W N N R
C S I R

SYMPOSIA · SIMPOSIA

S 15

SYMPOSIUM on MYCOTOXINS in FOODSTUFFS
SIMPOSIUM oor MIKOTOKSIENE in VOEDSEL

SESSION 1 *SESSIE*
HUMAN NUTRITIONAL ASPECTS
ASPEKTE VAN MENSLIKE VOEDING

SESSION 2 *SESSIE*
AGRICULTURAL ASPECTS
LANDBOUKUNDIGE ASPEKTE

Organised by

THE INDUSTRIAL RESEARCH DEVELOPMENT DIVISION, C S I R

On behalf of the

National Nutrition Research Institute,
National Chemical Research Laboratory and
The Microbiology Research Unit

THE DEPARTMENT OF AGRICULTURAL TECHNICAL SERVICES

Gereël deur

DIE AFDELING BEVORDERING VAN NYWERHEIDNAVORSING, W N N R

Namens die

Nasionale Voedingnavorsingsinstituut,
Nasionale Chemiese Navorsingslaboratorium en
Die Mikrobiologiese Navorsingsgroep

DIE DEPARTEMENT LANDBOUTEGNIESE DIENSTE

EXTRA-MURAL BUILDING
UNIVERSITY OF PRETORIA
25th AND 26th FEB., 1965

BUITEMUURSE GEBOU
UNIVERSITEIT PRETORIA
25 EN 26 FEBRUARIE 1965

Printed in the Republic of South Africa
by Minerva, Pretoria
G A D 283s15 N 243

C O N T E N T S

I N H O U D

Papers being presented at the second session are published in a separate booklet by the Dept. of Agricultural Technical Services.
Referate wat by die tweede sessie aangebied word, word afsonderlik deur die Departement Landboutegniese Dienste gepubliseer.

TOXIGENIC FUNGI ISOLATED FROM CEREAL AND LEGUME PRODUCTS

J.P. VAN DER WALT

M. Sc. (Pret) Drs. (Leiden) D. Sc. Tech. (Delft)

The study reported here was carried out by Mr. de B. Scott of the C S I R Microbiology Research Group and was commenced in January 1961. The investigation is fully reported in an issue of Mycopathologia et Mycologia Applicata now in press.

SYNOPSIS

An investigation of the toxigenicity of moulds recovered from cereal and legume crops is described. Two hundred and twenty-eight mould strains representing 59 species were tested by feeding Pekin ducklings on maize meal infected with pure cultures. Forty-six strains representing Aspergillus avenaceus, A. carneus, A. chevalieri, A. clavatus, A. flavipes A. flavus, A. fumigatus, A. mangini, A. nidulans, Fusarium moniliforme, F. roseum, A. niveus, A. ochraceus, A. ruber, Paecilomyces varioti, Penicillium islandicum, P. piceum, P. rubrum, P. urticae, P. variabile and Trichothecium roseum caused death within 14days. These 22 species were subsequently fed to weaned white mice and rats. Aspergillus avenaceus, A. flavipes, A. nidulans, A. niveus, A. ochraceus, Penicillium oxalicum, P. urticae caused the death of these animals within 14 days. Aspergillus carneus, Fusarium moniliforme, F. roseum, Penicillium islandicum, P. piceum and P. rubrum showed less severe toxic effects while the remaining 9 species showed no overt signs of acute poisoning.

SAMEVATTING

Navorsing oor die toksigeniteit van skimmel afkomstig van graan en peulgewasse word beskryf. Tweehonderd agt-en-twintig skimmelstamme, wat 59 species verteenwoordig, is getoets deur Peking-eendjies te voer met mieliemeel wat met suiwer kwekings besmet is. Ses-en-veertig stamme, verteenwoordigend van Aspergillus avenaceus, A. carneus, A. chevalieri, A. clavatus, A. flavipes, A. flavus, A. fumigatus, A. mangini, A. nidulans, Fusarium moniliforme, F. roseum, A. niveus, A. ochraceus, A. ruber, Paecilomyces varioti, Penicillium islandicum, P. piceum, P. rubrum, P. urticae, P. variabile en Trichothecium roseum, het binne 14 dae die dood veroorsaak. Hierdie twee-en-twintig species is vervolgens aan gespeende wit muise en rotte gevoer. Aspergillus avenaceus, A. flavipes, A. nidulans, A. niveus, A. ochraceus, Penicillium oxalicum, P. urticae, het binne 14 dae die dood van hierdie diere veroorsaak. Aspergillus carneus, Fusarium moniliforme, F. roseum, Penicillium islandicum, P. piceum en P. rubrum het n minder ernstige toksiese uitwerking gehad, terwyl die oorblywende 9 species geen waarneembare tekens van akute vergiftiging getoon het nie.

The production of toxic metabolites by moulds has been known to occur for well over seventy years, but the prevalence of mycotoxicoses was, except in the case of ergotism, largely unrecognized and unappreciated until SARGENT et al, (1961) established that, during 1960, 100,000 turkey poults had succumbed in Great Britain to poisoning by aflatoxin, a group of hepatotoxins produced by Aspergillus flavus. It is surprising today to realise that in spite of the discovery of a host of antibiotics of fungal origin, all extremely reactive against micro-organisms, no systematic thought was given before 1960 to the possible existence of fungal metabolites toxic to higher organisms.

The now recognised widespread occurence of hepatotoxic fungi on legumes and in cereal crops has led to considerable concern among animal and human nutritionists and has caused much speculation on the possible relationship between mouldy foodstuffs and the high incidence of primary hepatic carcinoma in Africa and the Orient (MIYAKE et al., 1960; DAVIDSON, 1963; ANON, 1963; BUTLER, 1964; OETTLé, 1964; KRAYBILL & SHIMKIN, 1964).

The first paper in which reference is made to a toxic mould was published as early as 1891, when WORONIN reported on the toxicity of bread prepared from grain infected with Gibberella zeae (perfect stage of Fusarium roseum) HOYMAN (1941) established that the feeding of cereals infected with this mould caused prolonged emesis and subsequently death in pigs. PRENTICE (1959) examined various species of Fusarium for the production of emetic substances on artificial media and the following species were found to be positive: F. culmorum, F. moniliforme, F. nivale and F. poae. STOBB et al (1962) recently succeeded in purifying a toxic substance from maize infected with Gibberella zeae.

At least eight species of fungi have been incriminated in toxicoses occurring among farm animals in America. Classical signs of bovine hyperkeratosis have been produced in calves fed on substrates inoculated with certain strains of Aspergillus clavatus, A. chevalieri and A. fumigatus (CARLL et al., 1954, 1955; FORGACS et al., 1954) while toxic strains of A. flavus and Penicillium rubrum have been found to be associated with mouldy corn toxicosis of pigs and cattle (BURNSIDE et al., 1957). The species connected with these two diseases, as well as toxic strains of Paecilomyces varioti, Penicillium purpurogenum and an unidentified species of Alternaria have been isolated from feed and litter collected from areas where the poultry haemorrhagic syndrome is enzootic (FORGACS et al., 1958).

Recently WILSON & WILSON (1964) pointed out that Aspergillus flavus could produce, under certain conditions, several other toxic metabolites in addition to aflatoxin. A strain of A. flavus was demonstrated by them to produce a hitherto unrecognised toxin which caused tremors in mice. In an earlier paper, WILSON & WILSON (1961) claimed that A. flavus as well as species such as Aspergillus niger and Aspergillus luchuensis could produce sufficient oxalate in foodstuffs to cause poisoning. It had previously been shown that rabbits developed pyrexia following the injection of culture filtrates of A. luchuensis (HARKNESS et al., 1950).

Toxic moulds have also been reported from Japan. YAMAMOTO (1954) isolated a toxic strain of Penicillium urticae from malt that had caused a large number of deaths in dairy cows. Recently IIZUKA & IIDA (1962) published a paper on the chemical structure of maltorysine, a toxic metabolite of Aspergillus oryzae var. microsporus which was likewise implicated in cases of animal poisoning. Since 1940, a series of papers

have been published on the toxicity of the so-called "yellow rice" infected with Penicillium islandicum (MIYAKE et al., 1960). It was established that toxic strains of this mould produced two different metabolites which caused marked degeneration of the liver followed by cirrhosis in white mice. Toxin-producing strains of P. islandicum have also been isolated from sorghum and millet-grains in Ethiopia (COADY, 1964) and from toxic barley in Britain (cited by SARGENT & CARNGHAN, 1963).

Russian workers have also called attention to the toxicity of various fungus species. LEVITSKII & KONIUKHOVA (1947) reported that Aspergillus nidulans, A. flavus, A. fumigatus and A. niger were toxic to rabbits. JOFFE (1962, 1964) presented evidence that the mycoflora of overwintered grain was responsible for alimentary toxic aleukia in man. Three species, Fusarium poae, F. sporotrichioides and Cladosporium epiphyllum, were notable for their frequency of occurrence on overwintered grains and soil and for the preponderance among them of highly toxic isolates.

In South Africa, MITCHELL (1918) reported that maize infected with Diplodia zeae caused symptoms of poisoning in cattle. THEILER (1927) found that maize which had been artificially infected with this mould was toxic to sheep and cattle but not to pigs and horses. According to WATT & BREYER-BRANDWIJK (1962) losses occurred among livestock which had ingested Fusarium moniliforme-infected maize. STEYN (1933) concluded that for all practical purposes mould-infested foodstuffs should be considered poisonous until the contrary had been proved by extensive feeding experiments.

FORGACS & CARLL (1962) critically reviewed the literature on mycotoxicoses and stressed that the importance of toxic fungi in foodstuffs had not been appreciated in the past, especially in countries of the Western World. They further emphasized that sufficient information had been forthcoming in recent years to warrant the more extensive study of the role of fungi in human and animal disease and particularly in diseases of unknown aetiology.

In this paper a preliminary investigation of the toxigenity of various cereal moulds, a few obtained from overseas collections but the majority recovered locally, is described. Of the former Penicillium islandicum, P. rubrum and Aspergillus flavus were already known to be toxigenic, while A. effuses and A. oryzae were suspected of being so. Of the locally recovered moulds, some strains of A. flavus were isolated from toxic groundnuts; the remainder were isolated from commercial products in general use.

MATERIALS AND METHODS

A number of isolates were randomly selected from various species of fungi recovered from domestic cereal and legume products. Not many of these fungi were obtained from foodstuffs actually known to be toxic. Most of these species are, however, widespread in nature; they occur on a variety of substrates and some are known as the causal organisms of deterioration of all kinds of stored seeds (CHRISTENSEN, 1957). Strains were maintained on potato-carrot extract agar or CZAPEK's solution agar containing 20 per cent sucrose.

In addition to the fungi isolated locally, strains of a few species were secured from sources abroad. Subcultures of Penicillium islandicum P. rubrum, Aspergillus effuses, A. flavus and A. oryzae were received

from the Centraalbureau voor Schimmelcultures in Baarn and a strain of P. rubrum, was kindly supplied by Dr. JAMES G. MILLER of the Georgia Coastal Plain Experimental Station, Tifton. A total of 228 isolates representing 59 different species was studied. Of these, 26 species belonged to the genus Aspergillus while Penicillium was represented by 28 species. The remaining fungi represented two species of Fusarium and one species each of the genera Paecilomyces, Trichoderma and Trichotheceum.

Maize meal was used as substrate for the large-scale cultivation of each fungus tested. The procedure finally adopted was as follows: Freshly prepared maize meal was weighed out in 250 g quantities which, after the moisture content had been adjusted to about 40% by the addition of water, were placed in cotton-plugged 5-liter Erlenmeyer flasks. The flasks were autoclaved for 20 minutes at 15 lbs overpressure. The sterilized substrates were then inoculated with an aqueous suspension of conidia prepared from well-sporulated agar cultures of each fungus. The period of incubation necessary for optimal development varied for the different species, but maximum growth and abundant sporulation were usually accomplished after 2 to 3 weeks incubation at 26°C. The mould-infested meals were thereafter placed in shallow layers on trays and dried in an air-circulating oven at 60°C. The product was reground to fine particle size in a grain mill. A composite of this material from the contents of several culture flasks was prepared for every isolate.

Day old white Pekin ducklings were used as test animals in a screen test for the production of toxic metabolites. The ducklings were caged in groups of three each and fed an appropriate commercial chicken mash for two to three days, at which stage the birds had an average body weight of about 50 g. Thereafter they were given a ration consisting of one volume of fungus-infected maize meal and three volumes of chicken mash. As control a mixture of unmoulded maize meal and mash was employed. The ducklings were allowed to feed ad libitum. Mortality was recorded over a period of 14 days.

Feeding trials on mice and rats were carried out, if a mould species proved fatal to ducklings, one strain of each toxic species being tested on newly weaned mice and rats. The percentages of ingredients (w/w) in the test diets were: mouldy maize meal, 50.0; egg powder, 32.0; sucrose, 9.0; wheat germ oil, 0.5; cod liver oil, 1.7; agar, 1.7, vitamin mixture, 1.7; salt mixture, 3.4. (For composition of vitamin and salt mixture see Food Enrichment in South Africa, 1959). Unmoulded maize meal was employed in the control diet. Groups of ten animals for both mice and rats were housed under controlled conditions. The initial weights of the mice and rats were respectively about 12 g and 50 g per animal. Feed and water were supplied ad libitum. The animals were weighed regularly during the first four weeks and mortality was recorded over the same period.

RESULTS AND DISCUSSION

The results of the toxicity tests on ducklings are given in Table 1. Feeds containing fungus-infected meals were not regarded as toxic unless they caused the death of all three ducklings in the test group within the trial period of 14 days. Fourty six strains of 22 species were toxigenic according to this standard. These species were: Aspergillus avenaceus; A. carneus; A. chevalieri; A. clavatus; A. flavipes; A. flavus; A. fumigatus; A. mangini; A. nidulans; A. niveus; A. ochraceus; A. ruber; Fusarium moniliforme; F roseum; Paecilomyces varioti; Penicillium islandicum; P. oxalicum; P. piceum; P. rubrum;

P. urticae; P. variabile and Trichotheceum roseum.

In the remaining groups most of the ducklings survived the experiment and gained weight. They appeared to be normal at the end of the test period and had apparently suffered no ill effects from the test ration. There were a few groups in which one duckling failed to survive, but this was usually a weak poult and such unconfirmed deaths were not considered an indication of toxicity.

The results of the toxicity tests on mice and rats are given in Tables II and III.

The diets which contain meals infected with Aspergillus avenaceus, A. flavipes, A. niveus, A nidulans, A. ochraceus, Penicillium oxalicum and P. urticae proved acutely toxic to both mice and rats. All ten animals of each group died. The animals at first refused the food but began to eat sparingly on the second day. They became progressively weaker from day to day and finally succumbed within two weeks after a very restricted consumption of the mouldy diets.

Feeds containing Aspergillus carneus, Fusarium moniliforme, Penicillium islandicum, P. rubrum and P. piceum also produced toxic effects but these were less severe and the animals survived for longer. The rats proved to be more resistant, with the exception of those in the Aspergillus carneus-group, where the mortality was higher in rats than in mice.

No deaths occurred among either mice or rats on the diets containing the other fungus-infected meals. All the animals gained weight, but in some groups, especially those receiving feeds contaminated with Aspergillus mangini and Penicillium variabile, they had an unhealthy appearance. In these two groups weight gain was also very much lower than in the other groups. Growth was much more rapid in both rats and mice on diets containing meals infected with Aspergillus clavatus, A. flavus, A. fumigatus and Trichotheceum roseum, and was approximately equal to that of the control groups in the animals of the A. chevalieri, A. ruber and Paecilomyces-groups

Variation in the susceptibility of different animal species to fungal toxins has been reported by other workers. Comparative feeding trials in Britain have shown, for instance, that ducklings are extremely susceptible to aflatoxin while mice are resistant to experimental poisoning over short periods (ALLCROFT & CARNAGHAN, 1963).

