas reported in line 7 of table 4, "Some (nodules) with areas of degeneration in the centre had a hepatoma-like appearance" and "nodules composed of hyperplastic cells, some probably hepatomata."

It is easy to find objections to the arguments advanced by both sides. The ground-nut meal in experiments (193–195) may have contained aflatoxin. This possibility ought to be dealt with by the control series of the experiments themselves. One such series (line 8) did show $^{2}/_{15}$ "other tumors." Other control series were negative. In view of the similarity of the acute action of aflatoxin and of the alkaloids on liver cell nuclear morphology (214, 215) one cannot rule out a synergystic action of these groups of compounds. Bull's long-term experiments did not continue long enough to exclude the possibility that tumors might appear. Similarly, in farm animals, survival after dosing might not be long enough for neoplasia. This cannot hold for man. It appears that some alkaloids (retrorsine, seneciphylline) are more carcinogenic than others (heliotrine, lasiocarpine, monocrotaline) and known human exposure is largely to the latter group.

Nevertheless, the author does not find it possible to dismiss either argument entirely. A difference worth exploiting in future experiments lies in the dosing schedules employed. In most of the experiments that produced tumours (table 4) alkaloid dosing was interrupted, or ceased altogether several months before death. In the experiments that failed to produce tumours (33) dosing was regular and continued until death. If neoplastic change is one of the side effects of liver regeneration, one cannot expect it to appear while a strong antimitotic influence is preventing liver regeneration.

Chickens, in Campbell's hands (47), developed liver tumours 2 and 3 weeks after an intravenous dose of Senecio alkaloids. Perhaps further experiments could exploit this peculiarity. Seneciphylline (47) and retrorsine (192, 193, 195) appear to be the alkaloids most likely to produce tumours.

In order to get over the morphological difficulties it would be helpful if suspected lesions were interpreted by a "blind" procedure by more than one pathologist (since pathologists' views on this problem are known to differ) and the resulting descriptions published so that the basis of any disagreement should be publicly available.

e. Summary. 1) Chronic administration of the alkaloids produces very large liver parenchymal cells.

2) These megalocytes show no obvious structural abnormality. Feulgen staining shows that the nuclei contain a quantity of DNA appropriate to their size.

3) Megalocytes appear both after long administration of repeated small doses and at long intervals after a single large dose of alkaloid.

4) The alkaloids have a pronounced antimitotic effect upon cells of the liver parenchyma and the duodenal mucosa.

5) Continuous administration of small doses of alkaloids produces small livers with abundant megalocytes. Interrupted dosing produces nodular livers in which areas of megalocytosis are interspersed with nodules of regenerating parenchyma.

6) Hepatomas are reported among the regeneration nodules resulting from interrupted dosing.

3. Mechanisms of toxicity in liver

a. Liver cell necrosis. In considering the onset of necrosis, it is clear that failure of protein synthesis, nucleolar segregation, and failure of DNA-mediated RNA synthesis are the earliest events so far recorded after pyrrolizidine poisoning. All the other biochemical lesions listed in section V A 1 follow these events in time, though we know very little about their causal relation. From experiments with other poisons, we know failure of the supply of adenosine triphosphate need not cause necrosis (80) and that quite severe restriction of protein synthesis can occur without causing necrosis (81). It is also known that changes in permeability of cellular organelles and the influence of calcium and other ion shifts (117, 152) are of importance in the genesis of liver cell necrosis. Apart from the work of Christie and Le Page (56, 57), who showed increased mitochondrial permeability at a late stage, we have no information on these points in the case of pyrrolizidine liver necrosis.

After the administration of the alkaloids, the sequence of events in time appears to be: failure of DNA-mediated RNA synthesis concurrent with failure of cytoplasmic protein synthesis and disaggregation of polysomes; failure of pyruvate oxidation; loss of glycogen; structural damage to mitochondria and appearance of fat; lysosomal activity; failure of mitochondrial NAD systems; failure of nuclear NAD synthesis; and necrosis.

Besides the objections already made, namely that we know neither which of these steps is essential to cell necrosis nor which of the very early changes is the primary one, other objections have to be met. Lasiocarpine causes nuclear changes in the reticuloendothelial cells of the spleen (216), in the duodenum, and in the Kupffer cells (32), but none of these suffers necrosis. The cells of the vascular endothelium in the liver do not show nuclear changes (217) but probably undergo necrosis (4, 6). After lasiocarpine the nuclear changes in the parenchymal and Kupffer cells occur throughout the lobule but necrosis is centrilobular only (214, 217). We seem to have a plethora of reasons why the liver cell might die, but no convincing evidence of why it does. An eventual explanation will have to take into account a significant protective effect described by Hayashi and Lalich (102). Cysteine (300 mg/kg body weight) injected 15 min before, and 2 hr after monocrotaline, gave significant protection against liver necrosis in rats.

The connection, if any, between the events that lead up to necrosis and the events leading up to megalocytosis is not understood. Bull *et al.* (39) and Schoental and Magee (197) have both established that megalocytosis arises after doses insufficient to cause liver cell necrosis. In comparing Sullman's results from tissue culture (211) with those of Svoboda and his group on nuclear changes (95, 176, 216, 217) a very interesting point emerges. Nucleolar abnormalities do not appear when cellular necrosis is not in prospect. If this could be confirmed, it could be a useful lead in the problem of untangling the megalocytic from the necrotic effect of the alkaloids. An experiment reported by Bull *et al.*

