[CANCER RESEARCH 28, 2237–2246, November 1968]

Toxicology and Carcinogenic Action of Pyrrolizidine Alkaloids

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SUMMARY

The studies of the hepatoxic and hepatocarcinogenic pyrrolizidine alkaloids (PA) have shown that (*a*) The conventional testing for toxicity and its evaluation on the basis of death occurring a few days after dosing is meaningless for carcinogenic agents. (*b*) A single dose of a water-soluble substance, the main part of which is metabolized and excreted in a few hours after ingestion and which does not cause immediately obvious illness, can induce tumors that become apparent a long time after its administration. The induction of tumors appears to resemble a ripening process, which can be accelerated by repeated dosage of the same or of certain other compounds (and presumably could also be delayed). (*c*) The first impact of a carcinogenic agent on a cell is the inhibition of its division, possibly through interference with a "mitotic factor." (*d*) The very young are more susceptible than adults to PA's; if ingested by lactating females, the latter may remain unscathed, while the suckling young will suffer the ill effects and may develop tumors when still very young. (*e*) The elimination of the toxin in the milk may be a factor in the greater resistance of the lactating female to such agents. (*f*) Sexual hormones affect the response to PA's, adult males are more susceptible than adult females to these hepatotoxins. (*g*) The hepatotoxic entity is probably a product formed from pyrrolizidine alkaloids in the course of their metabolism in the liver and possibly also in other tissues. The effects of age, sex, diet, and animal species may thus be related to the concomitant variations in the metabolism of PA's. (*h*) Hepatotoxic PA's induce in primates mainly pulmonary and venoocclusive lesions. (*i*) Man is also susceptible to the hepatotoxic PA's. Vascular lesions described as Chiari's syndrome in South Africa and as "venoocclusive disease" in Jamaica have been correlated with the ingestion of PA's.

About 20 years ago, the elucidation of the fundamental structure of Senecio alkaloids had been reported from the laboratories of Roger Adams, Menshikov, and Warren. The alkaloids proved to be esters of 1-hydroxymethyl pyrrolizidines with substituted butyric, glutaric, and adipic acids (*cf.* 22, 52, 53). The interest in pyrrolizidine alkaloids (PA) was due to the economic hazard to livestock in various parts of the world where plants containing hepatotoxic PA are present in pastures. Discussing the chemical developments in the field of PA, Mr. Felix Schwarz mentioned the use of Senecio plants by the Bantu in South Africa for medicinal and certain other purposes, as reported by Watt and Breyer-Brandwijk (54). This suggested to me the possibility that the high incidence of primary liver cancer reported from South Africa (*cf.* Ref. 6) might be related to the use of Senecio plants. Kennaway (20) showed that the incidence of this type of cancer among the Negroes in the U.S.A. does not differ much from that of the American whites and suggested that extrinsic factors were probably involved in the development of liver cancer among the Bantu.

Data on the incidence of primary liver cancer in various countries are fragmentary, as the diagnosis itself may present some difficulty in the absence of evidence from autopsy or biopsy. In such cases, estimation of blood lactic acid might help to distinguish between primary liver cancer and metastatic liver involvement. Very high values, 5 to 10-fold the normal, have been found in the blood of patients with primary liver cancer; patients suffering from other diseases, including tumors of various organs with metastases in the liver, had values only slightly higher than healthy people (33) (Table 1).

Alkaloids extracted from *Senecio jacobaea* (the common ragwort) obtained from a local herbalist were tested in mice and rats. The mice proved refractory, but 3 out of 11 rats that survived intermittent treatment with the alkaloids for more than 8 months developed hepatoma (13).

Table 1

	mg/100 ml
In health	9–15
In various diseases including those of the liver	8–30
In primary carcinoma of the liver	60–130

Lactic acid in human blood.

Alkaloids from South African Senecios, retrorsine (Chart 1), and its *N*-oxide, isatidine, obtained as gifts from Professor F. L. Warren, proved even more effective and induced liver tumors including malignant hepatocarcinoma that spread locally in the peritoneal cavity. One of the female rats treated with retrorsine developed a papillary adenoma of the lung (43), and a similar lung tumor developed in one rat among those treated with monocrotaline (Chart 2) (42).

The carcinogenic hazard due to plants containing the hepatotoxic PA's has not been recognized earlier, probably because the effects are insidious and follow even in the absence of immediate toxic manifestations. One single dose of PA given

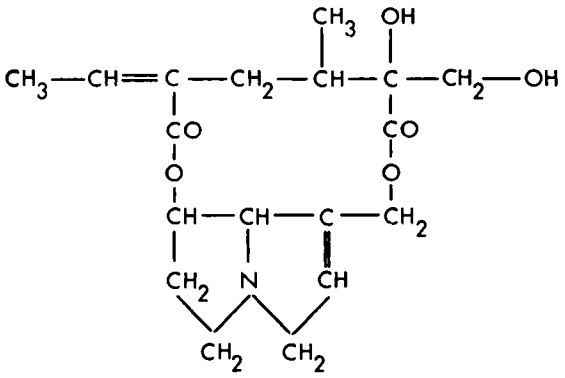


Chart 1. The chemical structure of retrorsine.

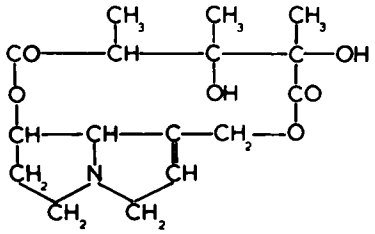


Chart 2. The chemical structure of monocrotaline.

Table 2

Boraginaceae	*Compositae*	*Leguminosae*
Amsinckia	Erechtites	Cytisus
Cynoglossum	Nardosmia	Crotalaria
Echium	Senecio	*Santalaceae*
Heliotropium	*Graminae*	Thesium
Lindefolia	Festuca	
Macrotomia	Lolium	
Rindera	Thelepogon	
Tournefortia		
Trachelanthus		
Trichodesma		

Botanical families and genera in which pyrrolizidine alkaloids have been found.