Toxin production by filamentous moulds probably occurs on a much greater scale than has heretofore been appreciated. This is evident from the fact that in the present investigation toxin production has been demonstrated in ten species concerning which no previous reports of toxicity have appeared in the literature.

Producers of balanced animal feeds have already been cautioned about the hazards associated with the use of materials affected with aflatoxin and it is now apparent that a similar watchfulness will have to be advocated in regard to other toxic mould metabolites. At present no chemical or physico-chemical methods for the determination of such metabolites are available, so that suspected feeds will have to be assayed by feeding trials.

It is self-evident that in the light of the information now available the possible effects of mould infestation of foodstuffs on

human health will have to be reconsidered. This question will be of particular importance in the Orient, where mould preparations e.g. of the Asp. flavus-oryzae group are used in a variety of foods and condiments.

Studies on the chemical structure and toxicological properties of the metabolites produced by the toxic moulds identified in this study as well as studies on methods for their determination, are in progress and will be reported on in due course.

Acknowledgements

This work was supported in part by a grant from the South African Oilseeds Control Board.

Table I

Species of fungi and number of strains tested for the ability to cause acute toxicoses in ducklings.

Fungus species	No. of strains examined	No. of strains found toxic
Aspergillus		
alliaceus THOM & CHURCH	1	0
amstelodami (MANG.) THOM & RAPER	6	0
avenaceus G. SMITH	1	1
candidus LINK	4	0
carneus (V. TIEGH.) emend. BLOCH	4	2
chevalieri (MANG.) THOM & CHURCH	6	2
clavatus DESM.	2	2
effusus TIRABOSCHI	3	0
flavipes (BAIN. & SART.) THOM & CHURCH	3	3
flavus LINK	10	6
fumigatus FRESENIUS	3	2
mangini (MANGIN) THOM & RAPER	2	1
nidulans (EIDAM) WINT.	5	3
niger VAN TIEGHEM	10	0
niveus BLOCH	1	1
ochraceus WILHELM	5	3
oryzae (AHLB.) COHN	2	0
repens (CDA.) DE BARY	3	0
restrictus G. SMITH	2	0
ruber BREM.	4	1

Table I (cont.)

Fungus species	No. of strains examined	No. of strains found toxic
sydowi (BAIN. & SART.) THOM & CHURCH	3	0
tamarii KITA	6	0
terreus THOM	5	0
ustus (BAIN.) THOM & CHURCH	3	0
versicolor (VUILL.) TIRABOSCHI	5	0
wentii WEHMER	5	0
Fusarium		
moniliforme (SHELD.) emend. SNYD. & HANS.	10	2
roseum (LINK) emend. SNYD & HANS	2	2
Paecilomyces		
varioti BAINIER	5	2
Penicillium		
aculeatum RAPER & FENNELL	2	0
brevi-compactum DIERCKX	5	0
charlesii G. SMITH	4	0
chrysogenum THOM	3	0
citrinum THOM	3	0
cyclopium WESTLING	5	0
expansum LINK	4	0
frequentans WESTLING	3	0
funiculosum THOM	5	0
herquei BANIER & SARTORY	5	0
implicatum BIOURGE	2	0

Table I (cont.)

Fungus species	No. of strains examined	No. of strains found toxic
islandicum SOPP	3	1
janthinellum BIOURGE	3	0
multicolor GRIGOR. - MANOIL. & PORADIELOVA	2	0
nigricans (BAIN.) THOM	3	0
notatum WESTLING	5	0
oxalicum CURRIE AND THOM	5	5
piceum RAPER & FENNEL	1	1
pulvillorum TURFITT	3	0
purporogenum STOLL var. rubrisclerotium THOM	5	0
raistrickii G. SMITH	3	0
rubrum STOLL	2	2
simplicissimum (OUD.) THOM	3	0
steckii ZALESKI	5	0
thomii MAIRE	1	0
urticae BANIER	2	2
variabile SOPP	5	1
viridicatum WESTLING	5	0
Trichotheceum viride PERS.	5	0
Trichotheceum roseum VAN BEYMA	3	1

Table II

Mortality in mice and rats fed on a diet containing fungi toxic to ducklings

Fungus species and strain no.		Deaths per 10 animals		Average day of death	
		Mice	Rats	Mice	Rats
Aspergillus avenaceus	G-826	10	10	7	6
A. carneus	G-243	3	8	-	-
A. flavipes	K-805	10	10	8	10
A. nidulans	G-106	10	10	7	7
A. niveus	G-132	10	10	6	6
A. ochraceus	K-804	10	10	9	8
Fusarium moniliforme	M-702	4	2	-	-
F. roseum	G-448	10	2	10	-
Penicillium islandicum	CBS-18	9	3	-	-
P. oxalicum	M-555	10	10	12	9
P. piceum	G-345	6	1	-	-
P. rubrum	P-13	10	2	11	-
P. urticae	G-391	10	10	6	7
Control (unmoulded diet)		0	0	-	-

Table III

Growth rate of mice and rats fed on a diet containing various toxic fungi which failed to cause death

Fungus species and strain no.	Average weight gain per animal (g.)							
	Mice				Rats			
	1 Week	2 Weeks	3 Weeks	4 Weeks	1 Week	2 Weeks	3 Weeks	4 Weeks
Aspergillus chevalieri G-54	5.1	9.8	11.7	12.8	28	62	83	102
A. clavatus M-61	3.5	7.3	9.6	10.9	23	57	74	95
A. flavus M-63	4.7	8.8	10.4	11.5	28	59	78	92
A. fumigatus M-567	4.0	6.8	9.0	10.4	24	52	71	93
A. mangini G-47	0.8	2.1	3.2	4.5	20	37	39	48
A, ruber G-27	5.8	10.4	12.8	13.7	35	67	93	108
Paecilomyces varioti M-565	4.6	8.5	10.2	11.4	29	52	78	99
Penicillium variable M-548	2.8	4.7	6.5	8.8	16	31	53	75
Trichotheceum roseum G-432	4.1	8.0	9.6	11.0	24	44	70	81
Control (unmoulded diet)	6.4	11.2	13.0	13.8	36	69	93	110

References

ALLCROFT, R. & CARNAGHAN, R.B.A. 1963. Toxic products in groundnuts I. . Biological effects. Chem. & Indust. 12th Jan. 1963.

ANON 1963. Moldy peanuts and liver cancers. J. Am. Med. Ass. 184: 57.

BURNSIDE, J.E., SIPPEL, W.L., FORGACS, J., CARLL, W.T., ATWOOD, M.B. & DOLL, E.R. 1957. A disease of swine and cattle caused by eating moldy corn II. Experimental production with pure cultures of molds. Am. J. vet. Research 18: 817 - 824.

BUTLER, W.H. 1964. Experimental toxicity and carcinogeniaty of aflatoxin. Paper presented at the Symposium on Mycotoxins in Foodstuffs, March 18th and 19th 1964, held at the Massachusetts Institute of Technology, Cambridge (Mass.), U.S.A.

CARLL, W.J.., FORGACS, J. & HERRING, A.S. 1954. Toxicity of fungi isolated from a food concentrate. Am. J. Hyg. 60 : 8-14

CARLL, W.T., FORGACS, J., HERRING, A.S. & MAHLANDT, B.G. 1955. Toxicity of Aspergillus fumigatus substrates to animals Vet. Med. 50 : 210-212.

COADY, A. 1964. Aflatoxin. Brit. Med. J. 1; 1510.

CHRISTENSEN, C.M. 1957. Deterioration of stored grains by fungi. Bot. Rev. 23 : 108-134

DAVIDSON, C.S. 1963. Plants and fungi as etiologic agents of cirrhosis. New. Eng. J. Med. 268 : 1072-1073.

Food enrichment in South Africa - a study of principles of food enrichment and their application to food policy in South Africa, with special reference to the use of fish flour for the protein enrichment of bread. 1959. C S I R Research Report 172, South African Council for Scientific and Industrial Research, Pretoria.

FORGACS, J. & CARLL, W.T. 1962. Mycotoxicoses. Adv. Vet. Science 7 : 273-382.

FORGACS, J., HERRING, A.S. & MAHLANDT, B.G. 1954. A toxic Aspergillus clavatus isolated from feed pellets. Am. J. Hyg. 60 : 15-26.

FORGACS, J., KOCH, H., CARLL, W.T. & WHITE-STEVENS, R.H. 1958. Additional studies on the relationship of mycotoxicoses to the poultry hemorrhagic syndrome. Am. J. vet. Research 19 : 744-753.

HARKNESS, W.D., LOVING, W.L. & HODGES, F.A. 1950-51. Pyrexia in rabbits following the injection of typical mold cultures. J. Am. Pharm. Ass. Sci. ed. 39-40 : 502-504

HOYMAN, W.G. 1941. Concentration and characterization of the emetic principle present in barley infected with Gibberella saubenetti. Phytopath. 31 : 871-885

IIZUKA, H. & IIDA, M. 1962. Maltoryzine, a new toxic metabolite produced by a strain of Aspergillus oryzae var. microsporus isolated from the poisonous malt sprout. Nature, Lond. 196 : 681-682

JOFFE, A.Z. 1962. Biological properties of some toxic fungi isolated from overwintered cereals. Mycopathol et Mycol. Appl. 16 : 202-221

JOFFE, A.Z. 1964 Toxin production by cereal fungi causing toxic alimentary aleukia in man. Paper presented at the Symposium on "Mycotoxins in Foodstuffs", March 18th and 19th, 1964, held at the Massachusetts Institute of Technology, Cambridge (Mass.) U.S.A.

LEVITSKII, B.G. & KONIUKHOVA, V.B. 1947. O toksichnosti kormov porazenni naalii pleseyani (On the toxicity of feed contaminated with common fungi). Veterinaria 24 : 40-43. (Cited by FORGACS and CARLL, 1962).

MITCHELL, D.T. 1918. A conditions produced in cattle feeding on maize infected with Diplodia zeae. 7th & 8th Reports of the Director of Veterinary Research, U. of S.A. 1918 : 425-437

MIYAKE, M., SAITO, M., ENOMOTO, M., SHIKATO, T. and ISHIKO, T. 1960. Toxic liver injuries and liver cirrhosis induced in mice and rats through long term feeding with Penicillium islandicum Sopp-growing rice. Acta Path. Jap. 10 : 75-123

PRENTICE, N. 1959. Production of emetic material by species of Fusarium. Nature, Lond. 184 : 1319.

SARGENT, K. & CARNAGHAN, R.B.A. 1963. Groundnut toxicity in poultry : Experimental and chemical aspects. Brit. vet. J. 119 : 178 - 184

SARGEANT, K., SHERIDAN, A., O'KELLY, J. & CARNAGHAN, R.B.A. 1961. Toxicity associated with certain samples of groundnuts. Nature, Lond. 192 : 1096-1097

STEYN, D.G. 1933. Fungi in relation to health in man and animals. Onderstepoort J. vet. Sci. 1 : 183-212

STOBB, M., BALDWIN, R.S., TUITE, J., ANDREWS, F.N. & GILLETTE, K.G. 1962. Isolation of an anabolic, uterotrophic compound from corn infected with Gibberella zeae. Nature, Lond. 196 : 1318

THEILER, A. 1927. Die Diplodiosis der Rinder und Schafe in Süd-afrika. Deutsch Tierärctl. Wschr. 35 : 395 - 399

WATT, J.M. & BREYER-BRANDWIJK, M.G. 1962. The medicinal and poisonous plants of southern and eastern Africa. E & S Livingstons Ltd., Edinburgh and London.

WILSON, B.J. & WILSON, C.H. 1961. Oxalate formation in moldy foodstuffs as a possible factor in livestock toxic disease. Am. J. vet. Research 22 : 461 - 969

WILSON, B.J. & WILSON, C.H. 1964. Toxin from Aspergillus flavus: Production on food materials of a substance causing tremors in mice. Science. 144 : 177-178.

WOTONIN, M. 1891. Ueber das "Taumelgetreide" in Sud-Ussurien. Botan. Zeit. 49 : 81-93

YAMAMOTO, T. 1954. Studies on the poison-producing mold isolated from dry malt. J. Pharm. Soc. Hapan 74 : 797-801.

OETTLé, A.G. 1964. Cancer in Africa, especially in regions South of the Sahara. J. Nat. Cancer Inst. 33 : 383-439

KRAYBILL, H.F. & SHIMKIN, M.B. 1964. Carcinogenesis related to foods contaminated by processing and fungal metabolites. Advances in Cancer Research 8 : 191-246

TOXIGENIC DEMATIACEAE

L. Gouws
M.Sc. (U.O.V.S)
National Nutrition Research Institute, C S I R

SYNOPSIS

Moulds belonging to the family Dematiaceae have usually been regarded as harmless saprophytes. Recent work has indicated, however, that they may be of importance in diseases of unknown aetiology. A few mycotoxicoses caused by Dematiaceae are briefly described and a short account is given of work on toxic Dematiaceae done in this laboratory

---oooOooo---

SAMEVATTING

Skimmels wat tot die familie Dematiaceae behoort word gewoonlik as onskadelike saprofiete beskou. Onlangse werk het egter aangetoon dat hul van belang mag wees by siektes van onbekende oorsprong. 'n Aantal mikotoksikoses wat deur Dematiaceae veroorsaak word is kortliks beskryf. 'n Kort oorsig is ook gegee van werk gedoen op toksiese Dematiaceae in hierdie laboratorium.

---oooOooo---

With the realization in recent years that moulds may play an important role in diesease of unknown aetiology much effort has been directed towards the study of toxigenic fungi. The majority of workers in the field of mycotoxicoses have, however, limited their investigations to the so-called "storage fungi", notably the genera Aspergillus, Penicillium and certain species of Fusarium. This was especially the case after British workers discovered that groundnut poisoning in poultry was caused by a toxin elaborated by the fungus Aspergillus flavus. Recently the field of study has been growing even narrower and papers are frequently published on the biological and pathological properties of Aspergilli and in particular Aspergillus flavus, while comparatively little is reported on other toxigenic moulds, for instance, the less known but widely distributed toxigenic mould belonging to the family Dematiaceae of the order Moniliales, class Fungi Imperfecti. The purpose of this paper is to review very briefly a few mycotoxicoses caused by toxigenic dematiaceae and to discuss our work on a few of these moulds.