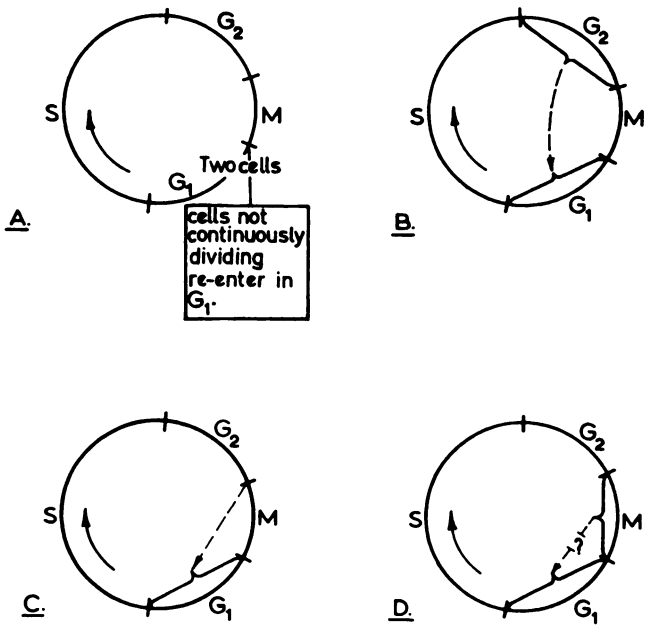


FIG. 2. A, the cell cycle; M = mitosis; G_1 = phase of preparation for S; S = phase of DNA synthesis; G_2 = resting phase; B, C, D, possible positions for "by-pass" of mitosis occurring in the pathogenesis of megalocytes; B, by-pass runs from an unknown point in G_2 to an unknown point in G_1; C, by-pass runs from early prophase (M) to G_1; D, cell enters metaphase but does not complete mitosis, re-entering G_1 without division.

(32) also lends support to the view that necrosis and megalocytosis arise through unrelated pathways. He showed that the LD50 at 2 days after a dose of alkaloid was the same for rats which had been on a long course of chronic administration of the alkaloid (0.1 LD50 3 times weekly), and which, therefore, had a high proportion of megalocytes in the liver, as for normal rats.

b. Megalocytosis. Detailed studies of cell division and its relation to DNA synthesis have led to the description of the "cell cycle" (109). Continuously dividing cells go through four phases as shown in figure 2A. Mitosis (M) is followed by an interval (G1) before the phase of DNA synthesis (S). This is separated by another interval (G2) from the ensuing mitosis. Cells that are not continuously dividing leave and rejoin the cycle somewhere in G1.

Considering the megalocyte in these terms, we assume that it does not divide, *i.e.*, it does not complete M. Occasionally it does enter M, since mitoses are seen in megalocytes, and since some contain centrioles. It is implicit in the histological descriptions of megalocytes (large nuclei, abundant chromatin) that they go repeatedly through the S phase, synthesising a new complement of DNA each time. This has been measured for the megalocytes of dimethylnitrosamine poisoning (55) but not for pyrrolizidine poisoning. It is thus apparent that megalocytes go round the cycle without completing M. The "by-pass" (fig. 2) which allows them to do so can exist in three forms:

Figure 2B, the by-pass omits M altogether, producing megalocytes with increased DNA but no centrioles.

Figure 2C, the by-pass runs from early in prophase to some part of G1, producing megalocytes with centrioles (1); this process can apparently occur more than once.

Figure 2D, the cell enters metaphase and appears in histological preparations in abnormal mitosis. What happens next is not known. Possibly these cells die. Perhaps they succeed in some resolution of metaphase other than cell division and so get back on a by-pass to G1. Perhaps occasional megalocytes do divide.

This view of the genesis of megalocytes immediately raises two questions, namely: will any antimitotic substance give rise to megalocytes? And, why do not the alkaloids act as antimitotic substances in other tissues? The answer to the first is "no." Bull *et al.* (33) have given repeated doses of colchicine and shown no liver damage and no rise in ploidy of hepatocytes. Clearly, simply stopping mitosis is not enough.

The answer to the second question is that the alkaloids do interfere with mitosis in other tissues, but only rarely give rise to necrosis and megalocytosis there. Interference with cell division has been reported in rat duodenum (32), mouse germ cells (183), Drosophila germ cells (61), human foetal liver cells in culture (104, 211), and various standard tumours (65, 131, 171). Abnormalities of the nucleus not specifically related to mitosis have been seen in Kupffer cells (32) and reticuloendothelial cells of the spleen (216). With the exception of the liver cells in culture, none of these lesions progressed to megalocytosis. Megalocytes are occasionally reported in kidney (30, 94) and lung (15, 223). It is tempting to argue from this that arrest of mitosis is a property of the parent alkaloid, but the subsequent development of megalocytosis occurs only in tissue where the alkaloid is metabolised. This, however, is stretching the evidence quite as far as it will go. If the present promising status of the pyrrolizidine pyrroles as the principal toxic metabolite of the alkaloids is confirmed, this argument would suggest that the pyrroles cause megalocytosis. The more widely distributed antimitotic effect must be looked for, either among reactions of the alkaloids themselves, or among some other derivatives of the alkaloids. The pyrrolizidine pyrroles, so far as is known, are available only to the liver, the lung and the heart (which they apparently reach from the liver), the spleen, and the kidney (144). Antimitotic effects in the duodenum (32) and the germ cells (61, 183), if not due to the alkaloids themselves, must be due to derivatives formed within reach of these tissues.

c. Carcinogenesis. In man, there is a strong connection between cirrhosis of the liver and primary carcinoma of the liver. Evidence from the study of alcoholics suggests that carcinoma becomes more common when the destructive process in the liver is arrested and the liver is allowed to regenerate (136). In laboratory animals, pyrrolizidine poisoning sometimes, but by no means always, produces a histological picture in the liver comparable to human cirrhosis. Regular continued dosing (37) produces small livers with a very high proportion of megalocytes and little regeneration. Hepatomas are not found on this regime.

Interrupted dosing, or a course of continuous dosing followed by an interval before the death of the animal (198) produces livers that contain large regeneration nodules. On this type of regime, hepatomas have been found. It appears therefore that, as in human cirrhosis, the appearance of liver cell carcinoma is associated with active regeneration of the liver after a prolonged bout of liver cell destruction. It seems to be worth investigating a possible summation of these two effects.

Mutagenesis is often discussed as a mechanism of carcinogenesis. After the demonstration of the mutagenic activity of the alkaloids in Drosophila, Schoental and Bensted (192) showed that large doses of whole body radiation (400 r), though themselves leukemogenic, did not increase the incidence of hepatoma after a single large dose (30 mg/kg by mouth) of retrorsine (see table 4, lines 1, 2, and 4). In this case, two known mutagenic stimuli did not add together to increase the yield of liver cancer. It would be interesting to know whether they would increase the yield of mutants.