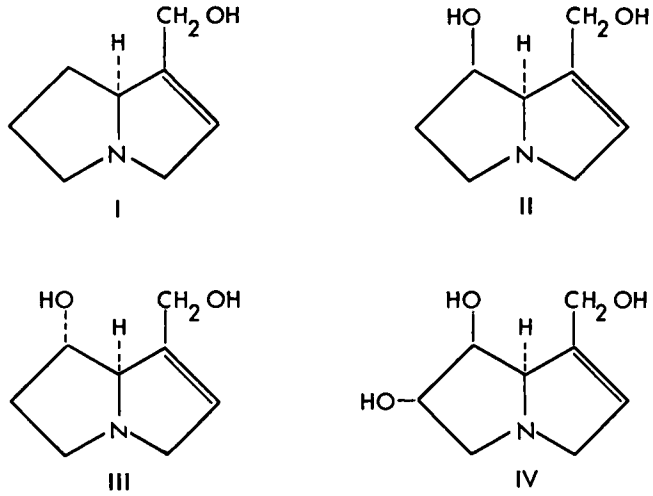


Chart 3. Chemical structures of the basic moieties of hepatotoxic pyrrolizidine alkaloids, the necins: I, Supinidine, 1-hydroxymethyl-1,2-dehydro-8α-pyrrolizidine; II, Retronecine, 1-hydroxymethyl-1,2-dehydro-7βhydroxy-8α-pyrrolizidine; III, Heliotridine, 1-hydroxymethyl-1,2-dehydro-7α-hydroxy-8α-pyrrolizidine; IV, Crotanecine, 1-hydroxymethyl-1,2-dehydro-6β,7β-dihydroxy-8α-pyrrolizidine.

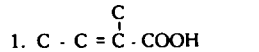
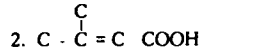
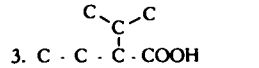
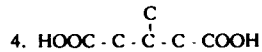
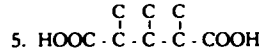
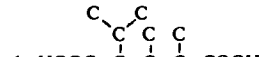
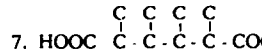
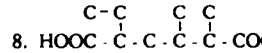
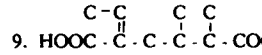
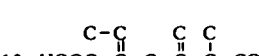
orally to a rat, which does not necessarily cause much upset, might induce progressive lesions in the liver or in the lung which will kill the animal several weeks or many months later (4, 40, 44, 45). Depending on the structure of the alkaloids, the dosing pattern, and on the length of survival, death will be due to liver necrosis, lung edema, pleural effusions, and other pulmonary and renal lesions (17), a kwashiorkor-like syndrome (34), venoocclusive disease (19, 31), liver cirrhosis, or primary liver tumors (*cf.* 37, 40).

Pyrrolizidine alkaloids are found in many plants of several unrelated botanical families (Table 2). However, not all are hepatotoxic. The hepatotoxic PA are allylic esters of the basic amino alcohols (Chart 3) with branched chain acids (Table 3); they occur among many Senecio, Crotalaria, Cynoglossum, Heliotropium, and Trichodesma plants. The relationship between the chemical structure and hepatotoxicity of the various alkaloids has been reviewed (35, 39).

The acute toxicity of various alkaloids may vary by a factor of more than 10 depending on the structure. The most toxic are cyclic diesters of retronecine with derivatives of adipic and glutaric acids (their LD_{50} is less than 100 mg/kg body weight). Among the open ester alkaloids, the diesters are more toxic than the monoesters, and those of heliotridine more so than those of retronecine. Toxicity depends on the stereochemistry of the basic, as well as of the acidic moieties, and on the number of hydroxyl groups, which by increasing water solubility affect the rate of elimination from the body. The acute toxicity of these insidiously acting PA is, however, not necessarily related to their chronic and carcinogenic effects which usually become apparent a long time after the ingestion of the alkaloids. The basic and the acidic moieties obtained by hydrolysis of the ester alkaloids are not hepatotoxic.

Many of the plants containing such alkaloids have been, and are being, traditionally used as herbal medicines for various disorders, chronic or recurrent, as emmenagogues and abortifacients (54), and not only in primitive communities; *Martindale's Extra Pharmacopoeia* (25) refers to the following uses of Senecio and Cynoglossum plants: "The ragwort, *S. Jacobaea*, and in the U.S.A. the golden ragwort, *S. aureus* in the form of extracts (1:1; dose 1-4 ml) have been used as emmenagogues, but are of doubtful value. Ragwort in the form of a decoction or ointment, has also been applied externally as vulnerary. Netherland Pharmacopoeia includes the whole plants of *S. vulgaris*, groundsel .... *Cynoglossum officinale*, Hounds tongue Poot. It has been used as a demulcent and sedative, in coughs

Table 3

Acids	
1. 	Angelic, tiglic
2. 	Senecioic
3. 	Echimidic, heliotrinic, lasiocarpic, macrotomic, trachelantic, viridifloric
4. 	Dicrotalic
5. 	Fulvinic, crispatic, monocrotalic
6. 	Grantianic, incanic, junceic, trichodesmic
7. 	Sceleranecic, sceleratinic
8. 	Jacobinic, jacolinic, jacozinic
9. 	Integerrinecic, isatinecic, retronecic, senecic, usaramoensic
10. 	Riddellic, seneciphyllic, spartioidinecic.

Carbon chains present in hepatotoxic pyrrolizidine alkaloids.

and diarrhoeas. Narcotic properties were formerly ascribed to Cynoglossum ... ." The plants may be used in single or a few large doses or repeatedly in small doses over long periods.

There are no morphologic characteristics that distinguish the species of plants which are hepatotoxic. Though a method has been described that can identify the dehydropyrrolizidine moiety (26), this cannot distinguish between the ester alkaloids that are toxic and the basic moiety that is not toxic. Only testing in animals can give a definite indication of the hazard that a plant represents.