MYCOSES RECOGNISED TO BE DUE TO TOXIC DEMATIACEAE

a. Stachybotrys atra

One of the first mycotoxicoses associated with a dematiaceous fungus derives its name, Stachybotryotoxicosis, from that of the causative mould Stachybotrys atra. This fungus has usually been regarded as a harmless saprophyte. It is world-wide in distribution and is noted for its ability to decompose cellulose (Siu, 1951). Under certain conditions, and especially in Russia, hay or straw naturally infected with this mould may become extremely toxic to horses (Forgacs and Carll, 1962). Although stachybotryotoxicosis is primarily a disease of horses, other farm animals such as cattle, sheep and swine may also be affected. Equine stachybotryotoxicosis is seasonal The first cases appear in the winter when the horses are stabled and the disease generally disappears after the animals are turned out to pasture. The incidence of the disease may vary from year to year and from one season to another. Thus in one area many animals may be affected one year and only a few the next, while in another area the situation may be reversed. The rate of spread of this toxicosis may vary widely. On some farms the disease may afflict the entire herd within 6 to 8 hours after exposure to toxic hay, while on other farms it may take two to three weeks before all the animals become sick. Equine stachybotryotoxicosis is neither infectious or contageous and animals apparently do not develop an immunity since the disease can

recur in the same horses during a single season or in succeeding years in horses previously affected. The disease can take an acute or chronic form, and the mortality depends on the type of toxicosis developed. It appears to be highest among animals affected with the acute form of the disease and lowest in the first stages of the chronic form. The general pathological findings in the acute and chronic forms of the disease are essentially the same and consist of profuse haemorrhage and necrosis in many tissues.

b. Pithomyces chartarum

Recent work in New Zealand has established that facial eczema disease of sheep in that country is cause by a toxin produced by the fungus Pithomyces chartarum (Percival, 1959; Done et al. 1961; Mortimer 1963). As in the case of stachybotryotoxicosis, facial eczema disease of sheep is seasonal. The disease usually occurs in the autumn, especially if this is preceded by a hot dry summer followed by an abundance of rain. The pastures on which facial eczema most commonly occurs are perennial rye grass (Lolium perenne) and white clover (Trifolium repens). Like other mycotoxicoses facial ezcema is neither infectious nor contageous and tends to be localised within a given area, so that one particular pasture may be toxic while adjoining pastures are non-toxic. The principal clinical signs of facial eczema in sheep are loss of condition, icterus and photosensitization. The toxic metabolite of Pithomyces chartarum was isolated by Synge and White (1959) and named sporidesmin. The empirical formula of the substance was reported by these authors to be $C_{19}H_{21}O_6N_3S_2Cl$.

c. Periconia minutissima

A disease strikingly similar to facial eczema occurs in the U.S.A. in cattle that have grazed on mouldy Bermuda grass (Forgacs & Carll 1962). The predominant fungus associated with mouldy Bermuda grass toxicosis is a strain of Periconia minutissima. This toxicosis also occurs chiefly in the autumn, especially after frost and rain, when the dead grass becomes infected with various fungi. The toxicosis may, however, occur at other seasons if a factor such as drought kills the Bermuda grass and this event is followed by rain. Cattle fed on Bermuda grass contaminated with Periconia minutissima develop hepatogenous photosensitization, but control studies with pure culture media on which this fungus has been grown have not yet been carried out.

d. Alternaria and Cladosporium species

Classical signs of poultry haemorrhagic syndrome have been produced in young chickens fed on grain infected with an unidentified Alternaria species isolated from feed and litter collected from the major broiler areas in the United States (Forgacs & Carll, 1962). In addition, toxic strains of Alternaria tenuis, Cladosporium epiphyllum and Cladosporium fagi have been isolated from samples of overwintered grain known to cause alimentary toxic aleukia in humans (Joffe, 1964). The toxins secreted by Cl. epiphyllum and Cl. fagi are very similar and have been named in accordance with the species that produce them, viz.:

$CH_3(CH_2)_9CH = CH(CH_2)_{13}-C^{O}_{SH}$ Epicladosporic acid

$CH_3(CH_2)_9CH = CH(CH_2)_9-C^{O}_{SH}$ Fagicladosporic acid

TOXIGENIC DEMATIACEAE ISOLATED IN SOUTH AFRICA

In South Africa there occurs a disease of sheep that bears a striking resemblance to all the abovementioned mycotoxicoses and especially to facial eczema. This disease, namely "Geeldikkop", is also seasonal. It usually makes its appearance in the summer months shortly after the first rains have fallen, especially if these are followed by a period of hot dry weather. The disease is prevalent in the Karoo, the North Western Cape and the Southern Free State and is responsible for extremely high annual losses to the sheep-rearing industry of this area. In certain years, however, the disease may occur only on isolated farms or in isolated camps on affected farms. "Geeldikkop" disease of sheep is neither infectious or contageous and all attempts to transmit the disease have failed (Brown, 1959 a & b). The Karoo annual Tribulus terrestris is generally considered to play a role in the aetiology of the disease. However, no toxic principle capable of inducing "Geeldikkop" symptoms in healthy sheep has yet been isolated from this plant.

Dr. P.R. Enslin of the National Chemical Research Laboratory, who was responsible for the major proportion of the work on the chemistry of Tribulus terristris, has suggested, after careful consideration of the climatic and edaphic factors in "Geeldikkop" and facial eczema, that "Geeldikkop" disease may also be caused by a fungus. He stresses, however, that in view of the differences in the pathology of the two diseases it is unlikely that the same mould can be responsible for facial eczema and "Geeldikkop". In collaboration with Dr Brown of

Onderstepoort, Dr Enslin has isolated thirty pure cultures of fungi from Tribulus plants. These were fed to sheep but the results were negative.

Recent Experimental Work

In a recent study conducted at the N N R I the work of Enslin and Brown was repeated, but in addition moulds were isolated from Tribulus litter, and fed to experimental animals.

a. Source of samples

Tribulus terrestris plants and litter were collected from two farms in the Victoria West district in which frequent outbreaks of "Geeldikkop" had occurred in the past. Plants were also collected from a farm in the Bethuli district (O F S) where a severe outbreak of "Geeldikkop" had occurred the week before our arrival. This outbreak was confined to an old land on which Tribulus plants were growing in abundance. Microscopic examination of these plants together with those collected in the Karoo revealed that Alternaria type spores commonly occurred on the dry and dead leaves and twigs. In all, 130 pure cultures of fungi were obtained. The majority of these isolates were Alternaria tenuis. The remainder consisted of a few unindentified Helminthosporium and Stemphylium species and an assortment of Mucors, Aspergilli and Penicillia.

b. Oral toxicity tests (small animals)

In view of the proponderance of dematiaceae, twenty eight of these isolates were selected for oral toxicity tests on day-old Game Hampshire chicks. All tests were run in triplicate. The rations consisted of a mixture of one volume of fungus-infected maize meal and three volumes chicken mash. The results are shown in Table 1. Histopathological examinations of the livers of chicks which succumbed revealed areas of necrosis.

Table 1

Effect of mould cultures on day-old chicks

Culture	Code. No	Deaths per total no. in group	Days on which death occurred
Helminthosporium sp.	K 6	3/3	12, 13, 15
Alternaria tenuis	BFB 27	3/3	14, 16, 16
A. tenuis	SGB 4	2/3	8, 14
A. tenuis	BFB 9	1/3	5
Stemphylium sp.	BFB 24	1/3	9
Stemphylium sp.	BFG 16	1/3	7

Although only two of the twenty eight cultures tested were definitely toxic to chicks, a number of other isolates apparently also produced small amounts of toxin as chicks fed on these moulds were slightly smaller than the controls at the end of the feeding trial, i.e. after 20 days. It was therefore decided to repeat this experiment on day-old Pekin ducks, as these have been shown to be highly susceptible to mycotoxins. The results of this experiment are shown in table 2.

Table 2

Effect of mould cultures on day-old ducklings

Cultures	Code No.	Deaths per total no. in group	Days on which death occurred
Helminthosporium sp.	K 6	3/3	4, 4, 5
Alternaria tenuis	BFB 27	3/3	1,1, 2
A. tenuis	BFB 22	3/3	1, 3, 3
A. tenuis	BFB 30	3/3	5, 5, 5
A. tenuis	BFB 31	3/3	2, 3, 5
A. tenuis	SGB 4	3/3	1, 3, 4
A. tenuis	BFB 9	1/3	4
A. tenuis	K 3	3/3	1, 2, 5

The toxicity of two of the cultures (Helminthosporium sp.; K 6 and Alternaria tenuis; BFB 27) was also tested in newly weaned rats. Groups of 10 rats were used for each of the cultures tested. The diets consisted of 50% mould-infected maize meal mixed with a suitable ration. At the end of one week 6 out of the 10 rats fed on the Alternaria-infected meal and 9 out of 10 fed on the meal infected with Helminthosporium sp. had died. The remaining rats were killed. Histopathological examinations of the livers revealed areas of necrosis.

c. Toxicity of Alternaria tenuis (BFB 27) and Helminthosporium sp. (K 6) to sheep.

Five adult merino sheep were used for this experiment. They were fed lucerne hay ad libitum and dosed daily with 5 g moulded maize meal per kg body weight. After two weeks the dose was increased to 10 g mould-infected meal per day. The results are given in table 3.

Table 3

Effect of mould cultures on adult merino sheep

Culture	Code No.	Sheep no.	Day of death or discharge
Alternaria tenuis	BFB 27	9846	24 (died)
A. tenuis	BFB 27	15473	34 (discharged)
Helminthosporium sp.	K 6	14493	15 (died)
" " "	K 6	15516	30 (slaughtered)
" " "	K 6	12633	42 (discharged)

Two sheep were discharged after 34 and 42 days in an apparently healthy condition. One (15516) was slaughtered and tow died after 15 and 24 days. Post mortem examination revealed necrosis in many tissues. The symptoms were not those of "Geeldikkop".

d. Toxigenic Alternaria sp. from cereal and legume products.

Recent work in this laboratory has shown that Alternaria sp. are among the most numerous moulds occurring on kaffir corn seed and malt (Scott, 1964). The question therefore arose whether these moulds could produce toxic metabolites. Seven pure cultures of Alternaria tenuis were isolated from kaffir corn seed and tested for toxicity on day-old ducklings. A few cultures of Alternaria previously isolated from maize meal and groundnuts were also tested. The results are given in Table 4.

Table 4

Effect of mould cultures on day-old ducklings

Source	Culture	Code No.	Deaths per total no. in group	Day on which death occurred
Kaffir corn	Alternaria	AS 2	3/3	3, 5, 6
"	"	AS 5	3/3	3, 4, 4
"	"	AS 6	3/3	4, 4, 5
"	"	K 287	3/3	3, 4, 5
Groundnuts	"	G 468	3/3	3, 4, 4
Maize meal	"	37	3/3	2, 4, 7

GENERAL CONCLUSION

Too little experimental work has yet been done to justify any definite conclusion, but the findings suggest that toxic Dematiaceae may be an important factor in diseases of unknown origin characterised by liver necrosis and haemorrhage. It is of special interest that toxic strains of these moulds have been isolated from kaffir corn seed in view of the large quantities of kaffir corn consumed by human beings in South Africa and elsewhere.

REFERENCES

BROWN, J.M.M. (1959a) Advances in "Geeldikkop" (Tribulosis ovis) research 1. The history of "Geeldikkop" research. J.S. African Vet. Med. Assoc. 30: 97-111.

BROWN, J.M.M. (1959b) Advances in "Geeldikkop" (Tribulosis ovis) research 3. The epizootology of "Geeldikkop". J.S. African Vet. Med. Assoc. 30: 403-417

DONE, J., MORTIMER, P.H., TAYLOR, A. & RUSSEL, D.W. (1961). The production of sporidesmin and sporidesmolides by Pithomyces chartarum. J. gen Microbiol. 26: 207-222

FORGACS, J. & CARLL, W.T. (1962). Mycotoxicoses. Advances in Vet. Sci. 7: 273-382.

JOFFE, A.Z. (1964). Toxin production by cereal fungi causing toxic alimentary aleukia in man. Paper presented at the Symposium on "Mycotoxins in Foodstuffs", held at the Massachusetts Institute of Technology, Cambridge (Mass.) U.S.A.

MORTIMER, P.H. (1963). The experimental intoxication of sheep with sporidesmin, a metabolic product of Pithomyces chartarum. Res. vet. Sci. 4: 166-185.

PERCIVAL, J.C. (1959). Photosensitivity diseases in New Zealand XVII. The association of Sporidesmium bakeri with facial eczema. N.Z. J. Agric. Res. 2: 1041-1056.

SCOTT, DE B (1964). Fungus infestation of cereal and legume products. Progress report for the period 1.4.64 - 30.9.64, Project 9131-4203. South African Council for Scientific and Industrial Research, Pretoria.

SIU, R.G.H. (1951). Microbial decomposition of cellulose. Reinhold Publishing Corp., New York.

SYNGE, R.L.M. & WHITE, E.P. (1959). Sporidesmin: a substance from Sporidesmium bakeri causing lesions characteristic of facial eczema. Chem & Ind. 1959, p. 1546.

GIFTIGE SKIMMELMETABOLIETE

K.J. van der Merwe
Dr. rer. nat. Göttingen
Nasionale Chemiese Navorsingslaboratorium

SAMEVATTING

'n Aantal chemiese aspekte verbonde aan die probleem van mikotoksikose word behandel. Na aanleiding van die aflatoksiene, 'n groep lewergifstowwe met kankerverwekkende eienskappe afkomstig uit die skimmel Aspergillus flavus, word daar tans op groot skaal deur verskeie navorsingsgroepe na ander giftige skimmelmetaboliete gesoek. 'n Kort opsomming word gelewer van die giftige skimmelmetaboliete wat vandag bekend is, asook van werk wat in hierdie verband aan die Nasionale Chemiese Navorsingslaboratorium van die W.N.N.R. gedoen word.

TOXIC FUNGAL METABOLITES

SYNOPSIS

Certain chemical aspects connected with the problem of mycotoxicosis will be discussed. As a result of the discovery of the aflatoxins, a group of carcinogenic hepatotoxins produced by the fungus Aspergillus flavus, several research groups are now carrying out an extensive search for new toxic fungal metabolites. A short summary will be given of the toxic fungal metabolites which are known today and of work being carried out in this connection at the National Chemical Research Laboratory of the C.S.I.R.

Alhoewel dit reeds vir meer as 70 jaar bekend was, dat skimmels tot die vorming van giftige metaboliete in staat is, was die verband tussen hierdie gifstowwe en sekere siekteverskynsels, met die enkele uitsondering van ergotisme,

in die algemeen nie erken nie. Dit is vandag moeilik om te verklaar dat, nieteenstaande die ontdekking van 'n groot aantal antibiotika uit skimmels, geen sistematiese ondersoek na die effek van skimmelmetaboliete op die gesondheid van hoër organismes deurgevoer is nie.

Die onlangse ontdekking van die aflatoksienprobleem en die besef dat daar in alle waarskynlikheid ander giftige skimmelmetaboliete van vergelykbare belangrikheid mag bestaan, het nie alleen wêreldwye belangstelling vir die probleem van mikotoksikose uitgelok nie, maar het tot 'n grootskaalse soektog na nuwe giftige skimmelmetaboliete gelei. Voor 'n bespreking van werk wat in hierdie verband aan die Nasionale Chemiese Navorsingslaboratorium gedoen word, is dit wenslik om enkele chemiese aspekte van mikotoksikoses, wat by 'n sodanige ondersoek van belang is, aan te dui.

Die chemiese strukture van 'n aantal bekende mikotoksiene word in Figuur 1 weergegee. Dit wissel van relatief eenvoudige stowwe soos die oksalaat ioon (volgens literatuur o.a. vir die giftigheid van Aspergillus niger verantwoordelik) en β-nitropropioonsuur ('n kankerverwekkende gifstof wat deur verskillende skimmels gevorm word) tot ingewikkelde molekule soos byssochlamiensuur en die peptied-agtige islanditoksien. Byssochlamiensuur, wat 'n relatief ongewone negelid ring en twee anhidried groeperings bevat, word deur die skimmel Paecilomyces varioti gevorm en staan in verband met sg. haemorrhagiese siekte by kuikens. Islanditoksien is 'n kankerverwekkende stof wat deur die skimmel Penicillium islandicum gevorm word en hoofsaaklik vir die giftigheid van sg. Islandia geel rys in Japan verantwoordelik is. Drie van die boustene in hierdie molekuul is van 'n baie buitengewone aard. Behandeling met 'n baie verdunde ammoniak oplossing verwyder die chloor atome en ontneem die stof sy giftigheid.