Svoboda and his collaborators (177, 216) have shown that several liver carcinogens cause acute changes in the nucleus of the liver parenchymal cell. Similar changes occur after lasiocarpine, which they bracket with other well established liver carcinogens (aflatoxin B_1, 3′-methyl-4-dimethylaminoazobenzene, dimethylnitrosamine, and tannic acid). In attempting to relate them to carcinogenesis one encounters two difficulties. The changes described in the case of lasiocarpine all revert to normal over 72 hr (217). The common latent interval for pyrrolizidine carcinogenesis (table 4) is at least 1 year. In order to connect the two, one must carry the change across the latent interval in a form in which it is not detectable by present techniques. This dilemma is common enough in oncology, but until it has been dealt with, one cannot sensibly claim any relevance for acute actions of carcinogens. A second difficulty is that similar changes occur in the nuclei of reticuloendothelial cells of liver (32) and spleen (216) and an unspecified nuclear fragmentation is recorded in cells of duodenal mucosa (32). In none of these tissues does carcinogensis follow. There is, at present, no evidence from *in vivo* studies that lasiocarpine is a liver carcinogen.

4. The vascular lesion

The major impetus behind the investigation of liver vascular lesions derives from the study of veno-occlusive disease of the liver in man (see section II D 1). Vascular lesions were, however, well documented (72) before veno-occlusive disease was first described. It is now becoming clear that liver vascular lesions and lung lesions run similar courses in their early stages, and it is artificial to separate the description of one from the other. However, because of the history of the subject, it is necessary to deal with the literature on the two problems separately. Those interested must read section V A 4 and section V B together.

a. Veno-occlusive disease in animals. Successful efforts have been made to produce veno-occlusive disease in animals with pyrrolizidine alkaloids and with plant extracts. Venous occlusions practically indistinguishable from the human lesion have been described in horses (106), cows (23), rats (28, 156), and monkeys

(3–5, 7). Some alkaloids produce a much higher incidence of this lesion than others. Monocrotaline and fulvine yield the highest incidence, retrorsine and senecionine a lower one, while venous occlusions are rarely reported after lasiocarpine and heliotrine (191). The type of dose used is also very important. A single large dose (for rats, one approximating the acute LD50) is the most effective for producing this type of lesion (156). Repeated small doses of the same plant extract produce a different pathological picture leading (in rats) to cirrhosis, but never producing the typical venous occlusion (27). This effect probably accounts for the relative absence of venous occlusions from grazing animals chronically exposed to the toxic plants. Man, who is not habitually exposed to the alkaloids but probably drinks them only as medicine, and therefore occasionally and in large doses, often experience this reaction.

b. Pathogenesis of the venous occlusion. A single dose of *C. fulva* extract or dimethylnitrosamine will produce venous occlusions in the livers of rats (156, 157). Investigation of the sequence of events after a single dose (156) revealed that the collagenous venous occlusions appear late after dose. They are first seen between 7 and 10 days. Centrilobular necrosis is present at 1 day and is therefore not secondary to the occlusions. Though it was suggested in early descriptions of veno-occlusive disease that necrosis and haemorrhage in the liver were secondary to the collagenous occlusive lesions, it is clear from many other studies (section V A 1) that the alkaloids have a primary lethal action on liver cells. Centrilobular haemorrhage is seen from 2 days. Signs of hepatic outflow block, namely, portal hypertension, ascites, and engorgement of the liver, appear between 2 and 5 days (158). These effects likewise precede, and therefore cannot be secondary to, the collagenous venous occlusions seen histologically. These studies suggest that some other lesion is stopping the blood from getting out of the liver in the period 2 to 7 days after a single dose of Crotalaria or dimethylnitrosamine. For the purposes of the ensuing discussion I shall refer to hepatic outflow block in this period as "early outflow block" to distinguish it from "the collagenous occlusions" which cause outflow block from 7 days on, and which are visible in histological preparations. The collagenous occlusions probably represent repair of the lesion responsible for "early outflow block."

c. Mechanisms for the production of "early outflow block." Four possible mechanisms for the early outflow block are proposed and will be considered in the following order: centrilobular necrosis; sinusoidal outlet block; necrosis of vascular endothelium; and platelet agglutination.

Centrilobular necrosis itself was at one time thought to be a sufficient cause for obstruction to liver blood flow, on the grounds that the swollen cells would press upon, and shut off, the sinusoids.

Stoner (209) showed elegantly that this was not so in the case of liver necrosis from carbon tetrachloride, by demonstrating that liver blood flow, measured by a thermocouple implanted in the liver tissue, was not depressed before or at the time of maximal cell necrosis. This point, however, has not been widely appreciated and one still encounters the idea, based upon the mild sinusoidal congestion sometimes seen histologically after carbon tetrachloride poisoning, that large

cells must squash the sinusoids. The point takes a new importance from the current interest in enzyme induction in the liver by phenobarbitone. Sufficient doses of phenobarbitone increase liver weight, partly by production of new liver cells and partly by hypertrophy of existing liver cells. The effect may be of use in the treatment of some diseases of the liver. It is thus crucially necessary to know whether the cell hypertrophy compromises the liver circulation in any way. Studies of this precise point are lacking, but the changes of liver weight produced by doses of the order given to patients are less than the change (up to 10%) produced by the normal diurnal ebb and flow of glycogen. Stoner's studies with carbon tetrachloride have shown conclusively that large (necrotic) cells need not impede liver blood flow.