For this purpose, the dried ground whole plant should be used and given in powdered diet to weanling male rats which are the animals of choice for this purpose. If the rats abstain from eating such a diet, force-feeding of concentrates from crude alcoholic and aqueous extracts is necessary. Isolated pure alkaloids may not represent the toxicity of the plant or of its crude extracts. This can be illustrated on *Heliotropium indicum*, one of the most widely used medicinal herbs in Asia, Africa, and South America, where it is known by local vernacular names. [*Heliotropium indicum*, Linn. (Cock's comb, turnsole); Spanish: Yerba de Borrajou, Yerba de Cotorra; Sanscrit: Srikastini, suryavarta; Hindi: Hattaguri; Malay: Jinkiukala, rumput olet, seri-bumi; French Guinea: Crête coq, herbe a malingre; Cambodia: Dramoi, damrey, etc.]. According to Ayurweda, *H. indicum* cures all intractable fevers, wounds, local inflammation, and acts as diuretic. It is also used for urticaria. The juice of the fresh herb is used for external application in snake bite and in scorpion bite. Flowers are emmenagogue in small doses, abortive in large (21). In Porto Rico *H. indicum* is used as a cure for ulcers and sore throat. In Gambia, Mandingo use it as cure for venereal diseases. In the Gold Coast, as enema, to cure erysipelas, gonorrhea, and local sores. Ashanti women boil the leaves and mix them with clay to stop abortion. In Guiana it is used for yaws, ulcers; infusions of flowers are used for menorrhagia. It is evidently used both as an abortifacient and to prevent abortion, and is used in many diseases of childhood.

Our experience with *H. indicum* illustrates that it is not possible to assess the toxicity of a plant from the toxicity of the isolated alkaloid. When weanling rats were given the dried ground whole plant (received from Ghana through the courtesy of Dr. Ashimoto) 15% in powdered diet (MRC 41B) for a month, a few died with striking liver lesions at the end of this period (Fig. 1). The rats that survived longer developed fibrotic livers and liver tumors. When the same batch of plants (kept in the laboratory in a dry state) was tested 5 years later, its feeding as 15% in powdered diet had to be extended to about 3 months in order to induce similar lesions in rats (Figs. 2–4). Evidently the alkaloids became partly hydrolyzed and less toxic on keeping. The alkaloid, indicine, which has been isolated as the main constituent of *H. indicum*, is a monoester, (-)trachelantoyl-retronecine, (29) and shows very low toxicity compared, for example, with heliotrine, a monoester of heliotridine (Chart 4). A single dose of 1000 mg/kg did not induce significant liver lesions. Another monoester, indicine, acetylated in the acidic moiety, is a minor constituent (27) and would not account for the toxicity of the plant. Paper chromatography of the crude alkaloidal extracts indicated the presence of diesters, but these, being open diesters of retronecine in which the two acidic moieties face the same side, appear to hydrolyze readily and to give monoesters on isolation.

Nonester pyrrolizidine alkaloids devoid of hepatotoxic activity have been isolated from several Crotalaria and other plant species. The presence of 1-hydroxymethyl-and 1-methoxymethyl-1,2-epoxypyrrolizidines (Chart 5) has been reported in *Crotalaria trifoliastrum* Willd and *C. aridicola* Domin., which have been suspected as the cause of esophageal lesion in horses known as Chillagoe disease in Northern Australia. It would be of interest to know whether these epoxyalkaloids can induce esophageal lesions in experimental animals.

Recently a neurolathyrogen, α-amino-β-oxalylaminopropionic acid has been found in two species of Crotalaria (5).

If force feeding of plant extracts does not cause acute death from liver necrosis, the presence of greatly enlarged parenchymal cells "megalocytosis" (8), in liver sections of rats killed after 4–5 weeks (Fig. 1) would indicate the potential hepatotoxicity of the plant, and gross lesions may develop after a longer time. On this basis 10 species of plants have been found to be hepatotoxic among 40 collected by Dr. A. Coady in Ethiopia (Table 4) (41).

We have been informed (J.B. Gillett, The Herbarium, Nairobi, personal communication) that some of these plants are being used medicinally in Eastern Africa for various purposes, including the use by women in pregnancy and parturition. The use of such plants during pregnancy and lactation might present particular hazard to the fetus or to the suckling baby.

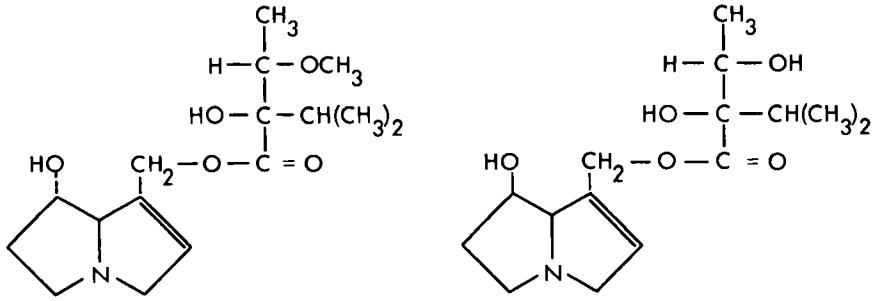


Chart 4. Chemical structures and LD_{50} of heliotrine and indicine.

It has been shown experimentally in rats that while PA is not very effective as an abortifacient, the fetus and the very young are highly susceptible to its action and may develop acute or chronic liver or lung lesions, though the mothers may remain free of ill effects (16, 36, 49). Similar observations have been made recently using other hepatocarcinogens, e.g., cycasin (48).

It is worth considering how far the widespread use of hepatotoxic herbs by women in pregnancy and lactation might be related to the occurrence of liver disease and of kwashiorkor among children in certain developing countries (34). It may be significant that *no* kwashiorkor-like syndrome has been reported among children of inmates of German concentration camps in Europe, where malnutrition was of the extreme kind.

The name "kwashiorkor" means "red boy" in Ga, one of the languages of the present Ghana and was first described as a deficiency disease by Cicily Williams (55). The name illustrates the fact that in this condition the hair of the children becomes decolorized, reddish, or almost white and very thin. This syndrome is seen mainly among 1–3 year old children, often of unmarried mothers or of those that died in childbirth. It is still considered a purely nutritional deficiency syndrome. The connection with malnutrition may indeed be significant as the effects of hepatotoxins are often more pronounced if superimposed on protein deficiency. It is of interest that regardless of the treatment given in kwashiorkor, whether of a high protein or of a high fat diet, 20% of the children admitted to hospitals still die.