Verdere voorbeelde word in Figuur 2 weergegee. Sporidesmin, 'n ingewikkelde organiese molekuul wat beide chloor en swawel bevat, is 'n hepatotoksiese metaboliet wat deur die skimmel Pithomyces chartarum gevorm word en vir die voorkoms

van sg. „facial eczema" onder skape in Nieu-Seeland verantwoordelik is. Een van die beter bekende voorbeelde van mikotoksikoses onder mense is ergotisme, 'n toestand wat sporadies in Sentraal en Noord Europa aangetref is en nog so onlangs as 1953 as 'n epidemie in Frankryk geraporteer is. Dit volg op die gebruik van brood berei uit rog waarop die skimmel Claviceps purpurea voorkom. Die aktiewe komponente is alkaloïede wat afgelei kan word van die basiese skelet Lisergiensuur.

Hierdie voorbeelde toon dat mikotoksiene nie tot enige bepaalde klas van organiese verbindings behoort nie, maar chemies grootliks van mekaar kan verskil. Dit verklaar nie alleen gedeeltelik die uiteenlopende aard van siekteverskynsels wat hul veroorsaak nie, maar maak dit onmoontlik om in die geval van 'n onbekende mikotoksien vooraf die geskikste isolasieprosedure te bepaal. Sodanige metode kan slegs uit 'n sistematiese ondersoek, waarin elke stap van skeiding met behulp van giftigheidsproewe gevolg word, verkry word.

Die konsentrasie waarin mikotoksiene op skimmelbesmette voedsel aangetref word, is gewoonlik in die orde van een deel per miljoen en laer. Alhoewel dit vandag met behulp van moderne chromatografiese skeidingsmetodes moontlik is om die gifstof selfs by hierdie groot verdunning te isoleer, is die opbrengs uit hierdie bron gewoonlik ontoereikend vir 'n uitgebreide chemiese en toksikologiese ondersoek. Daar word dus in die reël gepoog om reeds op 'n vroeë stadium van die ondersoek verskimmelde materiaal van 'n hoër giftigheid met behulp van suiwer skimmelkulture in die laboratorium te berei. By sodanige stap moet egter in gedagte gehou word dat die veranderde toestande, waaronder die skimmel in die laboratorium gekweek word, gifstofproduksie in sommige gevalle sterk kan beïnvloed.

Die invloed van omgewingsfaktore op gifstofproduksie word duidelik in geval van die sg. „alimentary toxic aleukia" geïllustreer. Hierdie siekte, wat veral tussen 1942 en 1947 onder mense in Rusland voorgekom het, het sy toppunt in 1944 bereik toe dit in sekere dele van die Orenburg distrik vir die dood van meer as tien persent van die bevolking verantwoordelik was. Dit volg op die gebruik van brood

berei uit graan wat geruime tyd deur sneeu bedek was en deur die skimmels Fusarium sporotrichoides, Cladosporum epiphylum en Cladosporum fagi besmet is. Die gifstowwe wat dié siekte veroorsaak is in Figuur 3 aangetoon. Dit is gevind dat geen van hierdie gifstowwe by gewone kamertemperatuur gevorm word nie, maar wel wanneer die skimmel by lae temperature, veral rondom $0^{o}C$ groei. Van dié gifstowwe is so stabiel dat dit na 6 jaar nog in opgebergde graan voorkom en ook nie tydens die bak van brood vernietig word nie.

Nog 'n voorbeeld word in Figuur 4 aangegee. Dikumarien is die mikotoksien wat vir die sg. haemorrhagiese soetklawer vergiftiging onder beeste verantwoordelik is. Dit word deur 'n Aspergillus uit kumarien gevorm, 'n stof wat in die klawer self voorkom. Daar die skimmel self geen kumarien kan sintetiseer nie, is mikotoksienproduksie streng van die teenwoordigheid van kumarien in die voedingsmedium afhanklik.

Die giftigheid van mikotoksiene is nie noodwendig altyd tot mens en dier beperk nie, maar affekteer soms ook die groei van mikro-organismes of plante. Dit word weerspieël in die feit dat sekere antibioties aktiewe stowwe, wat gedurende die soektog na antibiotika gevind en as te giftig bewys is, vandag van ons bekende mikotoksiene is. 'n Enkele voorbeeld hiervan is patulien (Figuur 4), 'n bekende antibioties aktiewe metaboliet uit Penicillium urticae wat verantwoordelik is vir moutvoervergiftiging by beeste.

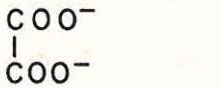
Dit moet egter onthou word dat „giftige" antibioties aktiewe metaboliete en „giftige" phytotoksiene in 'n ander verband geïsoleer is en dus nie noodwendig altyd met betrekking tot mikotoksikose van groot belang hoef te wees nie. Sekere skimmels is naamlik in staat om meer as een gifstof te sintetiseer, soos duidelik in die geval van Aspergillus flavus geïllustreer kan word (Figuur 5). Die belangrikheid, wat elkeen van hierdie stowwe met betrekking tot mikotoksikose besit, hang van die relatiewe hoeveelhede waarin hul voorkom en hul relatiewe giftigheid af.

Aspergillus ochraceus is 'n skimmel wat wyd verspreid in die natuur voorkom

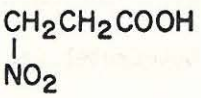
en waarvan die giftigheid onlangs deur die Mikrobiologiese Navorsingsgroep van die W.N.N.R. aangetoon is. Aspergillus ochraceus maak 'n deel uit van die mikroflora van „katsuo bushi" en ander gefermenteerde vispreparate wat in die Verre Ooste gebruik word, terwyl sy vermoë om 'n gewensde geurverandering tydens die fermentering van koffie te bewerkstellig, deur 'n patent gedek word. Daar is aan die einde van 1963 aan die Nasionale Chemiese Navorsingslaboratorium van die W.N.N.R. met 'n chemiese onder soek na giftige skimmelmetaboliete uit hierdie skimmel begin.

'n Giftige stam van die skimmel is op groot skaal in die laboratorium op gesteriliseerde mieliemeel gekweek, en die ekstraksie prosedure en fraksioneringsmetodes wat aangewend is om die gifstof suiwer te isoleer, word in Figuur 6 opgesom. Giftigheidsproewe, wat beide kwalitatief en semi-kwantitatief van aard is, is na elke stap gebruik om die suiwering van die gifstof te volg en seker te maak dat die aktiewe komponent nie gedurende hierdie behandeling ontbind nie. Dit kon uiteindelik aangetoon word dat slegs een metaboliet, waaraan die naam ochratoksien A toegeken is, vir die giftigheid van hierdie skimmel verantwoordelik is.

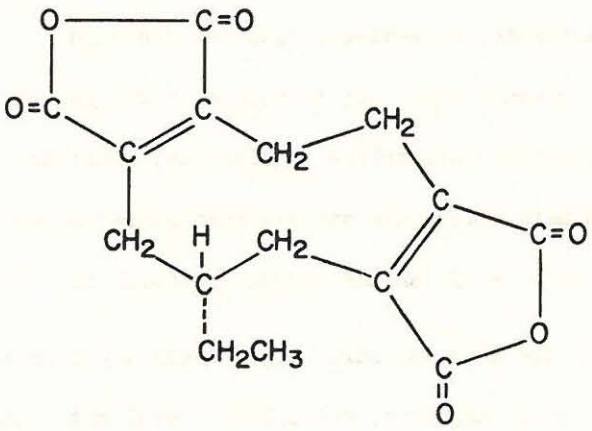
In die laaste figuur word die chemiese strukture van ochratoksien A en twee newe komponente, ochratoksiene B en C, aangegee, wat onlangs veral met behulp van fisies-chemiese metodes soos massaspektrometrie, kernmagnetiese resonans, infrarooi- en ultravioletspektroskopie alhier opgeklaar is. Slegs ochratoksien A is giftig en sy toksikologiese aspekte is reeds deur dr. Theron behandel. Die strukture van ochratoksiene B en C toon dat die verwydering van chloor of die verestering van die karboksielgroep ochratoksien A sy giftigheid ontneem.



Oksalaat
(Aspergillus niger)



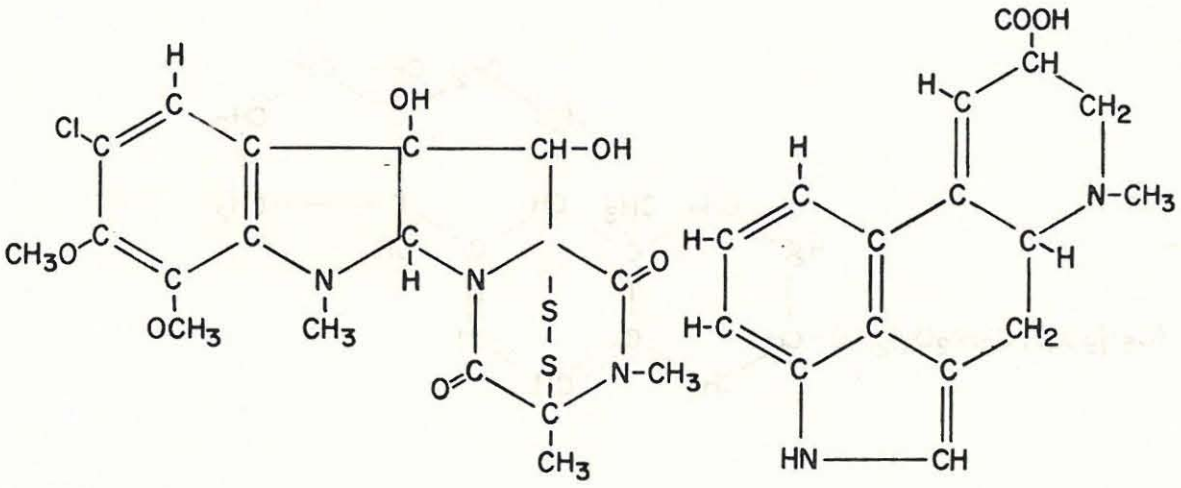
β-nitro-propioonssuur



L-seriel-L-seriel-L-dichloroproliel-β-feniel-β-aminopropioniel-L-α-aminobottersuur anhidried.

Islanditoksien
(Penicillum islandicum)

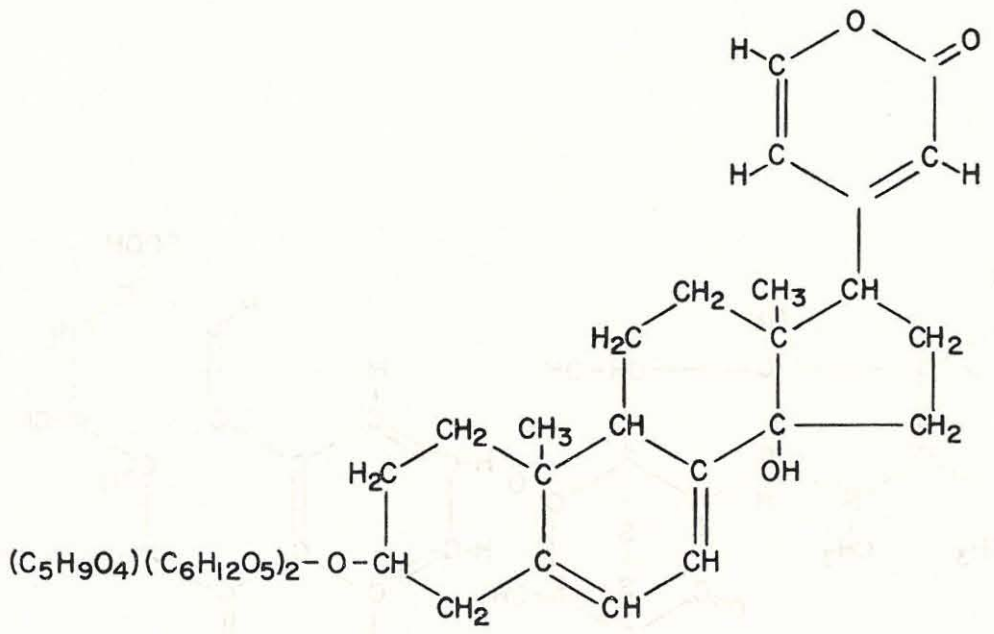
FIGUUR 1.



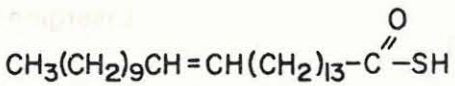
Sporidesmin
(Pithomyces chartarum)

Lisergiensuur

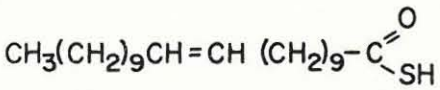
FIGUUR. 2.



Fusariogenien (Fusarium sporotrichoides)

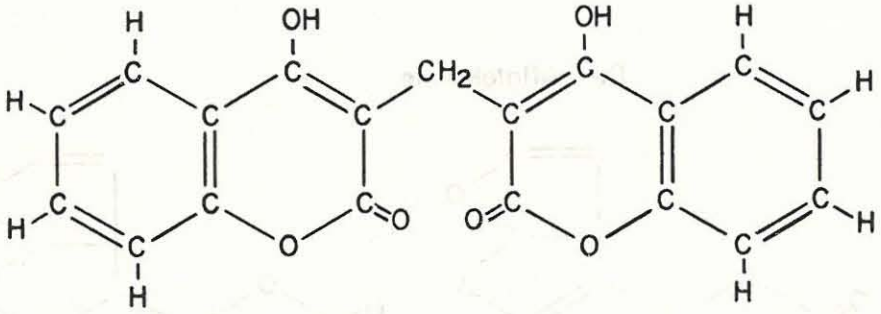


Epicladosporiensuur (Cladosporum epiphylum)

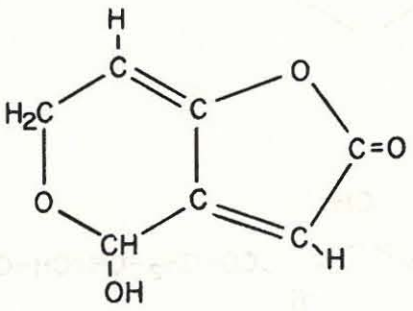


Fagicladosporiensuur (Cladosporum fagi)

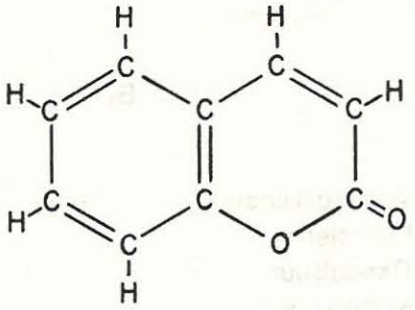
FIGUUR 3.



Dikumarien



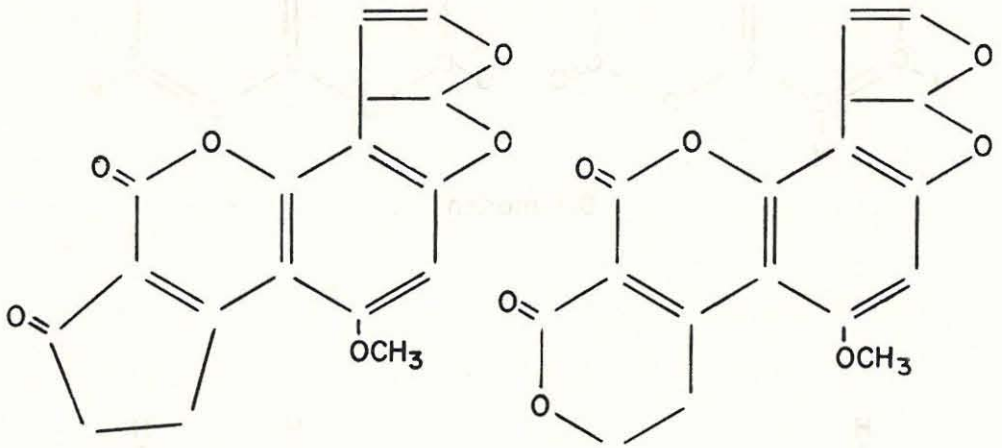
Patulien
(Penicillium urticae)



Kumarien

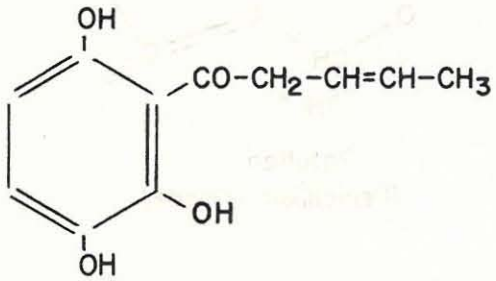
FIGUUR 4.