There are two recent accounts of studies of the early stages of veno-occlusive disease in rats, made by transillumination. Rappaport used fulvine (172, 173) and McLean (154) used dimethylnitrosamine. The findings are almost identical. Between 16 and 24 hr after dose the outlet end of the sinusoid is blocked by stationary columns of red cells. In McLean's description this lesion is typically patchy, groups of blocked sinusoids alternating with groups in which blood flow persists. Stasis and extravasation of red cells spreads backwards from the centre of the lobule towards the portal acinus until, at 3 days and later, no circulatory detail can be seen by transillumination. Portal blood flow (measured at laparotomy) is 60% below normal 3 days after a single dose (173). Portal pressure is significantly raised 3 days after fulvine and 5 days after dimethylnitrosamine (158, 173). In both cases this is before the first appearance of collagenous venous occlusions at 7 days. McLean has followed the later course of the lesion by transillumination. Five days after dose the centrilobular haemorrhage has receded enough for some detail to be visible. Between 6 and 10 days a new pattern of vessels emerges. Unlike the vascular pattern of normal liver, which is very regular and fan-like, reminiscent of the patterns seen in a leafless tree, the new pattern is irregular and tortuous. Some vessels show sharp angular changes of direction. Others join to form circles. This is the vascular pattern corresponding to the "by-pass channels" seen in histological preparations (fig. 1, section II D). McLean (154) postulated that they are formed by the persistence of blood flow in the groups of sinusoids remaining open during the early sinusoidal lesion at 24 hr. Segments of the central veins served by the groups of closed sinusoids are gradually abandoned in favour of these by-pass routes, and suffer occlusion by fibrosis.

It is not clear what sort of lesion blocks the affected sinusoids at 24 hr. Pre- and postsinusoidal sphincters, having their basis in expansile lining cells (148) have been described in the rat. A toxic action on sinusoid or vein endothelium which then swells and occludes the lumen seems more likely in the light of other evidence.

Allen's electronmicroscopic evidence from monkeys (4, 6) and Brooks' *et al.* from children (27a) shows that the endothelial cells of sinusoids and central veins suffer necrosis from 6 hr after a dose of monocrotaline. Fluid, blood cells, and debris penetrate the "rather loose structures of the vein wall," making it swell and shutting off the blood flow. This description is compatible both with

the transillumination story and with the action of pyrrole derivatives discussed below. Allen did not state how widespread the lesion is. If it were typical of every central vein, liver blood flow would have to stop. If it is patchy it would fit well with the transillumination findings.

Carman Davies (73) reported platelet masses in small and medium-sized hepatic veins of the rat in the period 1 to 4 days after a dose of Crotalaria extract. The existence of these masses is not in doubt, but it is impossible to say whether they are a primary cause of vascular obstruction, or a physiological reaction to other damage in the vessel wall.

d. Pyrroles and other metabolites of liver, in the production of vascular lesions. Venous occlusions in the liver are also seen after a number of other poisons. They are described in rats, mink, and ruminants after dimethylnitrosamine (48, 129, 157), in cattle (but not so far in other species) after aflatoxin (9a, 179), and in man after therapeutic doses of abdominal radiation (178). With the exception of radiation, these poisons have two points in common: they are metabolised by the liver, and they cause necrosis of liver cells.

A recent paper by Butler, Mattocks, and Barnes (46) suggested strongly that it is the pyrrole metabolites of the pyrrolizidine alkaloids that are responsible for vascular lesions in the liver and the lungs. A pyrrole derivative of retrorsine, prepared by chemical means, was given by slow injection into the mesenteric vein of rats. From 24 hr onwards, the left lobe of the liver suffered multiple infarcts arising from areas of necrosis and associated thrombosis of the wall of small branches of the portal vein. In the process of repair of the infarcted areas, fibrosis and parenchymal cell megalocytosis appeared in the left lobe while a compensatory hypertrophy of essentially normal liver tissue occurred in the right lobe. These findings fit remarkably well with the view that *alkaloid* arriving at the liver in the portal vein is harmless. In the centrilobular cells it meets an enzyme system capable of converting it to the pyrrole derivative, which then attacks the centrilobular cells themselves and the blood vessels immediately downstream of them. Conversely, pyrrole arriving at the liver attacks the lining of the first small blood vessel it meets, is mostly absorbed by these vessels, and reaches the liver cells in amounts sufficient to cause megalocytosis but insufficient to cause acute necrosis. Administration of the chemical pyrroles of retrorsine and monocrotaline into the tail vein gave rise to lesions in the lungs and nowhere else. With large doses a fatal pulmonary oedema developed within 24 hr. With smaller doses lung lesions closely similar to those which follow retrorsine and fulvine (15) appeared between 3 and 4 weeks. These findings will be discussed in the following section (V B). In 1935 Davidson wrote: "It may be that in the process of destruction of the alkaloid by the liver some substance is produced which is toxic to the vascular system." It seems that Mattocks and his collaborators (46) have now provided the evidence fulfilling this prophecy. Whether this is a matter for congratulation, on the grounds that the scientific community has hammered out techniques adequate to provide proof in an area previously inaccessible to experiment, or whether it is cause for gloom, on the grounds that it has taken 35 years to progress from reasonable hypothesis to proof, must rest with personal inclination.

Two further points can be raised in connection with this work. The pyrrole derivative seems to attack vascular endothelium only when the rate of blood flow slows down. It travels from the tail vein to the lungs without affecting any of the endothelium it passes on the way, but attacks the lung capillaries. Similarly, even with slow mesenteric injection, it spares the mesenteric and portal veins but attacks the small branches of the portal tree. It does not attack the red cells with which it must be in contact for quite as long as it is in contact with the endothelium of the small vessels.

In considering vascular lesions in the liver one sometimes sees described a centrilobular fibrosis with thickening of the walls of central and sublobular veins as late as 1 or 2 months after dose (15, 48). This is probably a reaction to chronic congestion of the liver secondary to pulmonary hypertension and cor pulmonale (121), as is often seen in human chronic heart failure. It is not part of the normal progression of veno-occlusive disease, and it lacks the abnormal vascular pattern of the by-pass channels inherent in that syndrome.

e. Summary. 1) Single, or few, large doses of the alkaloids produce veno-occlusive disease of the liver in several species including man.

2) A closely similar clinical syndrome is produced in the liver of experimental animals by a number of other poisons.

3) The lesion responsible for the syndrome is a block to the outflow of blood from the liver, arising at the outlet end of the simusoid 24 hr after dose.

4) Among several mechanisms suggested for this block to blood flow, electron microscopic evidence favours necrosis of the walls of the central veins.