Epidemiologically there does not seem to be a direct relation between the incidence of kwashiorkor, marasmus, veno-occlusive disease, and primary liver cancer. This is understandable. The more serious and acute the liver damage, the more likely will the patient die before tumors have time to develop. On the other hand, the incidence of primary liver cancer is often related to the incidence of the more chronic lesion, liver cirrhosis (*cf.* 6).

Since the discovery of the hepatotoxic and hepatocarcinogenic effects of aflatoxins, the metabolic products of the ubiquitous *Aspergillus flavus* and of certain other fungi (*cf.* 38), the tendency has developed to ascribe liver disease, including liver cancer, to mold products which are likely to contaminate food in the hot and damp climate of the tropic's rainy seasons. Fungal contamination has indeed to be considered as a possible factor, which either *per se* or in conjunction with hepatotoxins of other origin may be responsible for chronic liver disease in the tropics and subtropics. However, liver diseases of childhood are more likely to be the result of the use of hepatotoxic herbs as emmenagogues and abortifacients.

Low (6%) protein diet has been found to sensitize rats to the acute toxic effects of pyrrolizidine alkaloids and to decrease the LD_{50}(44). The survivors showed extremely fatty livers but did not develop liver tumors if they continued to be

Table 4

1. *Crotalaria laburnifolia L.*
2. *Crotalaria recta* Steud ex A. Rich.
3. *Cynoglossum caeruleum* Forsk.
4. *Cynoglossum geometricum* Bak. et C. H. Wright
5. *Heliotropium cinerascens* Steud ex DC.
6. *Heliotropium supinum* L.
7. *Senecio gigas* Vatke.
8. *Senecio myriocephalus* Sch. Bip. ex A. Rich.
9. *Senecio Schimperi* Sch. Bip. ex A. Rich.
10. *Senecio Schultzii* Höchst ex A. Rich.

Plants from Ethiopia which proved hepatotoxic to rats.

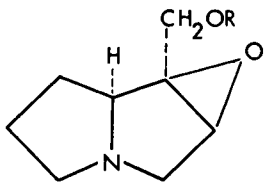


Chart 5. Chemical structures of epoxy alkaloids from the Crotalaria species. R = H; 1β,2β-epoxy-1α-hydroxymethyl-pyrrolizidine. R = CH_3; 1β,2β-epoxy-1α-methoxy-methyl-pyrrolizidine.

maintained on the low protein diet (Schoental, unpublished results). Controls, pair-fed the low protein diet showed neither fatty livers nor gross liver abnormalities. Similar effects of low (5%) protein diet on the liver lesions due to aflatoxins have recently been reported (23).

The Effects of PA in Various Species Including Primates

From field poisoning of livestock, it has been known that cows, horses, and sheep can die from liver damage when grazing on plants containing hepatotoxic PA. Similar lesions have been reproduced experimentally in livestock by feeding the toxic plants or their extracts (8, 24, 50).

Among laboratory animals, rats proved to be very susceptible and to develop acute and chronic lesions and hepatomas even after a single dose of retrorsine (40). Males are more susceptible than females and male hormones increase the susceptibility of the latter (32). Rabbits, guinea pigs, and mice are more resistant than rats, and liver tumors have not yet been induced by PA in these species. Chicken are susceptible and can develop liver tumors (9). Administration of estrogens to males was claimed to reduce late fibrotic changes (10).

Primates are susceptible to the acute and chronic effects of the alkaloids. It still remains to be shown whether they can develop tumors of the liver or of the lung

Rhesus monkeys have been reported to die with periportal liver necrosis 3–9 days after senecionine (30–75 mg/kg body weight *in toto*) given in divided doses by intravenous injections (51). Monocrotaline, given intragastrically (2 doses, 250 mg/kg each, at 14 days interval) killed 7 *Macaca speciosa* monkeys within 14–38 days, mainly from venoocclusive lesions (?).

We tested several alkaloidal preparations in monkeys *Macaca mulatta* in collaboration with Mr. D. J. Short, of the National Institute for Medical Research, Mill Hill. Two monkeys were given i.p. several doses of retrorsine and of a mixture of alkaloids from ragwort, *S. jacobaea* L, 10–30 mg/kg body weight/dose; 2 other young monkeys were given fulvine and its *N*-oxide, 10-20 mg/kg of body weight/dose i.p. or orally in sweetened drinking water (0.1 mg/ml). The treatment was intermittent; it was interrupted when the monkeys appeared to lose condition or weight. One monkey died after 3 months; the other monkeys were killed when they appeared ill. The times of survival and main lesions are summarized in Table 5. Venous occlusive lesions were present in all the monkeys (Figs. 5, 6).

So far, no evidence is available as to the susceptibility of man to aflatoxins, but a few instances of liver disease have been correlated with the ingestion of plant materials containing hepatotoxic PA (46, 56). In such cases the most striking lesions were usually vascular, and they have been described as Chiari's syndrome in 12 patients, who consumed bread contaminated with Senecio seeds (46) or as "venoocclusive" disease in children in Jamaica, where *Crotalaria fulva* L is often included in "bush teas" (7).

In rats, single doses of the PA only seldom induce venoocclusive lesions (44); the latter have been described in rats dying 9–10 days after the administration of *C. fulva* (31) or after monocrotaline (19). Such vascular lesions have been described in cows, in experimentally poisoned calves (24), and in horses (*cf.* 18, 46). It would appear that the vascular changes are one of the various manifestations of the action of PA and are more pronounced in the species that are much larger than rats. The relationship between lung congestion, changes in blood pressure, the vascular lesions and "cor pulmonale" requires further study. In Uzbekistan, U.S.S.R., a brain disease Dzhalangarsk encephalitis has been traced to the use of *Trichodesma incanum*. Feeding its seeds induced similar symptoms in rats and guinea pigs (47). The plant contains the alkaloids,trichodesmine and incanine and their *N*-oxides (57), which are likely to be hepatotoxic.