Die Aflatoksiene



B_1 G_1

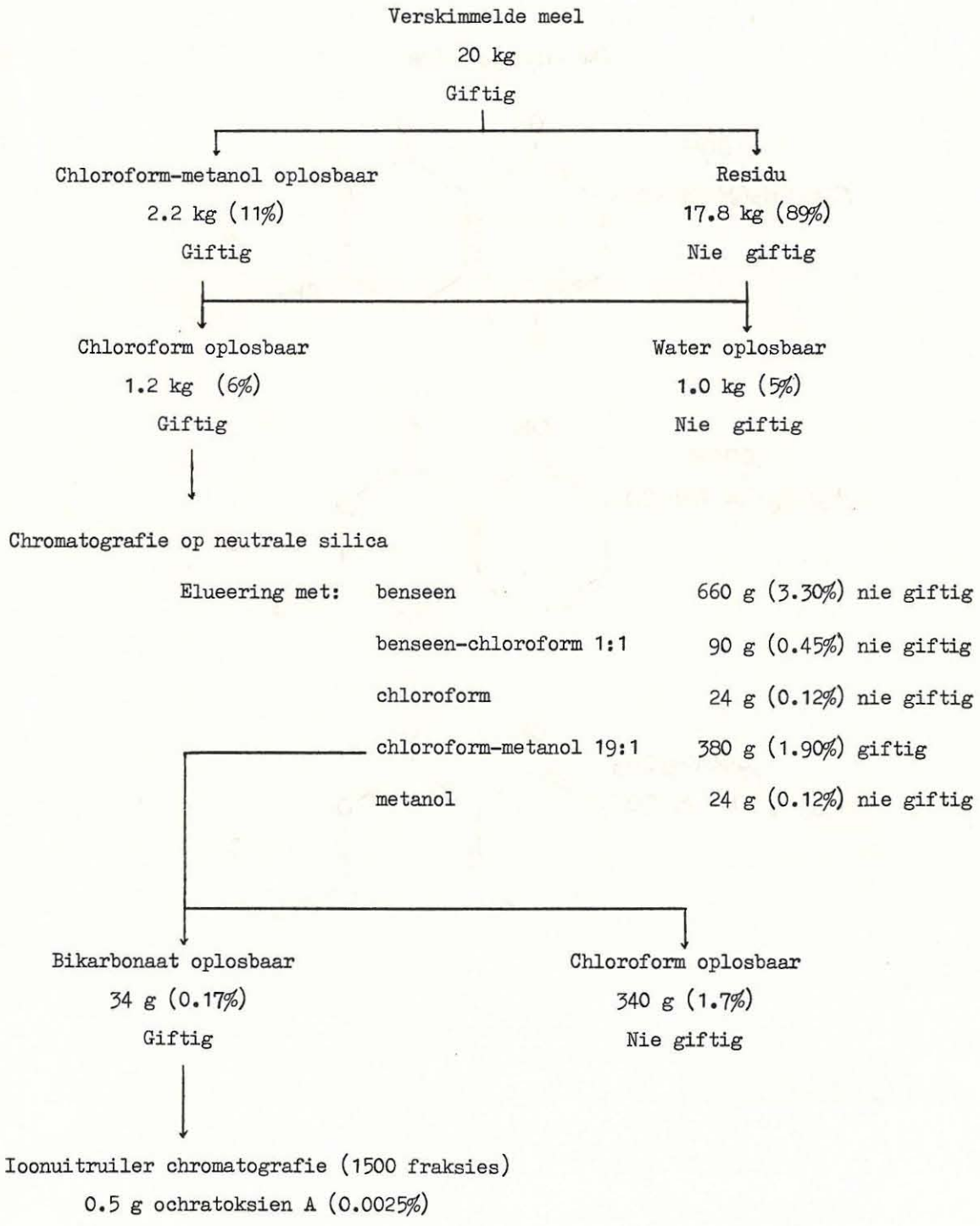
Aspergilliensuur
Flavisien
Oksaalsuur
Kojïensuur



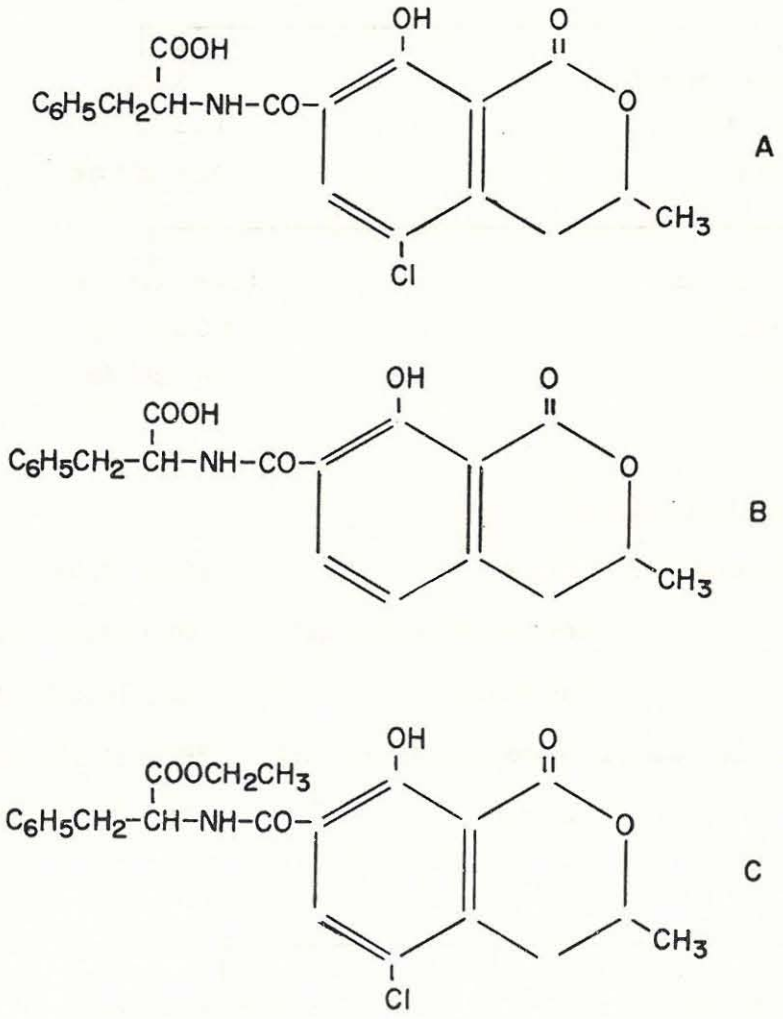
Maltoryzien
(Aspergillus oryzae var. microsporus)

FIGUUR 5.

Figuur 6


Verskimmelde meel
20 kg
Giftig
Chloroform-metanol oplosbaar
2.2 kg (11%)
Giftig
Residu
17.8 kg (89%)
Nie giftig
Chloroform oplosbaar
1.2 kg (6%)
Giftig
Water oplosbaar
1.0 kg (5%)
Nie giftig
Chromatografie op neutrale silica
Elueering met:
benseen
660 g (3.30%) nie giftig
benseen-chloroform 1:1
90 g (0.45%) nie giftig
chloroform
24 g (0.12%) nie giftig
chloroform-metanol 19:1
380 g (1.90%) giftig
metanol
24 g (0.12%) nie giftig
Bikarbonaat oplosbaar
34 g (0.17%)
Giftig
Chloroform oplosbaar
340 g (1.7%)
Nie giftig
Ioonuitruiler chromatografie (1500 fraksies)
0.5 g ochratoksien A (0.0025%)

Die Ochratoksiene



FIGUUR 7.

THE ESTIMATION OF AFLATOXIN : A CRITICAL SURVEY OF THE AVAILABLE PHYSICO-CHEMICAL METHODS

J.H. Lombard, M.Sc. and L.J. Vorster, B.Sc.

National Nutrition Research Institute, Council for Scientific and Industrial Research, Pretoria

SYNOPSIS

The characteristic blue-violet fluorescence of Aflatoxin B (B_1 and B_2) and the greenish fluorescence of Aflatoxin G (G_1 and G_2) under ultra-violet light from the basis for the estimation of these substances in all methods so far proposed for this purpose. Various procedures based on extraction followed by column and/or paper chromatography and the detection of aflatoxin under ultra-violet light have been described. The most satisfactory of these methods is that of de Iongh et al, a modification of which is at present in use at various laboratories in the Republic.

SAMEVATTING

Die kenmerkende blou-violet fluoressensie van aflatoksien B (B_1 en B_2) en die groenerige fluoressensie van aflatoksien G (G_1 en G_2) onder ultraviolet lig vorm die grondslag vir die bepaling van hierdie stowwe in al die metodes wat tot dusver vir hierdie doel aangewend is. Verskillende metodes wat gegrond is op uitloging, gevolg deur kolom- en/of papierchromatografie en die opsporing van aflatoksien onder ultra-violet lig word beskryf. Die beste metode is dié van de Iongh e.a. Hierdie metode word tans in gewysigde vorm in verskeie laboratoriums in die Republiek gebruik.

INTRODUCTION

The outbreak of Turkey-X disease in the UK in 1960 led to an intensive search for the cause of death of the 100,000 or so turkey poults affected. Toxicologists in due course traced the origin of the disease to a toxic component produced by a fungus present in the groundnut meal used to make up the rations fed to the birds. In the course of efforts to establish the chemical structure of the toxic substance, which was given the name of "aflatoxin", it was found that extraction was optimal with methanol or chloroform and that extracts containing aflatoxin fluoresced in ultra-violet light. Chromatography of the extract on a column of neutral alumina resolved the aflatoxin into two bands, one of which gave a blue-violet fluorescence (aflatoxin B_1 and B_2) and the other a greenish fluorescence (aflatoxin G_1 and G_2) under ultra-violet irradiation.

This preliminary work paved the way for the elaboration of both qualitative and quantitative methods for the estimation of aflatoxin. To date all methods proposed are based on its fluorescence under ultra-violet light.

METHODS

1. The first publication describing a method for the semi-quantitative estimation of aflatoxin was one of Coomes and Sanders (1963)[1] of the Tropical Products Institute, London. The method may be summarised as follows: After defatting, the material to be examined is extracted with methanol. The methanol extract is freed from all traces of fatty material by partition extraction with light petroleum, and the aflatoxin is then removed from the methanol extract by continuous liquid-liquid extraction with chloroform. The chloroform extract is thereafter chromatographed on a column of neutral alumina. After the elution of aflatoxin the portion containing the aflatoxin is subjected to paper chromatography and the chromatogram is then examined under ultra-violet light. The smallest quantity of toxin that can be detected in this way is 0.2γ g.

2. In a second method, proposed by Broadbent, Cornelius and Shone (1963)[2] of the Tropical Products Institute, London, greater precision is obtained by spotting dilutions of the chloroform extract on a thin-layer chromatoplate until fluorescence ceases to show up in the spots. The extract for this method is obtained in much the same way as that described by Coomes et al.[1] After concentration, the extract is spotted in suitable quantities on thin-layer chromatography plates, coated with neutral alumina. The plates are then developed in chloroform containing 1.5% methanol. The developed plate is

viewed under UV light at 365 mμ. If aflatoxin is present in the aliquot it shows up as a blue-violet fluorescent spot in the case of aflatoxin B and a greenish spot in the case of aflatoxin G. The smallest quantity of aflatoxin B, which is detectable under these conditions is 0.006 μg.

3. In a later publication[3], the group at the Tropical Products Institute, London, stated that their method could be improved by introducing the following modifications:

   (a) Substituting Kieselgel G for alumina on the TLC plates.
   (b) Increasing the methanol content of the eluent from 1.5% to 5%.

   The higher resolution obtainable with the kieselgel plates makes it impossible to detect 0.002 μg aflatoxin.

4. A provisional description of a third method was published by Nesheim et al[4] in January 1964 and later confirmed in a private communication received from the USA. The basis of the method is as follows: The material to be examined is blended at high speed either with methanol if it is defatted or with a mixture of methanol and hexane in the case of undefatted material. A suitable aliquot of the clarified extract is chromatographed on a column of Celite 545 and eluted successively with hexane and chloroform to separate the aflatoxin-containing portion from any fatty material present. The resultant extract is chromatographed on thin-layer plates as described under 2. and 3.

5. In an effort to bring the determination of aflatoxin onto a more exact basis, the TPI group (Nabney and Nesbitt, 1964[5]) suggested the following additional modification of their procedure: The final extract is applied to the thin-layer plate as a band on the baseline. After elution of the plate, the band of aflatoxin is scraped off carefully, desorbed and made up to a suitable volume. The fluorescence of this solution can be accurately determined in a spectrophotometer of fluorimeter at 363 mμ.

6. The final method to be discussed is one described in a paper by de Iongh and van Pelt of the Unilever Research Laboratories in Vlaardigen, and Ord and Barrett of the Unilever Laboratories at Welwyn.[6] The method is basically the same as that proposed by the TPI group, but with the following refinements:

   (a) The extraction time with methanol is shortened from four hours to one hour, and this is followed by extraction with chloroform for one and a half to two hours. After evaporation of the chloroform the residue is taken up in methanol and transferred to the methanol extract.

(b) The concentration of the methanol is adjusted to 85% and all traces of lipids are removed by partition extraction with light petroleum. This step ensures that no interfering light blue fluorescent streaks due to lipids will appear on the chromatogram.

(c) Satisfactory separation of the components is obtained by the use of 2% methanol in pure chloroform.

(d) A standard solution of aflatoxin B_1 (0.1μg per ml.) is used as a reference standard.

DISCUSSION

The merits and demerits of the available methods may be summed up as follows:

The paper chromatography method[1] is rather time-consuming and thus not very suitable for routine analysis. Furthermore, the resolution is unsatisfactory and the sensitivity of 0.2μg relatively low.

The me ıod proposed by Cornelius et al[2] has been used extensively in this country. It has two major shortcomings. Firstly, the alumina used for the TLC plates is actually intended for column chromatography and hence gives poor resolution. Secondly, the proposed extraction method leaves traces of fat in the extract which produce interfering streaks of light blue fluorescence. These in turn enhance the intensity of the fluorescence due to aflatoxin.

The shortcomings of the alumina method were largely eliminated when, in their later publication[3], the TPI group recommended the use of kieselgel on the TLC plates. The sensitivity of the method is improved by this modification but the presence of lipids in the final extract still interferes with the assessment of the chromatogram.

The method described by Nesheim et al[4] is unacceptable for the following reasons:

(a) Extraction in a Waring blendor with inflammable solvents is dangerous.

(b) Elution of interfering substances by column chromatography is almost impossible with this method since hexane will not soak into the Celite.

(c) The method does not have any particular advantage over the others.

The quantitative method proposed by TPI[5] is very time-consuming and is therefore unsuitable for routine analyses. It also lacks sensitivity in the range

below 1μ g/g. The authors admit in their summary that "this method is less sensitive than the one depending on dilution to extinction of fluorescence but it provides a more reliable means of assaying meals containing 1μg/g or more aflatoxin".

6. The method of de Iongh et al[6] has the following definite advantages:

(a) Extraction with methanol followed by extraction with chloroform removes all the aflatoxin from contaminated material.

(b) Partition extraction of lipids from the methanol extract yields a chromatoplate free from interfering fluorescent substances.