B. Lung damage

Studying reports of investigations aimed at the action of alkaloids in other tissues, one frequently comes across references to a lesion in the lung. The lesions most commonly referred to include hydrothorax, pulmonary oedema and congestion, epithelialisation of the alveoli, and pulmonary arteritis. Lung lesions of this general type have been reported in rats (72, 99, 175, 186, 194, 202), mice (96), frogs (70), turkeys (2, 9), pigs (94), sheep (134), rabbits (33), and horses (219a), but not in man. The literature has been reviewed by Kay and Heath (123). Experiments specifically designed to investigate the lung pathology have been reported mainly by three groups of workers: Lalich and his collaborators in Wisconsin; Kay and his co-workers at Birmingham and Liverpool; and Barnes and his colleagues at Carshalton. I shall summarise the work of these groups separately, as differences in the dosing schedules employed make direct comparison of results impossible.

1. Lung experiments

Lalich and Merkow (133) started the present wave of interest in the lung lesions when he showed that 0.1 % of *C. spectabilis* seed in the diet caused a fatal pulmonary arteritis in rats. The same lesion followed monocrotaline, 0.003 % in the diet (132). They stressed the arteritis, but alveolar haemorrhage and oedema are

also mentioned in these reports. The lesions were found at death 4 to 6 weeks after the beginning of the feeding. A further group of papers (101, 219, 221) reported that these lungs and their mediastinal lymph nodes were full of mast cells, while other tissues contained normal numbers; that the right ventricle was hypertrophied, suggesting cor pulmonale; and that a single dose of monocrotaline caused similar fatal lesions at about 4 weeks. Turner and Lalich (221) concluded that the mast cell hyperplasia was responsible for the release of 5-hydroxytryptamine, which was causing pulmonary hypertension, cor pulmonale, and a resultant pulmonary arteritis. In all these experiments the lungs were examined at spontaneous death 3 or more weeks after the beginning of treatment. Epithelial hyperplasia in the alveoli (221), capillary thrombosis (101), and intimal hyperplasia of the lung vessels (221) were recorded.

Two series of electron microscopic studies have been made by this group. Merkow and Kleinerman (159, 160) examined the lungs after 3 weeks feeding and showed a "necrotising vasculitis of small pulmonary arteries." These vessels showed endothelial hyperplasia and an accumulation of debris under the endothelium. They did not describe the alveolar wall. Valdivia *et al.* (222, 223) examined the lungs both early (4 to 48 hr) and late (1, 2, and 3 weeks) after a single dose of monocrotaline. The earliest finding was oedema of the alveolar interstitial tissue accompanied by mast cell invasion. By 24 hr both capillary endothelial and alveolar epithelial cells were altered. At 48 hr there was a gross defect of capillary permeability (demonstrated by thorotrast leakage), and giant forms of granular pneumocytes appeared in the lungs. Valdivia *et al.* (223) concluded that the capillary lesion is the earliest change and the pulmonary hypertension is probably secondary to it.

Kay and his group have used continuous dosing, 0.1 % of *C. spectabilis* seed in the diet, throughout. This kills the animals between 5 and 8 weeks. The lungs and lung circulation were examined at 5 weeks or later. In these experiments (103, 122) all the rats had a thickened pulmonary trunk, medial hypertrophy of the small pulmonary arteries, and hypertrophy of the right ventricle. A minority ($^3/_{10}$) had pulmonary arteritis. Many animals had thickening of the fibromuscular pads on the intima of the pulmonary vein. This lesion is not described elsewhere. It suggests some kind of chronic spasm on the outflow side of the pulmonary circulation, reminiscent of the sinusoidal outlet block reported in the liver. Right atrial and ventricular pressure measurements (121) confirmed pulmonary hypertension 6 weeks from the onset of feeding. (Since the right atrial pressure was raised, there must have been back pressure on the hepatic vein and hence on the liver parenchyma at this time.) As in other reports, dilated perivascular lymphatics, alveolar exudate, large cells in the alveolar wall, and proliferation of the alveolar epithelium were recorded. This group investigated the role of mast cells and 5-hydroxytryptamine (119, 120). They found that mast cell levels correlated with other exudative changes in the lung and that the highest levels occurred terminally. They concluded that mast cell invasion is associated with right heart failure and not with the genesis of pulmonary hypertension. Measurement of plasma free, and platelet bound 5-hydroxytryptamine in rats after a 5 weeks

course of feeding showed no rise over controls. Kay *et al.* (119) concluded that the alveolar lesions and the pulmonary hypertension may follow either from an increase in capillary permeability, or from constriction of the pulmonary veins.

Barnes and his collaboratores have used single doses of Crotalaria extract, fulvine (a near relative of monocrotaline) and its N-oxide (15), and the pyrroles of retrorsine and monocrotaline (46) to produce lung lesions. Animals were killed and examined at intervals. Other animals were allowed to die spontaneously. On this regime, lesions in the lung are demonstrable from the earliest sampling. They cause death from 3 weeks onwards. Barnes *et al.* (15) reported in the first week after fulvine, dilated lymphatics and a perivascular exudate; in the second and third weeks, abnormality of the alveolar septal cells, congestion and collapse of the alveoli, and an inflammatory infiltrate around the vessels; and between 3 and 6 weeks inflammation of the vessel coats themselves and a mast cell infiltrate.

The histological picture after a single dose of monocrotaline or retrorsine pyrrole (46) follows the same course in the early stages but does not include arteritis or mast cell invasion even at the time of death (4 weeks). The earliest lesion (2 to 3 days) is oedema and congestion of the alveoli with dilated lymphatics. Later than this (1 to 2 weeks) the alveolar wall is increasingly abnormal with oedema of the interstitium, simplification of the alveolar pattern and increased cellularity of the wall. At 3 and 4 weeks abnormal large cells are seen in the septa. Electron microscopic studies of this lesion at 3 weeks after dose are still in progress (44). Preliminary results suggest that the excess cellularity of the alveolar wall is due to proliferation of capillary endothelial cells and possibly to macrophage infiltration. Butler (45) does not think there is an excess of granular pneumocytes nor a proliferation of alveolar interstitial cells. The alveolar basement membrane is abnormally thick. The megalocytes appear to be large granular pneumocytes.