Mutagenic and Teratogenic Effects of PA

PA has been found to be mutagenic in *Drosophila* (11, 12, 14) and to cause chromosome breaks in the plant, *Allium cepa* (3); the latter could be prevented by cysteine.

Heliotrine has been shown to have teratogenic effects in the rat and to give rise to skeletal malformations (16). When senecionine was given on the 15th day of pregnancy, liver damage was induced in the fetus (49).

Some of the alkaloids have anticholinergic action and an inhibitory effect on the muscle tonus of rat and rabbit intestines. There is no parallelism, however, between the hepatotoxic, the mutagenic, and the anticholinergic action, as pointed out by McKenzie (30) (Table 6).

The active hepatotoxic entity is probably one of the products of metabolic degradation of PA. Formation of pyrrol derivatives by ring dehydrogenation (?) may be one of the early stages in metabolic transformation (28), and such products would be expected to react with sulphydryls more readily than does PA. Carbon-oxygen fission by sulphydryl compounds has been suggested to be able to transform PA into alkylating agents (15). However, it is not yet clear how alkylation could be related to the process of carcinogenesis. The mode of action of none of the known carcinogens can as yet be explained in biochemical terms.

The morphology of liver cells gives a clue to the action of PA. The immediate effect on the liver parenchymal cell is inhibition of its division. The cells grow large ("megalocytosis," (8)), they can synthesize proteins and nucleic acids, but they do not appear able to divide (1). Studies of the ultrastructure of such enlarged cells by electron microscopy confirmed that these enlarged hepatocytes are not dying cells. They have abundant rough endoplasmic reticulum studded with ribosomes; hence, protein synthesis can actively go on. These cells may have several centrosomes, but usually fewer microbodies. The distribution of intracellular organelles is irregular, and accumulations may be present in one part of the cell with deficiency in other parts, as if a factor were missing that is responsible for the organization of the cell. Whether this is a hormone or a specific intracellular agent that regulates cell division can at present only be surmised (1).

ACKNOWLEDGMENTS

My thanks are due to Professors G. Bras and P. N. Magee for the evaluation of some of the venoocclusive lesions in the livers of monkeys.

Table 5

Alkaloid	Route	Initial body weight (kg)	Survival in months	Main lesions
Retrorsine and *Senecio jacobaea*	i.p.	2.40	3.0 D	Liver: fatty, venoocclusive disease, large cells Lung: congested
Retrorsine and *Senecio jacobaea*	i.p.	7.05	3.7 K	Liver: necrosis, venoocclusive disease Lung: bronchopneumonia
Fulvine and fulvine-*N*-oxide	i.p. and p.o.	1.45	20.5 K	Liver: congested, venoocclusive disease, enlarged cells Lung: congested Pancreas: hemorrhagic
Fulvine and fulvine-*N*-oxide	i.p. and p.o.	1.25	21.0 K	Liver: necrosis, venoocclusive disease, large cells Lung: congested, mast cells

Survival time and main lesions found in monkeys treated with pyrrolizidine alkaloids. D, died; K, killed.

Table 6

	Mutagenic[a]	Anticholinergic[a]	Hepatotoxicity to rats[b]
Retrorsine	0.4		35
Senecionine	0.4	3.4	40
Fulvine	0.6		40
Monocrotaline	1.6	0.07	60
Jacobine	0.08	0.3	60
Lasiocarpine	1.00	1.0	80
Heliotrine	0.9	3.0	300
Supinine	0.2	78.0	400
Platyphylline	0.07	1000.0	not hepatotoxic

Biologic activities of several pyrrolizidine alkaloids.
[a] Activity relative to lasiocarpine = 1.
[b] Approx. LD_{50} in mg/kg body weight.

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Fig. 1. Liver section from a male rat killed one month after the beginning of feeding *Heliotropium indictum L.*, 15% dried ground plant in powdered diet. Note the enlargen parenchymal cells with deeply staining nuclei, bile duct proliferation, and infiltration of the portal zone and the hyperplastic nodule. H & E, × 200.

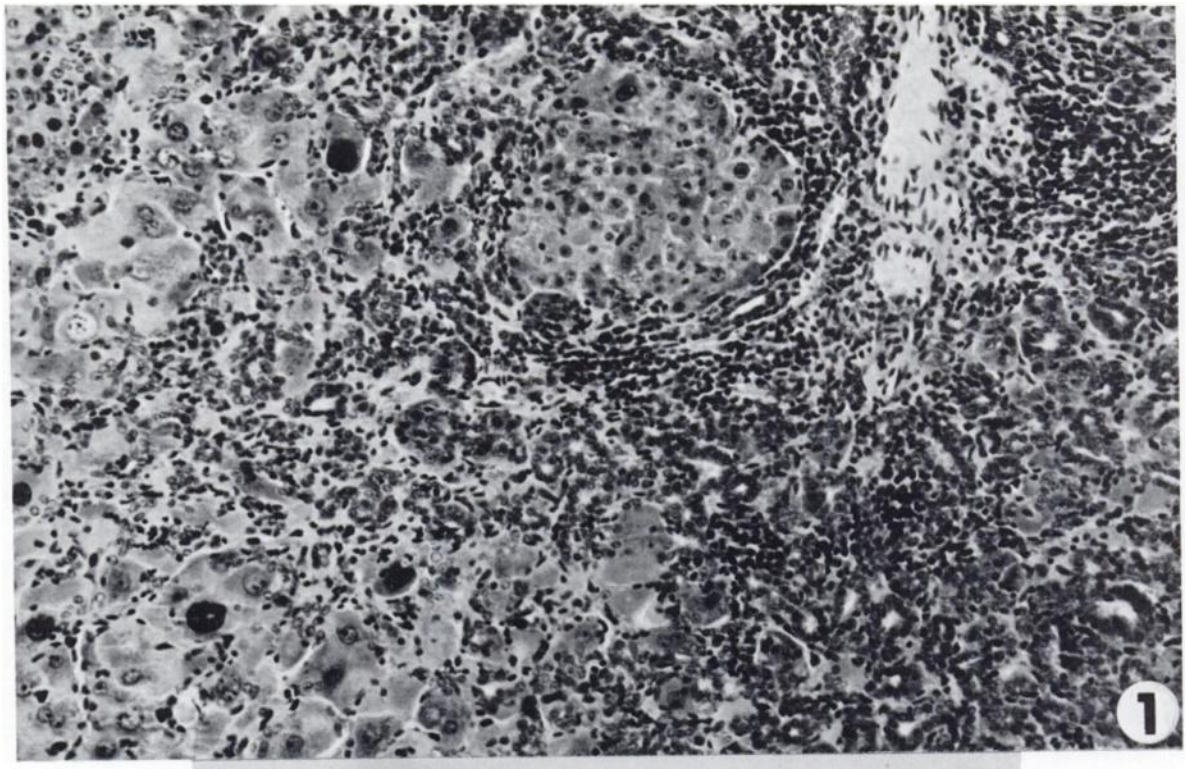
Fig. 2. Liver of a male rat that died 21 months after the beginning and 18 months after the completion of feeding 15% of *H. indicum* in diet. × 2.