(c) The use of a standard solution of aflatoxin B_1 enables the operator to evaluate the chromatogram with much greater confidence.

All the methods proposed except that of Nabney and Nesbitt can at best be termed semi-quantitative since the estimation of the toxin content of a sample depends on the naked-eye assessment of the intensity of the fluorescence produced by the toxin. The toxicity level is classified on the basis of the presence or absence of fluorescence at certain Rf values on the chromatoplates after chromatography of (usually) three aliquots of differing concentrations. The results are reported as:

very high	$> 2.0\mu g/g$.
high	0.5-2.0 $\mu g/g$.
medium	0.1-0.5 $\mu g/g$.
negative	$< 0.1\mu g/g$.

In view of the greater accuracy, speed and ease of manipulation afforded by the method of de Iongh et al this method is clearly the most satisfactory for the purpose of routine analyses. The NNRI has been applying this method in conjunction with a standard solution of 0.002μg pure aflatoxin B_1/10μl. as a reference standard.

REFERENCES

1. COOMES, T.J., SANDERS, J.C. 1963. The Detection and Estimation of Aflatoxin in Groundnuts and Groundnut materials. Part 1: Paper chromatographic procedure. The Analyst, vol. 88, March 1963, pp. 209-213.

2. BROADBENT, J.H., CORNELIUS, J.A., SHONE, G. 1963. The Detection and Estimation of Aflatoxin in Groundnuts and Groundnut materials. Part 11: Thin-layer chromatography method. The Analyst, vol. 88: March 1963, pp. 214-216.

3. TROPICAL PRODUCTS INSTITUTE, April 1964, Report G6.

4. NESHEIM, S. et al. 1964. Note on Aflatoxin Analysis in Peanuts and Peanut products. J.A.O.A.C., vol. 47, No. 3, p. 586.

5. NABNEY, J., NESBITT, B.F. 1964. Determination of the Aflatoxins. Nature, vol. 203: p. 862.

6. DE IONGH, H., VAN PELT, J.G., ORD, W.O. and BARRETT, C.B. 1964. The semi-quantitative determination of Aflatoxin B_1 in groundnut meal, groundnuts and peanut butter. The Vet. Rec. vol. 76, No. 34, pp. 901-3.

ACUTE LIVER INJURY IN DUCKLINGS AS A RESULT OF AFLATOXIN AND OCHRATOXIN POISONING

J.J. THERON D.Sc. M.B.C.H.B. (Pta)

N. LIEBENBERG Dip. M.T. (Pta)

H.J.B. JOUBERT

SYNOPSIS

In short-term experiments with ducklings aflatoxin B_1, one of the toxic metabolites of the mould Aspergillus flavus, caused necrosis of the parenchymal liver cells with haemorrhage. Histochemical preparations showed a progressive decrease in the activity of the enzymes succinic dehydrogenase, alkaline phosphatase, adenosime triphosphatase, inosine diphosphatase and thiamine pyrophosphatase during the development of the lesions, but an increase in the activity of acid phosphatase. Ultrastructural changes in the parenchymal hepatic cells are described and it is suggested that the toxic principle (aflatoxin B_1 or possible a modified but closely related substance) was transported by the red blood cells and that at least one its cytotoxic effects was due to a direct action on the liver cell membrane and the membranes of the various intracytoplasmic structures.

Under the same experimental conditions ochratoxin A, a toxic metabolite of the mould aspergillus ochraceus, caused a mild fatty infiltration of the liver.

SAMEVATTING

In korttermyn-eksperimente met eendjies het aflatoksien B_1, een van die toksiese metaboliete van die skimmel Aspergillus flavus, nekrose van die parenchiemale lewerselle met bloeding veroorsaak. In histochemiese preparate is 'n progressiewe vermindering in die aktiwiteit van die ensieme barnsteensuurdehidrogenase, alkaliese fosfatase, adenosientrifosfatase, inosiendifosfatase en tiamienpirofosfatase gedurende die ontwikkeling van die letsels gevind, terwyl suurfosfatase vermeerderde aktiwiteit gedurende dieselfde periode getoon het. Ultrastrukturele veranderings in die parenchiemale lewerselle is beskryf en daar word gemeen dat die toksiese stof (aflatoksien B_1 of moontlik 'n veranderde maar nou-verwante metaboliet) deur die rooibloedselle vervoer is en dat ten minste een van die sito-toksiese effekte van die stof te wyte was aan 'n direkte werking op die membraan van die lewersel en die membrane van die verskillende intrasitoplasmiese strukture.

Onder dieselfde eksperimentele toestande het okratoksien A, 'n toksiese metaboliet van die skimmel Aspergillus ochraceus, a geringe vetinfiltrasie van die lewer veroorsaak.

In 1960 the unexplained deaths of large numbers of turkey poults and ducklings in England were eventually traced to the feeding of a peanut meal diet which had become contaminated with strains of the common mould Aspergillus flavus Link[27]. Subsequently, the toxic principles were isolated from this mould and their structural formulae determined. It has been shown that certain strains of Aspergillus flavus produce a group of toxins collectively termed aflatoxins, which on the basis of their blue or green flourescence can be divided into aflatoxins B_1 and B_2 or G_1 and G_2[2,19,32,34]. These findings have stimulated widespread interest in the pathological effects of mould toxins. A recent report from this laboratory indicates that twenty-two species of moulds recovered from domestic cereal and legume crops are toxic to ducklings[25]. From one of these moulds (Aspergillus ochraceus Wilh.) the toxin, which was given the name of ochratoxin A, has been isolated in pure crystalline form[33].

The aflatoxins are essentially hepatotoxins and produce a wide variety of lesions ranging from liver cell necrosis and proliferation of bile ducts to malignant hepatomas in rats[8]. In ducklings the basic lesions are necrosis of hepatic parenchymal cells with haemorrhage and bile duct proliferation[20]. No studies of the pathological effects of ochratoxin have yet been published.

It is the purpose of the present paper to describe ultrastructural and certain histochemical changes in the liver cells of ducklings after the administration of pure crystalline aflatoxin B_1 or ochratoxin A in short-term experiments.

MATERIALS AND METHODS

The experimental animals were day-old Pekin ducklings (average weight 50 g).

Aflatoxin experiment. Crystalline aflatoxin B_1 dissolved in wheat germ oil was administered by stomach tube to 30 ducklings in doses of 100 µg per duckling. Ten control animals each received an equivalent volume (0.5 ml.) of wheat germ oil.

Ochratoxin experiment. Thirty ducklings each received through a stomach tube 100 µg crystalline ochratoxin A dissolved in 0.5 ml. 0.1M sodium bicarbonate. Ten control animals were given an equivalent volume of sodium bicarbonate. All the animals in both experiments were fed chicken mash and water ad libitum throughout the experimental period.

One experimental animal and one control from each experiment were killed by decapitation at intervals of 1, 2, 4, 8, 16 and 24 hours ater administration of the toxins. All surviving animals were sacrificed 72 hours after the start of the experiments.

Liver tissue obtained from the left lobe of the liver immediately after decapitation was divided into four parts. Unfixed cryostat sections from one block were stained for succinic dehydrogenase (SDH) according to the Nitro-BT method as described by Burstone[7]. A second block of liver tissue was fixed in cold (4°C) formol-calcium[4] for 12-24 hours and frozen sections were incubated in the appropriate substrates at 37°C for the demonstration of the following enzymes: alkaline phosphatase (Alk P)[11], acid phosphatase (Acid P)[11], adenosine triphosphatase (ATP-ase)[36], inosine diphosphatase (IDP)[21] and thiamine pyrophosphatase (TPP)[21]. For electron microscopy a third cube of liver tissue was immersed in cold (0-4°C) phosphate-buffered 1% osmium tetroxide[17] and embedded in Epon 812[16]. Sections were cut on Porter-Blum ultramicrotomes with glass knives, stained with lead hydroxide[13] and examined in a Philips EM-200 elctron microscope at 60 kv. In addition thin sections (approximately 0.25-0.5 μ) of epon-embedded material were stained for light microscopy with (a) 1% toluidine blue 0 in 1% borax[23] and (b) the periodic acid-Schiff reaction (PAS)[18]. The remaining portion of liver tissue was processed in a routine manner for paraffin sections and stained with hematoxylin and eosin (H & E).

RESULTS

Aflatoxin Experiment

Light Microscopy. Our findings in general confirmed those of Newberne et al[20]. Ducklings intubated with 100 μg crystalline aflatoxin B_1 developed severe liver cell necrosis which became evident in paraffin sections at time intervals of 12 to 24 hours after administration of the toxin. The lesions were always most severe in the periportal areas of the liver lobules. In these zones, in addition to parenchymal cell damage, haemorrhages were regularly observed (Fig. 1).

In thin sections stained with toluidine blue for light microscopy, a striking change was seen in ducklings sacrificed 2 hours after administration of aflatoxin. This was the occurence of two kinds of parenchymal liver cells, "light" and "dark" cells (Fig. 2). The dark cells were found in clumps mainly in the periportal zones.

Mitochondria and nuclei with prominent necleoli could readily be seen in the light cells, but it was difficult to distinguish these organelles, especially the mitochondria, in the dark variety. Minor variations in staining intensity were found in the normal liver cells of control animals, but the striking differences seen in the experimental animals were in no instance encountered in the controls.

In experimental animals sacrificed one hour after aflatoxin administration there was a decrease in the intensity of the staining reaction of Alk P in the periportal areas of the liver lobule (Fig. 3). Figure 4 is a section from a control animal which shows the presence of Alk P activity throughout the liver lobule. At this stage there was no change in the activity of the other enzymes. In experimental animals killed at later stages of the experiment (8th to 24th hour) a progressive decrease in staining intensity was noted in sections incubated for the demonstration of SDH, ATP-ase, IDP and TPP. In contrast to that shown by the control animal (Fig. 5), almost complete absence of ATP-ase activity is illustrated in Figure 6 in an experimental animal sacrificed 24 hours after aflatoxin administration. The only enzyme which did not show a decrease in activity was Acid P. In the case of this enzyme, increased activity was found in the peribiliary granules of liver cells in the periportal zones in experimental animals killed 16 and 24 hours after intubation with aflatoxin (Figs. 7 and 8).

Electron Microscopy. The hepatocytes of control animals showed a round or oval nucleus with prominent chromatin clumps and moderately dense karyoplasm (Fig. 9). The mitochondria were oval or elongated organelles with abundant cristae mitochondriales and a matrix which usually had approximately the same density as that of the ground cytoplasm. The rough endoplasmic reticulum (ER) occurred in the form of flattened cisternae which showed a tendency to encircle the mitochondria (Fig 9) The ER membranes had a full complement of ribosomal granules, and ribosomes were also present in the ground cytoplasm apparently unattached to any membranes. Prominent lipid vacuoles were always encountered in the liver cells of the control animals (Fig. 9).

In electron micrographs the presence of dark and light hepatocytes in experimental animals was obvious one hour after aflatoxin administration (Fig. 10). The increased density of the dark cells involved the cytoplasmic matrix and the organelles only, the nucleoplasm being of approximately the same density as that of nuclei in adjacent light cells (Fig. 10). At this stage of the experiment the ultrastructure of the

mitochondria showed no significant change when compared with that of control animals. The ER of the dark cells was, however, frequently seen in the form of extremely elongated parallel cisternae in the peripheral parts of the cytoplasm (Fig. 11).

Prominent changes were encountered 1 hour after aflatoxin administration at the sinusoidal surface of the liver cells in experimental animals. Red blood cells in the sinusoids were found to have formed pseudopodium-like projections and to have penetrated into the space of Dissé, where they lay in close contact with the sinusoidal surface membranes of the liver cells (Fig. 11). The adjacent Kupffer cells showed areas of focal cytoplasmic degradation[12] (Fig. 11). Those sinusoidal microvilli of the hepatocytes which were in intimate contact with the red blood cells were swollen and frequently club-shaped (Fig. 12). The cytoplasm of the adjacent hepatocytes often appeared vacuolated due to active formation of Golgi vesicles, but it is important to note that the ultrastructure of the mitochondria and ER of these cells was not significantly altered at this stage (Fig. 12).

During later stages of the experiment prominent structural changes were found in the mitochondria and the ER. Four hours after intubation with aflatoxin, red blood cells were seen in the cytoplasm of the liver cells (Fig. 13). Mitochondria in close contact with the red blood cells were extremely swollen. In the affected organelles, those portions of the external limiting membranes which were in most intimate contact with the red blood cells appeared as a single membrane, while the usual double limiting membranes were present at the opposite poles of the mitochondria away from the red blood cells (Figs. 13 and 14). Mitochondria from the same liver cell situated at some distance from the penetrating red blood cells showed no significant morphological changes (Fig. 13). In the affected hepatocytes the cisternae of the ER appeared dilated and certain segments of the ER membranes showed detachment of the ribosomal granules (Fig. 13).

It must be emphasized that during the initial eight hours of the experiment the most prominent structural changes were always found in membranous structures <u>in close contact with extravasated red blood cells</u>. In addition to the described alterations in the sinusoidal microvilli (Fig. 12) and the mitochondria (Figs. 13 and 14) changes were seen in the intercellular membrane. In Figure 15 is shown a portion of such a membrane in close contact with a red blood cell. It is obvious that certain parts of the cell membrane are fragmented, and the red blood cell appears to be

in direct contact with the ground cytoplasm. Although this apparent fragmentation and disappearance of a portion of the cell membrane may be due to oblique sectioning of the area of contact between the membrane and the red blood cell, it was only found in regions where the membranes were closely applied to the red blood cells. In adjacent areas where the membranes did not show such an intimate contact, the phenomenon was absent. Since this was such a consistent finding it is felt that it constitutes a clear pathological change.

Other effects of extravasated red blood cells on membrane-associated structures were also encountered. In Figure 16 a cisterna of the ER can be seen with one of its membranes in intimate contact with a red blood cell. This membrane showed a complete absence of ribosomal granules, while the opposite membrane of the same cisterna retained its full complement of ribosomes.

At later stages of the experiment (16 and 24 hours) structural changes were found in cytoplasmic organelles, especially mitochondria, which were not in obviously close contact with extravasated red blood cells. These changes differed in important respects from those encountered in the same organelles during the initial experimental period and associated with the proximity of extravasated red cells. The mitochondrial alterations found during the final stages of the experiment consisted in the first place of a marked increase in the density of the mitochondrial matrix (Fig. 17). At the same time the intracristal space and the external compartment appeared swollen, less electron-dense than the matrix and filled with finely granular material (Fig 18). An advanced degree of swelling between the leaflets of the cristae and in the external compartment gave rise to a lobulated organelle (Fig. 19) enclosed by a single membrane and consisting of three to four "lobules" of dense matrix with somewhat swollen cristae separated by electron-lucid oedematous areas (Fig. 19). In surviving ducklings sacrificed 72 hours after aflatoxin administration the mitochondria appeared to be entirely normal and occurred in groups of three to four organelles, each group being enclosed by a single membrane (Fig. 20; see also under Discussion).

The structural changes in the ER consisted mainly of detachment of ribosomes from the ergastoplasmic membranes and dilation of the cisternae of the ER (Figs. 17-19). These changes were first seen during the early stages of the experiment (4 hours after aflatoxin administration, Fig. 13). They became more prominent during the later stages, especially in the light hepatocytes (Fig. 21) and were still present in

experimental animals sacrificed 72 hours after intubation with aflatoxin (Fig. 20). In occasional cells the structural integrity of the mitochondria was maintained and the morphological alterations in the ER were the most prominent pathological change (Fig. 21).