Though the experiments reported by these three groups have received different emphasis in their interpretation, the actual abnormalities recorded are remarkably similar namely: I) dilated lymphatics, alveolar oedema, alveolar haemorrhage, and oedema of the alveolar interstitium, which imply an early gross increase in capillary permeability; II) proliferation of the cells of the alveolar wall and megalocytosis; and III) measurable pulmonary hypertension (thickened muscular coat of arteries, right ventricle hypertrophy, pulmonary arteritis, and terminal right heart failure).

2. Mechanism of toxicity in lung

The experiments of Barnes' group lead to the view that pyrroles are the proximate toxin responsible for lung lesions after ingestion of the alkaloids; that a capillary defect combined with, or precipitating, an abnormality of the alveolar wall is the primary lesion in the lungs; and that mast cell invasion and pulmonary arteritis are not essential stages in the genesis of the pulmonary lesion, nor in its progression to a fatal outcome.

The question of whether the capillary defect is primary, or whether it is sceondary to a rise of venous pressure due to pulmonary venous construction has not been adequately settled. From the effects of alkaloids and pyrroles upon blood

vessels in the liver it seems certain that pyrroles (and possibly other metabolites of the alkaloids) do kill capillary endothelium, and this is the most obvious explanation for the early capillary defect in the lung. However, obviousness is a poor guide to the eventual relevance of an explanation. The only evidence so far suggesting pulmonary venous constriction is the hypertrophy of muscular pads reports by Kay and Heath (122). Kay and Heath (123) found no alteration in right ventricular pressure in rats in the first 20 min after injection of small doses of monocrotaline (5 and 50 mg/kg body weight). Since the evidence of Butler *et al.* (46) suggests strongly that the lung lesion is caused by pyrrole derivatives, further experiments with pyrroles, or designed over a longer time course to allow for production of pyrroles, are necessary to settle this point.

The pyrrole experiments seem to have proved that mast cell invasion is an irrelevance. However, the measurements of 5-hydroxytryptamine levels (119) are not adequate to exclude a role of this substance because they were made between 6 and 7 weeks after the start of feeding. Relevant high levels could well occur in the first few days after a single dose, and this possibility should be investigated.

Kay and Heath (123) have stressed the role of *C. spectabilis* in the genesis of lung lesions, but this is a misleading emphasis. Monocrotaline is the active principle in this plant (101). Fulvine, not present in *C. spectabilis*, is equally active in the lungs (15). The chemical pyrrole of retrorsine is also active (46).

In assessing data on acute lung lesions, or acute death after pyrrolizidine alkaloids, some experiments of Gallagher and Koch (85) must be borne in mind. Large doses (greater than LD50) of heliotrine and lasiocarpine led to death in respiratory failure within 30 min. Large doses (1 to 6 μM/ml, $\equiv$ 200 to 400 mg/kg) of alkaloid block neuromuscular transmission in the rat phrenic-nerve-diaphragm preparation. Where large doses of alkaloid or metabolite cause a rapid fatal pulmonary oedema, respiratory paralysis could override any direct toxic action on the lung.

3. Endothelial proliferation

In studies of the vascular lesion in the liver (15, 72, 202, 217) and the lung (186, 194) there are frequent reports of partial or complete obliteration of vessels by masses of cells (for typical illustrations see ref. 15, 72, 206). This appearance is sometimes referred to as endothelial proliferation, on the assumption that the excess cells arise by division from the vessel endothelium. An alternative explanation is that they are invading macrophages. The only existing electronmicroscopic evidence (217) favours the latter.

It has been suggested that venous occlusions are first caused by proliferation of these cells followed by deposition of collagen. Other evidence (88, 230) suggests that vessel endothelium proliferates wherever blood flow is interrupted. Since present evidence (6, 46) is strongly in favour of a necrotizing action of the pyrrolizidine toxin on the walls of the small vessels, it seems probable that venous occlusions appear originally as fibrous scars marking the site of a destroyed vessel and that patches of endothelial proliferation in veins appear in histological sections cut through the area of "cul de sac" vein on either side of a collagenous oc-

clusion. The point could be settled by serial sections, but it hardly seems to merit the necessary work.

A disturbing feature emerging from an analysis of the publications on lung lesions is the practice of publishing the same, or very largely the same, experimental results in two different journals. Papers by Valdivia *et al.* (222, 223) and Heath and Kay (103, 122) cover large parts of the same ground twice. While this repetition may be necessary to reach the notice of specialists in different fields, one cannot regard it as desirable.

C. Actions on other tissues

The literature contains reports of lesions in a number of other tissues. Glomerular (6, 49a, 50, 101, 142a), tubular (33, 175, 183), and megalocytic (94) lesions are reported in the kidney. Lesions are described in brain (72, 142, 142a, 183, 203), muscle (2, 9), myocardium (133, 189), coronorary arteries (20), lymphoid tissue of thymus (99), and lymph nodes (49, 197). Failure of maturation of germ cells with progressive sterility are reported in Drosophila (61) and mice (183). None of these has been systematically investigated. The history of the lung changes, which were known but not pursued for 50 years, and which now turn out to be extremely interesting and relevant, is a cautionary tale in this respect. In particular, brain, which is so inconvenient to examine, and kidney, which is a candidate for metabolic activation of the alkaloids, should be investigated.

In 3 further directions some systematic studies have been made. These will be considered under the headings: anaemia (haemolysis and copper metabolism); mutagenesis and chromosome breakage; and antitumour activity.

1. Anaemia, haemolysis, and copper metabolism

Anaemia is reported among the late (4 or more weeks) effects of pyrrolizidine poisoning in rats (189, 197). Haemosiderin appears in the kidneys of rats and horses (101, 181) and in Kupffer cells of rats (101). It is thus likely that there is a haemolytic element in pyrrolizidine poisoning in these species. Sundareson (213) and McLean (155) both found an acute (2 to 3 days) loss of haematopoietic tissue from livers of newborn rats after senecionine and monocrotaline. Levin (137) reported bone marrow lesions in adult rats. There may, therefore, also be a failure of haematopoiesis behind the later anaemia.