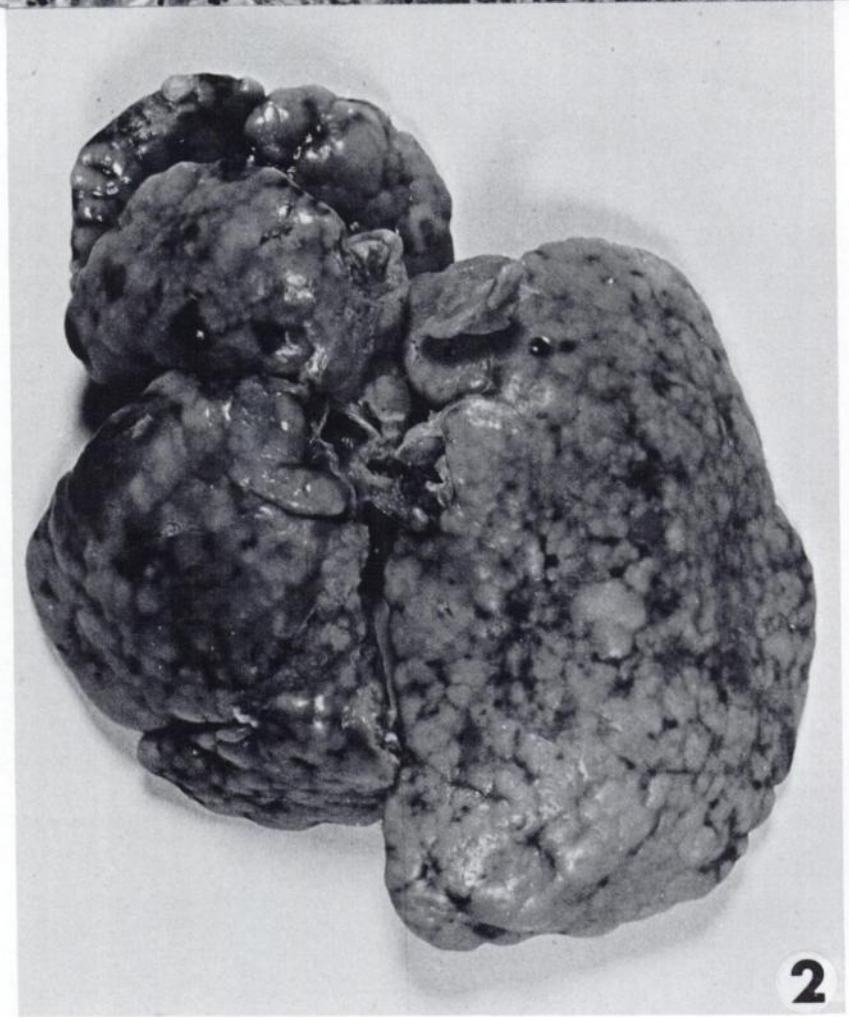
Fig. 3. Section from the liver in Fig. 2 showing a nodule of hepatocarcinoma. H & E, × 40.