The dark cells were found throughout the experimental period and frequently showed areas of focal cytoplasmic degradation[12] which assumed the form of dense osmiophilic membranous or granular material and vesicles in the ground cytoplasm (Figs. 13 and 21). In the later stages of the experiment localized areas of increased density were seen in the cytoplasm of the light cells (Fig. 22). These areas usually presented as round structures enclosed by a single membrane and filled with dense osmiophilic material presumably of cytoplasmic origin (Fig. 22).

The final appearance of the liver cells 24 hours after aflatoxin administration is illustrated in Figure 23, which shows several necrotic liver cells with dense swollen mitochondria, dilated ER vesicles, lipid vacuoles and localized areas of increased cytoplasmic density. Red blood cells and occasional lymphocytes were found within and between the necrotic hepatocytes.

Ocharatoxin Experiment

Light Microscopy The most prominent pathological change encountered in the livers of ducklings which had received 100 µg ochratoxin A was an increase fatty vacuolization of the hepatocytes. In epon-embedded material stained with the PAS procedure the liver cells of control animals showed large numbers of lipid vacuoles which varied in size (Fig. 24). In experimental animals there was an even greater degree of fatty vacuolization and the lipid was now present in the form of small discrete vacuoles of relatively uniform size throughout the cytoplasm of the hepatocytes (Fig. 25). The fatty infiltration was especially evident during the later stages of the experimental period (16-24 hours).

Evidence of structural damage to the mitochondria could be seen in PAS-stained sections examined under the light microscope. In the liver cells of control animals the mitochondria appeared as discrete negative images (Fig. 24) while in experimental animals they had a "blurred" appearance and were less sharply demarcated from the surrounding cytoplasm than in control ducklings (Fig. 25).

At no stage during the experimental period did the enzymes for which histochemical tests were performed show any evidence of change in activity. This is in marked contrast to the results obtained after aflatoxin

administration.

Electron Microscopy. In comparison with the liver cells of control animals (Fig. 26) the hepatocytes of experimental ducklings sacrificed 16 hours after ochratoxin administration showed clearly increased fatty vacuolization in low-power electron micrographs (Fig. 27). In addition to those within the cytoplasm, lipd vacuoles were regularly found in the liver sinusoids of the experimental animals (Fig. 27).

In sections examined in the light microscope presumptive evidence of structural damage to mitochondria was found. In electron micrographs morphological changes in mitochondria were clearly evident in ducklings killed 4 hours after ochratoxin administration (Fig. 28). At this stage the mitochondria were exclusively encountered as swollen round organelles (Figs. 27 and 28), the oval forms frequently seen in control animals (Fig. 26) being apparently completely absent in the experimental group. At later stages the degree of structural damage was more severe and the matirx of the mitochondria became coarsely granular with electron-lucid areas and compression of the cristae against the external membranes of the organelle (Fig. 29).

Morphological changes in the ER were found at an early stage. Four hours after intubation with ochratoxin the slender flattened cisternae seen in control animals were no longer encountered. The ER was reduced in amount and was exclusively present in the form of isolated dilated vesicles with focal loss of ribosomes from the ergastoplasmic membranes and increased numbers of free ribosomes in the cytoplasm (Fig. 28). This process of dilation of the cisternae eventually extended to the nuclear envelope (Fig. 29).

At later stages of the experiment (16 hours) liver cell mitochondria, dilated ER vesicles and lipid droplets were found free in the sinusoids (Fig. 30). These organelles were apparently discharged through gaps in the sinusoidal endothelium (Fig. 30).

In ducklings sacrificed 24 hours after ochratoxin administration patches of cytoplasm in most of the hepatocytes were seen to present a perculiar "empty" appearance and consisted mainly of finely granular ground cytoplasm with occasional dilated ER vesicles, inconspicuous Golgi lamellae and a marked reduction in the number of ribosomal ganules (Fig. 31). In these regions focal loci of cytoplasmic degeneration were regularly encountered (Fig. 31).

DISCUSSION

Aflatoxin Experiment

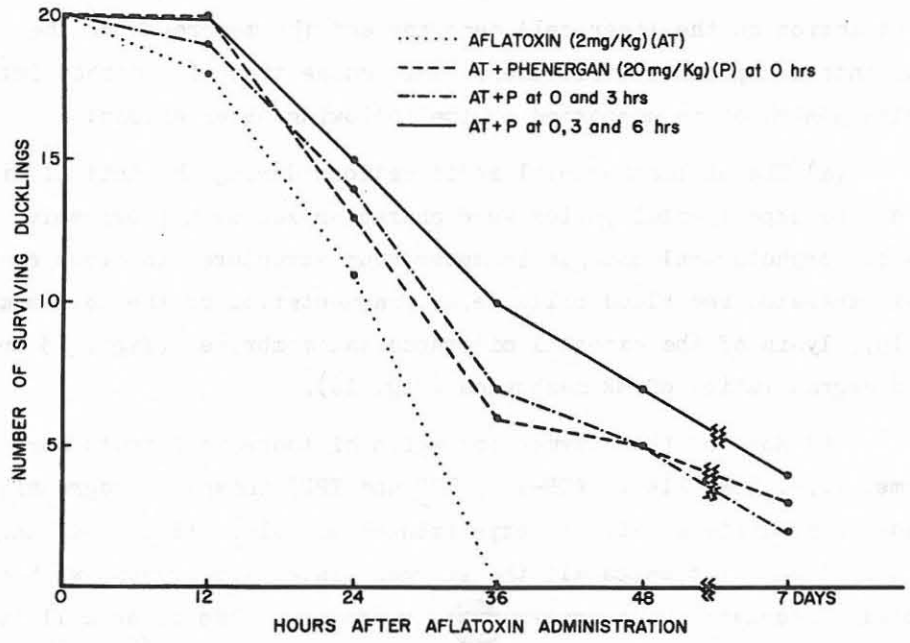
Mode of action of aflatoxin. The morphological changes in the liver cell organelles were always more severe in the immediate vicinity of extravasated red blood cells, especially during the earlier stages of the experiment. This suggests that the toxic principle (aflatoxin B_1 or possibly a modified closely related substance) was transported by the red blood cells and that at least one of its cytotoxic effects was due to a direct action on the liver cell membrane and the membranes of the various intracytoplasmic structures. This suggestion of a direct interaction with membranes is supported by the following observations:

(a) The ultrastructural modifications during the initial eight hours of the experimental period were characterized by a progressive series of morphological changes in membranous structures in close contact with extravasated red blood cells, e.g. fragmentation of the cell membrane (Fig. 15), lysis of the external mitochondrial membranes (Figs. 13 and 14) and degranulation of ER membranes (Fig. 16).

(b) Most of the enzymes for which histochemical tests were performed (i.e. SDH, Alk P, ATP-ase, IDP and TPP) showed a progressive decrease of activity in all the experimental animals. It is well known that in most animal species all the enzymes listed are present within or closely associated with membranous structures of the liver cell, e.g. SDH in mitochondria[5], Alk P mainly in sinusoidal membranes[35], ATP-ase in bile canalicular membranes[35], IDP and TPP mainly in Golgi vesicles[21]. It seems probable that structural aberrations in these membranes were responsible for functional changes in some of their enzyme activities. The only enzyme which showed increased activity during the latter stages of the experiment was Acid P. This increased activity may be related to the formation of dense bodies of the type illustrated in Figure 22. It has been shown that acid phosphatase is present in similar bodies commonly found in experimental hepatomas in rats[29].

(c) It is well known that carbon tetrachloride (CCl_4) induces liver cell necrosis in a wide range of experimental animals[24]. It has been suggested that some of the hepatotoxic effects of CCl_4 may be due to a direct interaction of the poison with the membranes of the cytoplasmic constituents of the liver cell[22]. Previous work[10] has indicated that "Phenergan" (10-(2-dimethylamino-isopropyl) phenothiazine hydrochloride) delays the onset of CCl_4- necrosis by preventing the leakage of pyridine

nucleotides and enzymes through mitochondrial and cell membranes. In day-old ducklings poisoned with aflatoxin B_1 (100 µg per 50 g duckling), administration of "Phenergan" also delays the onset of liver cell necrosis (Text - fig. 1)[30].



TEXT-FIGURE 1

Delaying effect of "Phenergan" on the onset of liver necrosis after aflatoxin B_1 administration.

This effect of "Phenergan" on aflatoxin toxicity may be due to a "stabilizing" action on cell and other membranes similar to that observed in CCl_4-experiments. It lends further support to the suggestion that under the present experimental conditions one of the toxic manifestations of aflatoxin was due to a direct effect on the membranes of the cell and cellular organelles.

Mitochondria. The mitochondrial changes observed during the earlier stages of the experiment (Figs. 13 and 14) were structurally different from those seen during the later stages (Figs. 17 to 20). The series of changes is shown schematically in Text-fig 2.

12. HRUBAN, Z., SPARGO, B., SWIFT, H., WISSLER, R.W., and KLEINFELD, R.G. Focal cytoplasmic degradation Am. J. Path. 42 : 657, 1963

13. KARNOVSKY, M.J. Simple methods for "staining with lead" at high pH in electron microscopy. J. Biophys. Biochem. Cytol. 11 : 729, 1961

14. LAFONTAINE, J.G., and ALLARD, C. A light and electron microscope study of the morphological changes induced in rat liver cells by the azo dye 2-Me-DAB. J. Cell Biol. 22 : 143, 1964

15. LIEBER, C.S. Pathogenesis of hepatic steatosis. Gastroenterology 45 : 760, 1963.

16. LUFT, J.H. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9 : 409, 1961.

17. MILLONIG, G. Further observations on a phosphate buffer for osmium solutions in fixation. In Electron Microscopy. Fifth International Congress. New York, Academic Press, Inc., 1962

18. MUNGER, B.L. Staining methods applicable to sections of osmium-fixed tissue for light microscopy. J. Biophys. Biochem. Cytol. 11 : 502, 1961.

19. NESBITT, B.F., O'KELLY, J., SARGENT, K., and SHERIDAN, A. Toxic metabolites of Aspergillus flavus. Nature 195 : 1060, 1962

20. NEWBERNE, P.M., WOGAN, G.N., CARLTON, W.W., and ABDEL-KADER, M.M. Histopathologic lesions in ducklings caused by Aspergillus flavus cultures, culture extracts, and crystalline aflatoxins. Toxicol. and Appl. Pharm. 6 : 542, 1964

21. NOVIKOFF, A.B., and GOLDFISCHER, S. Nucleoside diphosphatase activity in the Golgi apparatus and its usefulness for cytological studies. Proc. Nat. Acad. Sci. U.S.A., 47 : 802, 1961

22. REYNOLDS, E.S. Liver parenchymal cell injury. 1. Initial alterations of the cell following poisoning with carbon tetrachloride. J. Cell Biol. 19 : 139, 1963.

23. RICHARDSON, K.C., JARETT, L. and FINKE, E.H. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Tech. 35 : 313. 1960.

24. ROUILLER, Ch. Experimental toxic injury of the liver. In The Liver, Vol. 2, edited by Rouiller, Ch. New York, Academic Press. Inc., 1963.

25. SCOTT, De. B. Toxigenic fungi isolated from cereal and legume crops. Mycopathol. et Mycol. Appl. (in press).

26. SMUCKLER, E.A., ISERI, O.A., and BENDITT, E.P. An intracellular defect in protein synthesis induced by carbon tetrachloride. J. Exp. Med. 116 : 55, 1962.

27. SPENSLEY, P.C. Aflatoxin, the active principle in Turkey "X" disease. Endeavour 22 : 75, 1963.

28. STEINER, J.W., and BAGLIO, C.M. Electron Microscopy of the cytoplasm of parenchymal liver cells in α-napthylisothiocyanate-induced cirrhosis. Lab. Invest. 12 : 765, 1963.

29. SVOBODA, D.J. Fine structure of hepatomas induced in rats with p-dimethylaminoazobenzene. J.Nat. Cancer Inst. 33 : 315, 1964

30. THERON, J.J. Unpublished observations.

31. THOENES, W., and BANNASCH, P. Elektronen- und licht-mikroskopische untersuchungen am cytoplasma der leberzellen nach akuter und chronischer thioacetamidvergiftung. Virchows Arch. path. Anat. 335 : 556, 1962.

32. VAN DER MERWE, K.J., FOURIE, L., and SCOTT, De B. On the structure of the aflatoxins. Chem. and Ind. 41 : 1660, 1963.

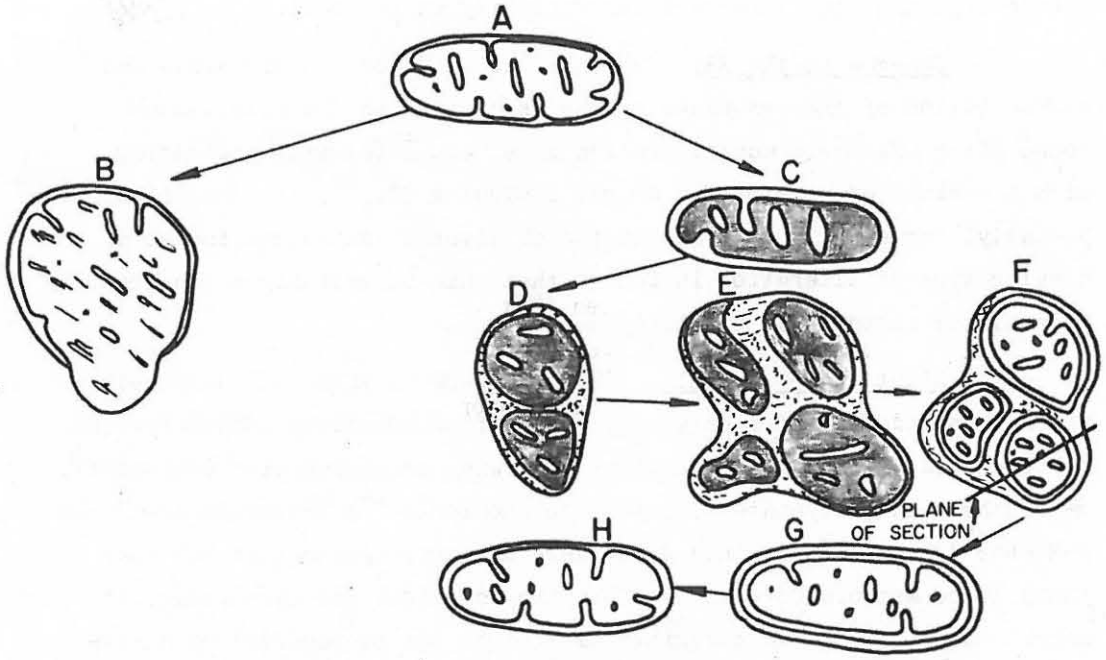
33. VAN DER MERWE, K.J., STEYN, P.S., FOURIE, L. SCOTT, DeB., and THERON, J.J. Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus Wilh. Nature (in press).

34. VAN DER ZIJDEN, A.S.M., KOLLENSMID, W.A.A.B., BOLDINGH, J., BARRETT, C.B., ORD, W.O., and PHILP, J. Aspergillus flavus and Turkey "X" disease. Nature 195 : 1060, 1962.

35 WACHSTEIN, M. Cyto- and histochemistry of the liver. In The Liver, Vol. 1, edited by Rouiller, Ch. New York, Academic Press, Inc., 1963.