Sheep occupy a special position with respect to haemolytic anaemia in pyrrolizidine poisoning. Bull *et al.* (38) reported that sheep grazing on heliotrope showed few ill effects in the first season. A small number dying in the first season had very high liver copper levels (4 to 10 times normal, 1000 ppm dry weight). When heliotrope again became available the following summer the same flock suffered very heavy losses, among whom 80% had high liver copper and haemoglobinuria. The signs of this illness were very like those of the haemolytic crisis of chronic copper poisoning. Bull concluded that the heliotrope deaths were due to chronic copper poisoning. Further very interesting experiments have shown this to be incorrect. With copper drenching to raise liver copper values, Bull *et al.* (35) have shown that sheep on grass pastures accumulate administered copper at the same rate as sheep on heliotrope pastures, but only the heliotrope groups suffer

haemolytic crises. Rats dosed chronically with heliotrine (0.1 LD50 twice weekly over months or years) (33) have liver copper levels 40 to 50 times the normal but do not develop haemolytic crises. Pigs dosed with Senecio (94) and cows dosed with heliotrope (40) do not develop high liver copper levels.

There is at present no explanation for these observations. The sheep is peculiar in breaking down pyrrolizidine to the 1-methylene derivative (section IV B). This metabolite is not recorded from other species. Perhaps it has a role in the precipitation of haemolytic crises.

2. Chromosome breakage and mutagenesis

The pyrrolizidine alkaloids are capable of causing chromosome breakage in the growing cells of the root tip of the onion (Allium) and of wild peas (*Vicia faba*) (11–14). They also do so in leucocyte cultures from the Tasmanian kangaroo, *Potorous tridactylus* (19a, b). Cysteine protects the plant tissues against this action. The alkaloids cause a significant number of mutants in successive broods of Drosophila and the effect is dose-dependent (59, 60, 64). Clark (unpublished) has shown recently (68) that pyrrolizidine pyrroles have mutagenic activity in Drosophila and Aspergillus and are capable of inhibiting the DNA virus, infectious bovine rhinobracheitis.

Theoretical mechanisms underlying chromosome breakage and mutagenesis. Mitomycin C causes breakage and reconstitution of chromosomes in mammals and is lethal to rats. Its mode of action is well documented as a cross-linking of the two strands of DNA (218). Because in chemical structure it is, in some ways, like the pyrrolizidine pyrroles, there is speculation that pyrrolizidine alkaloids may also cross-link the DNA. Experimental evidence on this point is scant. Culvenor *et al.* (68) showed that labelled alkaloid transferred its label to rat nucleic acids (not differentiated) in amounts "roughly equivalent to one molecule of alkaloid per molecule of DNA." Frayssinet and Moulé (84) showed that lasiocarpine, administered *in vivo*, caused a profound drop in DNA synthesis measured *in vitro*, and that it was the DNA itself which was effected. More work in this direction is badly needed.

Though evidence is lacking, it seems worth explaining the theoretical ways in which cross-linking of DNA might give rise to observed toxic actions of the alkaloids because the two stories can be made to fit rather well, and because of the current great leap forward in techniques for dissecting bacterial DNA.

In the language of the present day, mutation must mean altering or abridging the nucleotide sequence of the DNA; or altering the nucleotides themselves (*e.g.*, by alkylation) in such a way that they are "misread" at transcription. Somatic mutation, that is a heritable change in the behaviour of a line of cells within the organism, does not require alteration of the DNA but can arise from changes in differentiation. A change of this type can be viewed as an alteration in the distribution of "repressor protein" over the DNA.

Figure 3A represents DNA replication and separation of chromatids in a normal cell. During the S phase of the cell cycle (fig. 2., section V A 3 b) the two strands of DNA unwind to achieve replication. During mitosis the two complete new strands separate into the two new cells. Figure 3 illustrates that, where the

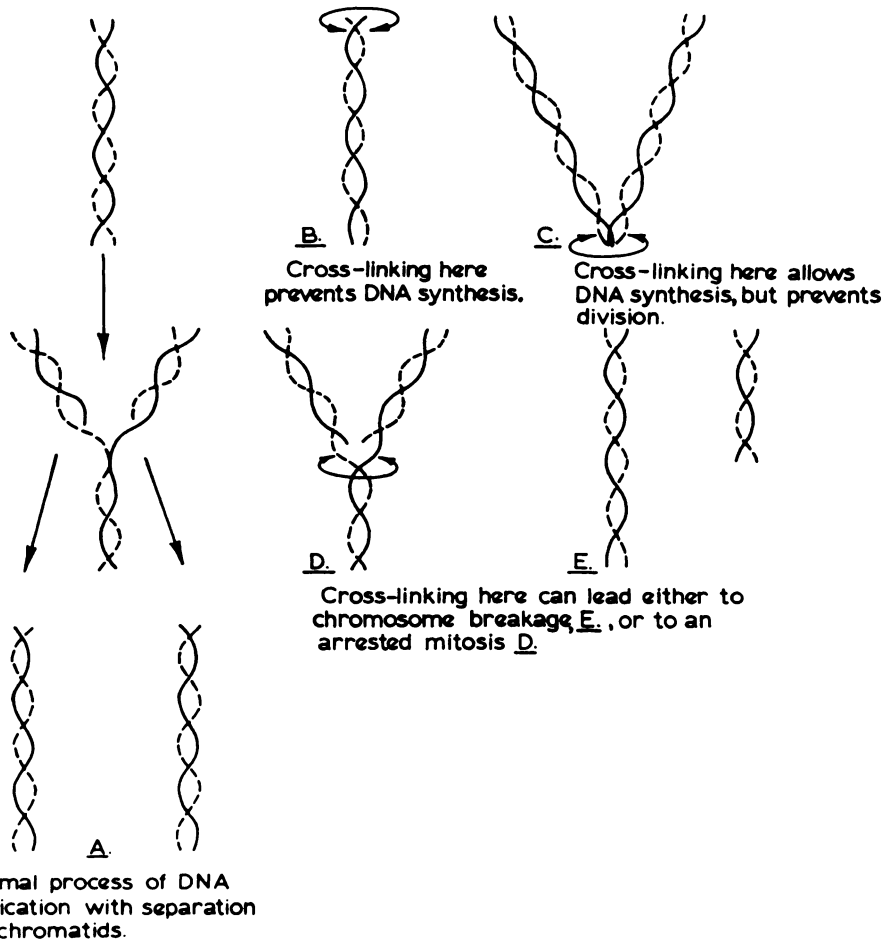


FIG. 3. A, normal replication of DNA and separation of chromatids; B, cross linking of DNA here prevents replication; C, cross-linking of DNA here allows replication but prevents separation of chromatids; D, cross linking here allows partial DNA replication and leads to arrest of mitosis or to chromosome breakage, E.

two strands are cross-linked, either unwinding or separation of chromatids, or both, will be impossible.