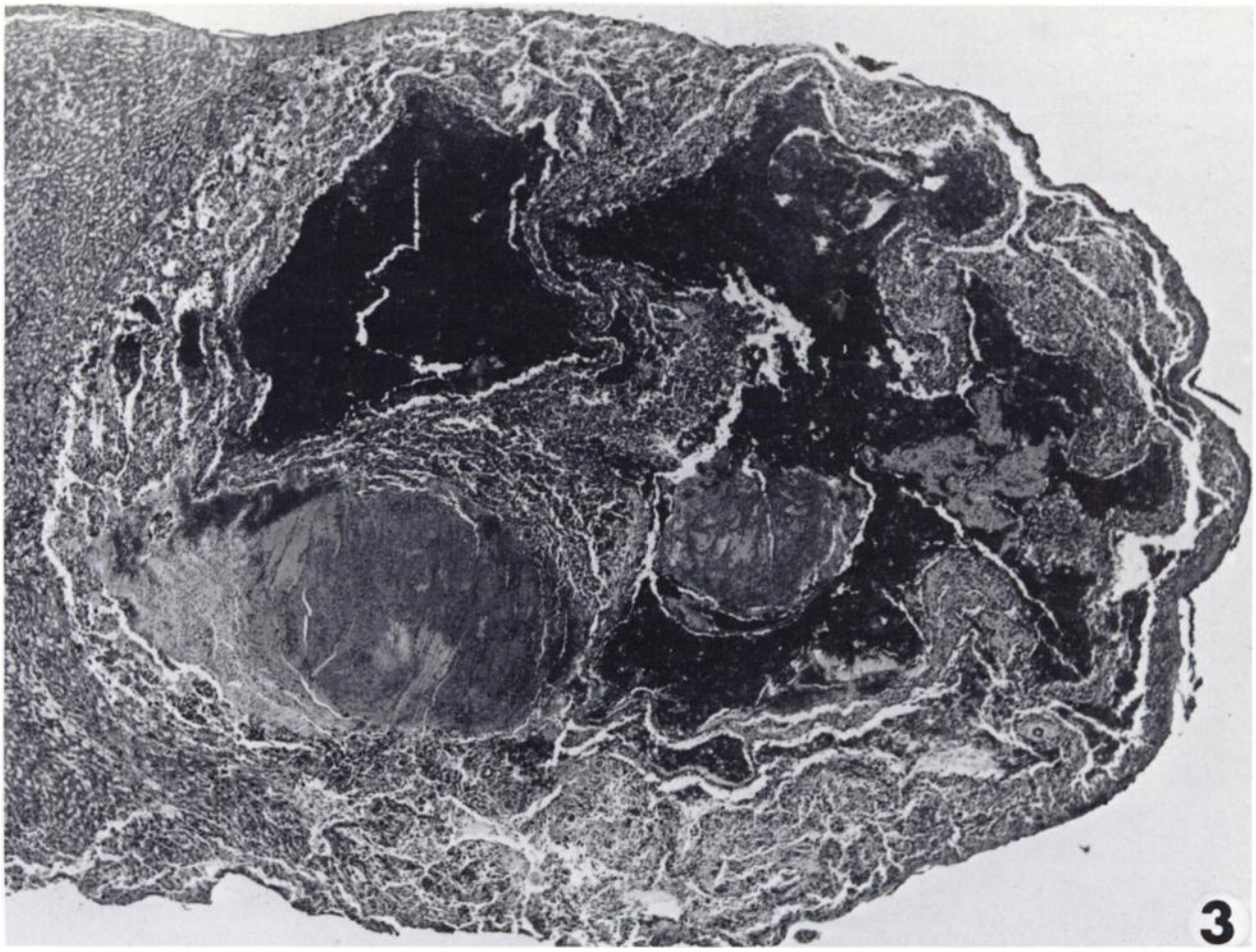
Fig. 4. Higher magnification of the tumor from Fig. 3. H & E, × 200.

Fig. 5. Liver section from a monkey killed 3.7 months after the beginning of i.p. treatment with retrorsine and *Senecio jacobaea* alkaloids showing narrowing of a central vein and blood pools. Mallory strain, × 500.

Fig. 6. Liver section from a monkey killed 21 months after the beginning of treatment with fulvine and its *N*-oxide. Note the narrowed lumen of the vein and the necrotic and fibrous changes. H & E, × 200.







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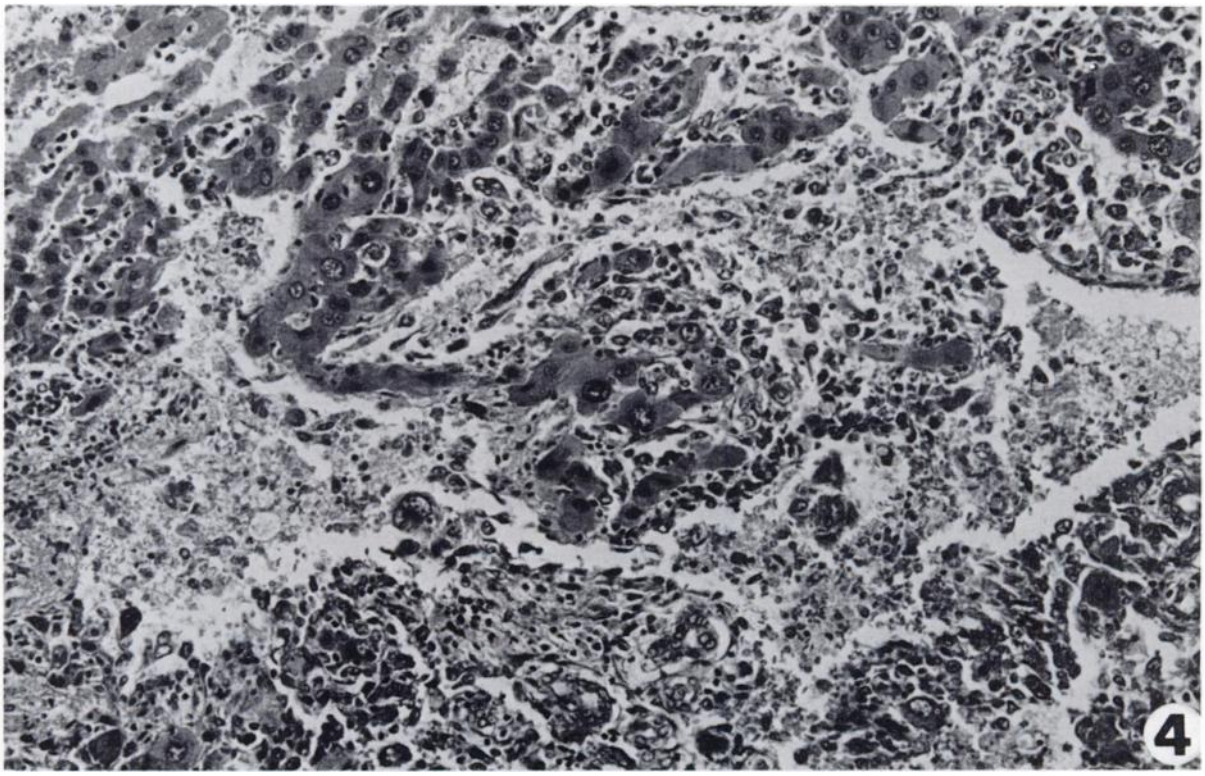