It is possible with this model to envisage four solutions to the problem of the cell cycle "by-pass" posed in figure 2 (section V A 3 b): Figure 3B, where the majority of chromosomes are cross-linked across the major groove close to the site or sites at which unwinding starts, DNA synthesis will be impossible and the cell will not go into mitosis. Figure 3C, where the cross-linking is such that DNA synthesis is possible but separation of chromatids is not, the cell will become a megalocyte. Figure 3D, where the cross-linking allows separation of some chromatids but others are bound too tightly to break, the cell will get "stuck" in mitosis and, presumably, die. Figure 3E, where the cross-linking is such that chromosomes

break, allowing cell division to reach completion, the daughter cells will have a complement of DNA different from that of the mother cell and will thus be mutants. Such mutations could be responsible for foetal malformations and for carcinogenesis. This model requires that the toxin bind to DNA at two sites on complementary strands of the same chromosomes, but in every other respect the binding can be random. Cross-linking at any point on the helix will produce one of the above results.

An interesting possibility arises out of figure 3C. This suggests that megalocytosis arises when the site of cross-linking is distant from the sites at which unwinding starts. It has been noted that megalocytes appear in few tissues and that these possess some items of metabolic machinery in common (liver, kidney, lung). The inference has been that the toxin responsible for megalocytosis is a metabolic product of these tissues. Another inference could be that the toxin can cross-link the DNA only in areas where it is free of repressor protein. Cells of similar metabolic capability must be transcribing from some of the same portions of the DNA. These portions of DNA will be free of repressor protein. If these portions are distant from the sites at which unwinding starts, then a cross-linking action of the pyrrolizidine toxin could give rise to megalocytosis in all such tissues.

3. Antitumour activity

The evidence for antimitotic activity led to the investigation of the alkaloids as antitumour substances. Significant antitumour activity has been demonstrated for seneciphylline, lasiocarpine, heliosupine (171), and monocrotaline (131) and in 10 of 22 alkaloids tested in a standard system, as specified by the Cancer Chemotherapy National Service Center, by Culvenor (65). There was no bias favouring inhibition of liver tumours. To ask a cancer patient to accept treatment with a relevant alkaloid would clearly be a very risky business in the present state of knowledge. The possible use of alkaloids in this direction lends a big impetus to work directed at the detailed understanding of their antimitotic action and its exact relation to their other actions. One must hope that alkaloid derivatives will be found in which antitumour activity can be isolated from other toxic effects.

VI. ACTIONS UNRELATED TO CELL DAMAGE

The effects the pyrrolizidine alkaloids were extensively investigated by Chen and his colleagues in the 1940's. They looked at carthemoidine, heliotrine, integerrimine, jacobine, lasiocarpine, longilobine, platyphylline, riddelliine, senecionine, seneciphylline, spartiodine, and supinine. The results were reviewed by Chen (50). Later papers are listed by Harris *et al.* (99) and Rose *et al.* (182). The consensus of these results was that the alkaloids have an action generally antagonistic to acetyl choline and that they lower cat (but not dog) blood pressure. McKenzie (149) investigated a large number of alkaloids and their derivatives. He found the intact ester side chains necessary for these actions. The 1:2 double bond in the pyrrolizidine ring was not necessary, platyphylline being the most effective of those he tested. N-Oxides were less effective than their parent alkaloids.

More recently, Snehalata and Ghosh (205) have looked at the newly identified alkaloid Crotalaburnine and found a similar series of actions. Jago *et al.* (114) reported hypotension (cancelled by atropine) following rapid intravenous infusion of heliotrine in sheep. Distension of the stomach in horses (33) and rats (155) suggests a possible pylorospasm. Scattered reports of convulsions after large doses (section II B 2) speak for an action in the central nervous system.

Since the systematic studies of Chen, the actions of these alkaloids, other than cell damage, have been very poorly documented. A new study is being undertaken by Raper at Melbourne (171a). One must hope this will yield results in keeping with present-day interests in pharmacology.

VII. CONCLUSION

The pyrrolizidine alkaloids give rise to a large number of toxic effects, many of them apparently unconnected with each other. They were for a long time regarded principally as liver poisons. In the past 10 years it has become clear that they also have a severe toxic action on the lung. The importance of making a systematic investigation of lesions in some other tissues has been stressed. Only one paper (42) deals with a possible autoimmune element among the toxic reactions. This aspect has been neglected and might repay investigation.

There are repeated, separate, reports of a protective effect of cysteine and methionine. Again, systematic investigation might turn these into a useful tool for investigating toxic mechanisms.

In assessing the veterinary problems and the results of feeding experiments with plants it is necessary to remember that plants can contain more than one toxin. Some plants containing pyrrolizidine alkaloids have been shown to contain a neurolathyrogen (19) and toxic levels of nitrites (127).

The study of the effects of the alkaloids upon cell division in mammals has now reached a point where it could profitably be linked up with the more detailed knowledge of DNA metabolism in bacteria. An investigation of the effects of the alkaloids and the pyrrolizidine pyrroles on DNA metabolism in *Escherichia coli* would be worthwhile.

According to the current evidence, it seems that these alkaloids present a hazard to man only in special circumstances. As a veterinary hazard, they remain important wherever pasture is poor or forage crops are likely to be heavily contaminated with the appropriate weeds. As tools for the student of biological mechanisms they are becoming increasingly interesting.

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