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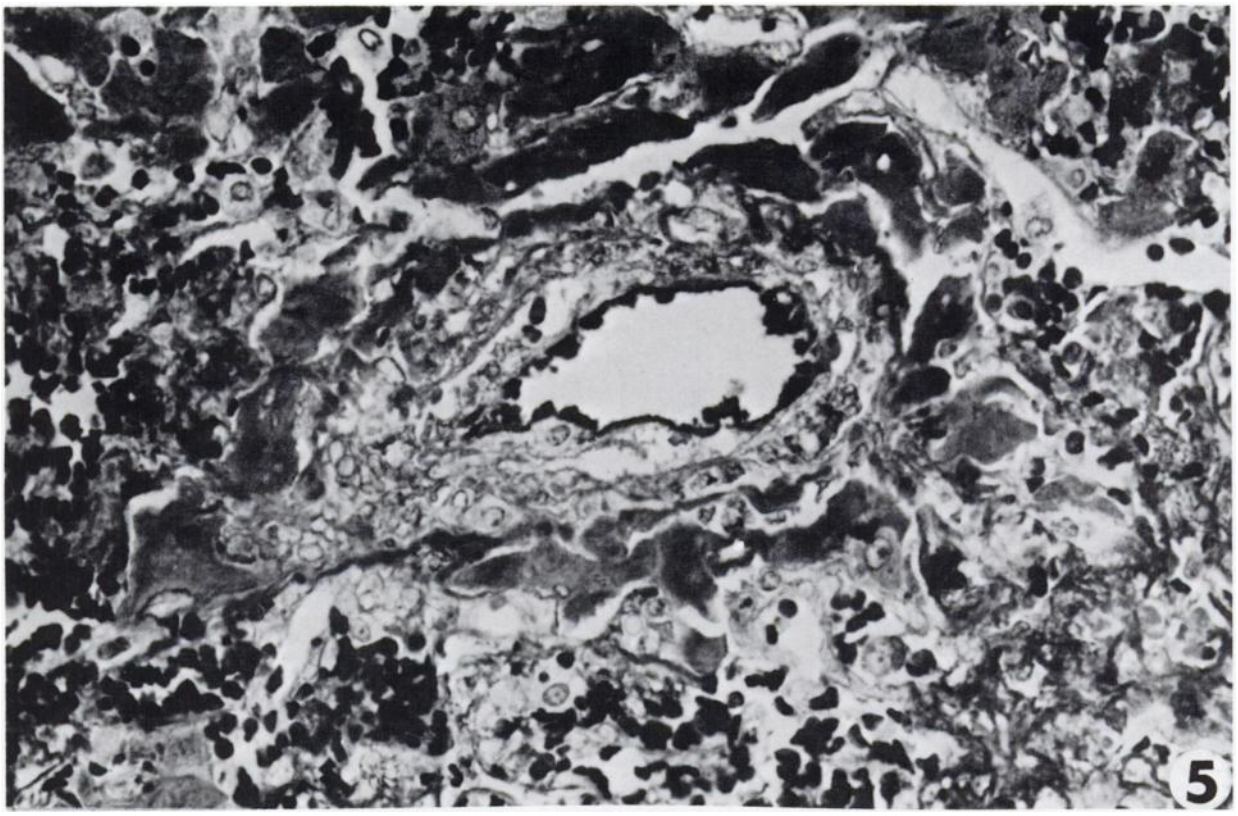

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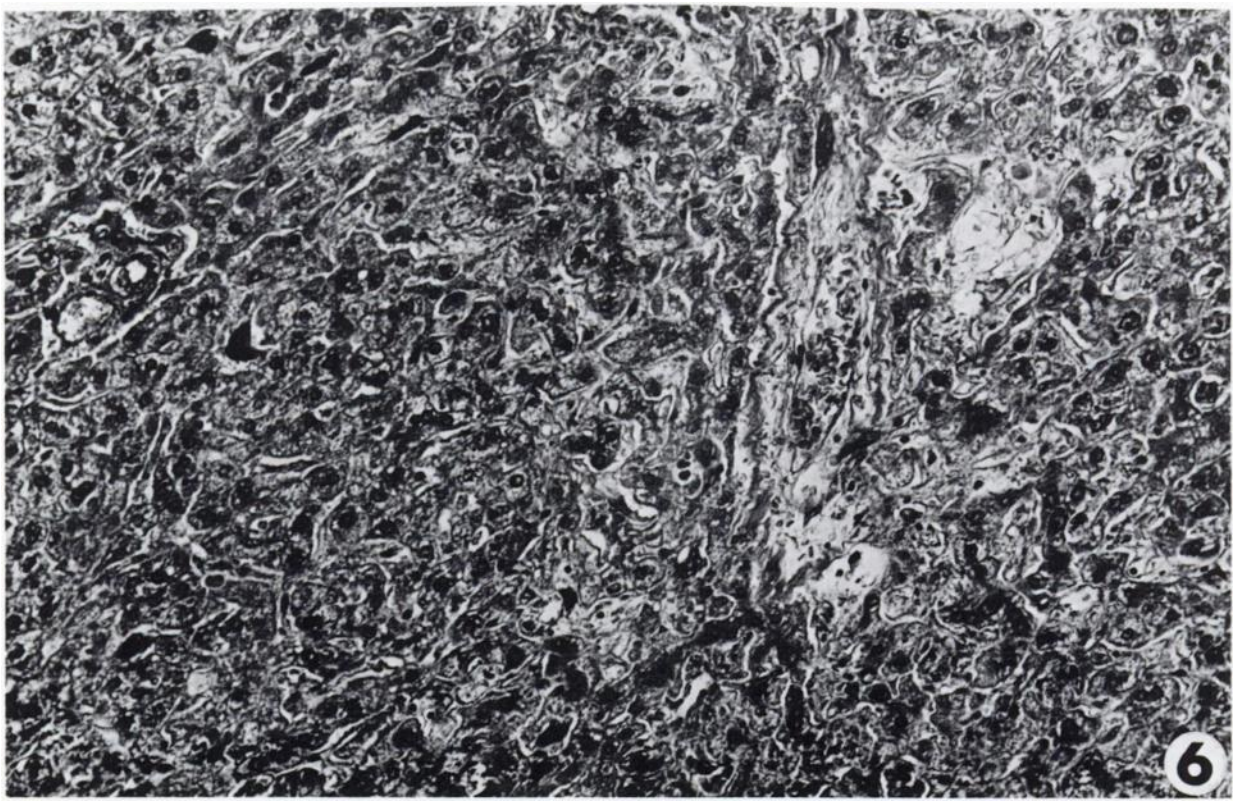

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