36. WACHSTEIN, M., and MEISEL, E. Histochemistry of hepatic phosphatases at a physiologic pH. Am. J. Clin. Path. 27 : 13, 1957

PHOTOGRAPHS



TEXT FIGURE 2

This diagram illustrates the structural alterations of the mitochondria in hepatocytes after aflatoxin B_1 administration. A: Structurally normal mitochondrion. B: shows swelling of the organelle with lysis of the external mitochondrial membrane. These changes were seen during the early stages of the experiment in mitochondria in close contact with extravasated red blood cells and were presumably due to a direct effect of the toxin on the structural integrity of mitochondrial membranes. In C to H, the structural mitochondrial alterations encountered during the later stages of the experiment are illustrated. C shows increased density of the mitochondrial matrix with slight swelling between the leaflets of individual cristae and in the external compartment. Progression of this process of swelling leads to the formation of a lobulated organelle enclosed by a single membrane with two (D) or four (E) lobules of dense matrix. In surviving ducklings structurally normal mitochondria are found in groups of three to four organelles enclosed by a single membrane (F). If the section pases through one organelle in the group a single structurally normal mitochondrion enclosed by a single membrane is encountered as illustrated in G (cf. Fig. 20, M_4 and M_5). H shows the appearance of a structurally normal mitochondrion after lysis of the enclosing membrane.

It seems possible that, while the lysis of the external mitochondrial membranes (Text - fig 2, B) was probably due to a direct effect of aflatoxin, the second group of mitochondrial alterations (Text - fig. 2, C to H) may have represented an attempt on the part of the cell to preserve the number and structural integrity of these organelles which

are so important for the continued viability of the cell.

Changes in the ER. Vesicular dilation of the cisternae and degranulation of the membranes of the ER similar to the alterations found after aflatoxin administration have been noted after poisoning with a variety of hepatotoxic drugs, including CCl_4[26], dimethylnitrosamine[9] and allyl formate[24]. The fact that such diverse toxins can induce a similar type of alteration indicates that this is probably a non-specific generalized response of the liver cell.

Light and Dark Cells. The occurence of light and dark cells which we noted in experimental ducklings after aflatoxin administration has been described in the rat liver following poisoning with ethionine[1], α- napthyl isothiocyanate[28], carbon tetrachloride[26], thioacetamide[31] and 2-Me-DAB[14]. In view of this wide range of toxic agents that has been found to be associated with the formation of light and dark cells, it seems probable that the described alteration can be regarded as a non-specific reaction of the liver cell.

Ochratoxin Experiment

The basic lesion in the livers of ducklings which received ochratoxin A was increased fatty vacuolization of the hepatic parenchymal cells. Multiple mechanisms (e.g. increased chylomicron uptake by the liver, excessive mobilization of free fatty acids from adipose tissue, a block in phospholipid synthesis etc.) have been implicated in the pathogenesis of hepatic steatosis[15]. The swollen mitochondria found in the livers of ducklings after ochratoxin administration closely resembled mitochondria seen in the livers of choline-deficient rats[3]. Some of the ultrastructural changes in the ER, especially the decrease in the amount of ergastroplasmic membranes and in the numbers of ribosomal granules, are also prominent features of choline-deficiency[3]. Decreased hepatic phospholipid synthesis has been described in fatty livers caused by choline deficiency[6]. The close resemblance between the fine cytological changes in the liver found after ochratoxin administration and those found in choline deficiency suggests that the toxin may also cause hepatic steatosis by interfering with phospholipid synthesis in the liver. However, other structural changes which were prominent in the present experiments (e.g. dilation of ER cisternae, focal degeneration of the cytoplasm) have not been encountered in choline deficient rats[3] and further correlated cytological and biochemical studies are required before it will be possible to determine which mechanism plays the primary role in the

pathogenesis of the hepatic steatosis induced by ochratoxin administration.

SUMMARY

In short-term experiments with ducklings aflatoxin B_1, one of the toxic metabolites of the mould Aspergillus flavus, caused necrosis of the parenchymal liver cells with haemorrhage. Histochemical preparations showed a progressive decrease in the activity of the enzymes succinic dehydrogenase, alkaline phosphatase, adenosine triphosphatase, inosine diphosphatase and thiamine pyrophosphatase during the development of the lesions, but an increase in the activity of acid phosphatase. Ultrastructural changes in the parenchymal hepatic cells are described and it is suggested that the toxic principle (aflatoxin B_1, or possibly a modified but closely related substance) was transported by the red blood cells and that at least one of its cytotoxic effects was due to a direct action on the liver cell membrane and the membranes of the various intracytoplasmic structures.

Under the same experimental conditions ochratoxin A, a toxic metabolite of the mould Aspergillus ochraceus, caused a mild fatty infiltration of the liver.

REFERENCES

1. ARAKAWA, K. An electron microscopic observation on hepatic cells of albino rats after DL-ethionine administration. 1. Changes in the rough surfaced elements of endoplasmic reticulum. J. Electronmicroscopy 8 : 54, 1960.

2. ASAO, T., BUCHI, G., ABDEL-KADER, M.M. CHANG, S.B., WICK, E.L. and WOGAN, G.N. Aflatoxins B and G. J. Am. Chem. Soc. 85 : 1706, 1963,

3. ASHWORTH, C.T., SANDERS, E., and ARNOLD, N. Hepatic lipids. Fine structural changes in liver cells after high-fat, high-cholesterol and choline-deficient diets in rats. Arch. Path. 72: 33, 1961.

4. BAKER, J.R. The structure and chemical composition of the Golgi element. Quart. J. mic. Sci. 85 : 1, 1944.

5. BARRNETT, R.J. The combination of histochemistry and cytochemistry with electron microscopy for the demonstration of the sites of succinic dehydrogenase acitivity. Proc. Fourth. Internat. Conf. on Electr. Micr., p. 91. Berlin, Springer-Verlag, 1960.

6. BEST, C.H. The lipotropic agents in the protection of the liver, kidney, heart and other organs of experimental animals. Proc. Roy. Soc. B. 145 : 151, 1956

7. BURSTONE, M.S. Enzyme Histochemistry, p. 512. New York, Academic Press, Inc., 1962.

8. BUTLER, W.H., and BARNES, J.M. Toxic effects of groundnut meal containing aflatoxin to rats and guinea-pigs. Brit. J. Cancer 17 : 699, 1963.

9. EMMELOT, P., and BENEDETTI, E.L. Some observations on the effect of liver carcinogens on the fine structure and function of the endoplasmic reticulum of rat liver cells. In Protein Biosynthesis, edited by Harris, R.J.C. New York, Academic Press Inc., 1961.

10. GALLAGHER, C.H., and REES, K.R. Levels of pyridine nucleotides in liver poisoning. Nature, 187 : 148, 1960.

11. GOMORI, G. Microscopic Histochemistry. Chicago, Univ. of Chicago Press, 1952.

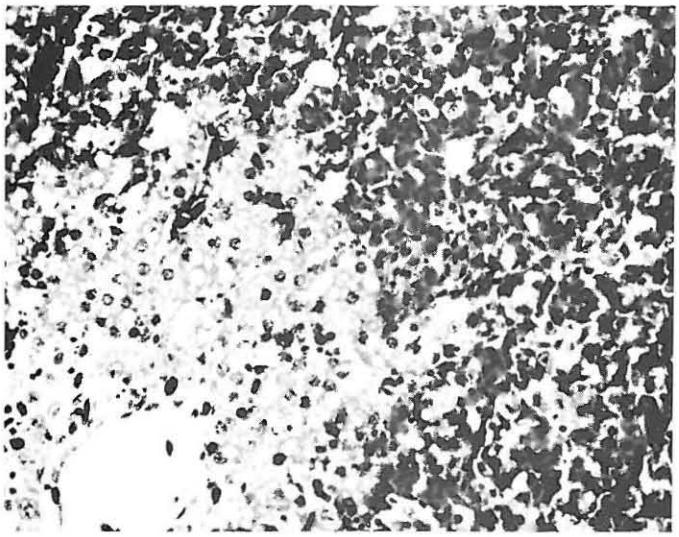


FIGURE 1. Necrosis of parenchymal liver cells and hemorrhage in peripheral areas of liver lobule in a duckling 12 hours after administration of aflatoxin B_1 (Hematoxylin and eosin) (x200).

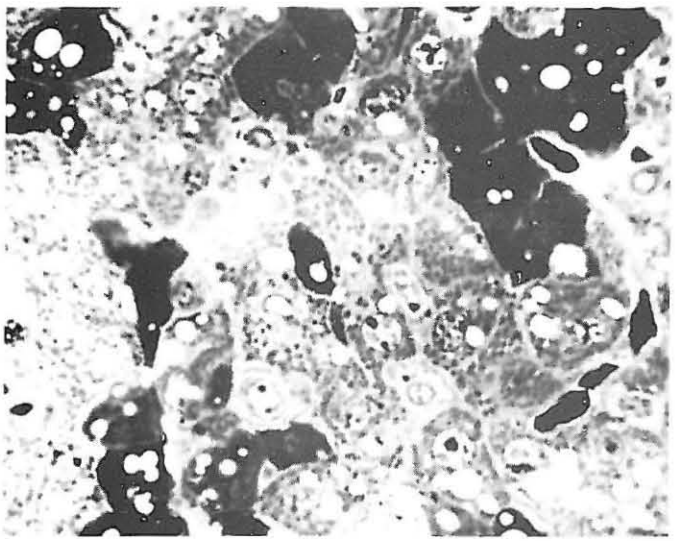


FIGURE 2. "Light" and "dark" liver cells 2 hours after aflatoxin administration. Note the dark granules (mitochondria) in the cytoplasm of the light cells (Toluidine blue) (x500).

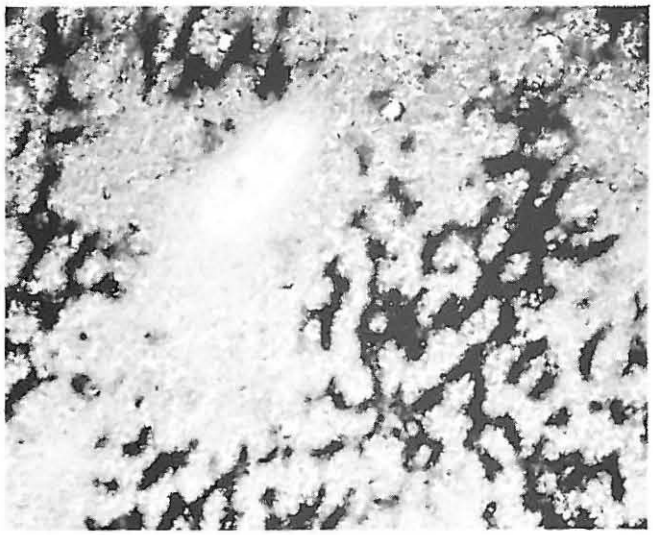


FIGURE 3. One hour after aflatoxin administration: Decreased activity of alkaline phosphatase in periportal zones of liver lobule. Compare with Figure 4 (x200).

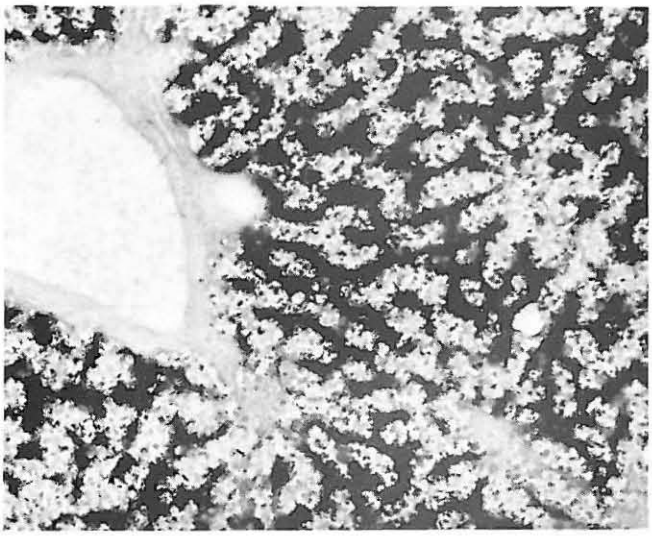


FIGURE 4. Alkaline phosphatase activity in liver lobule of control animal one hour after administration of wheat germ oil (x200).

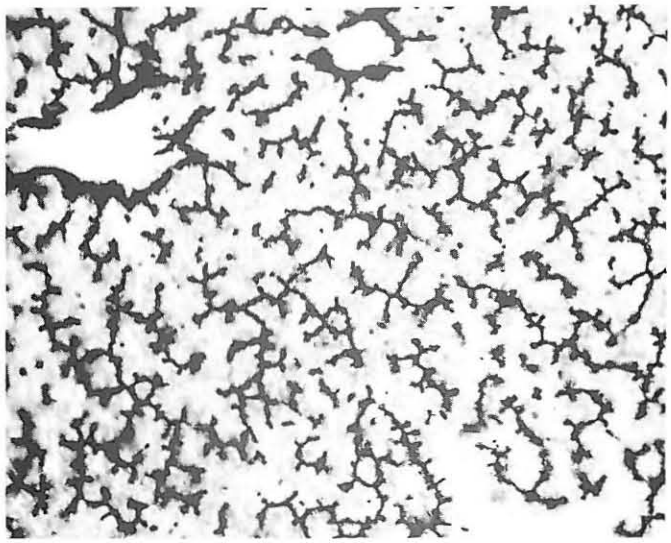


FIGURE 5. Adenosine triphosphatase activity in liver lobule of control animal 24 hours after administration of wheat germ oil (x200).

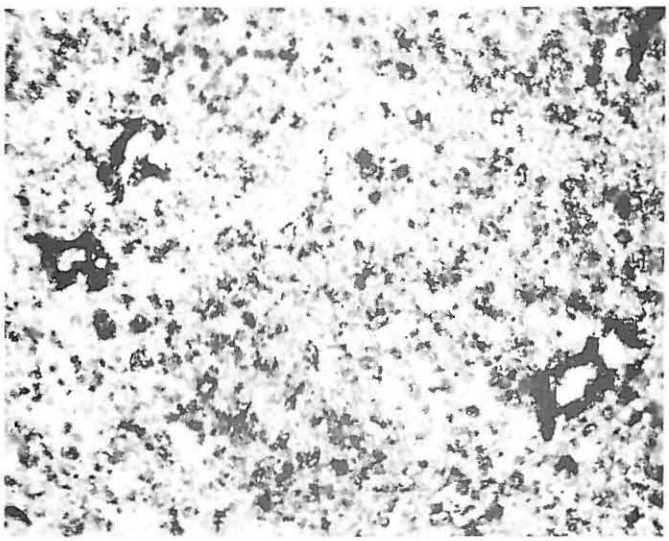


FIGURE 6. Twenty-four hours after aflatoxin administration: Decreased activity of adenosine triphosphatase throughout liver lobule. Some activity is still evident in portal tracts. Compare with Figure 5 (x200).

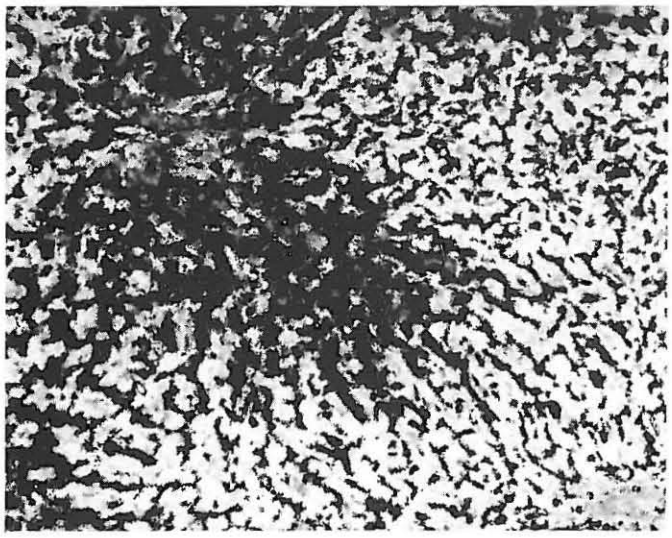


FIGURE 7. Sixteen hours after aflatoxin administration: Increased activity of acid phosphatase in peribiliary granules of liver cells in periportal zones. Compare with Figure 8 (x200).

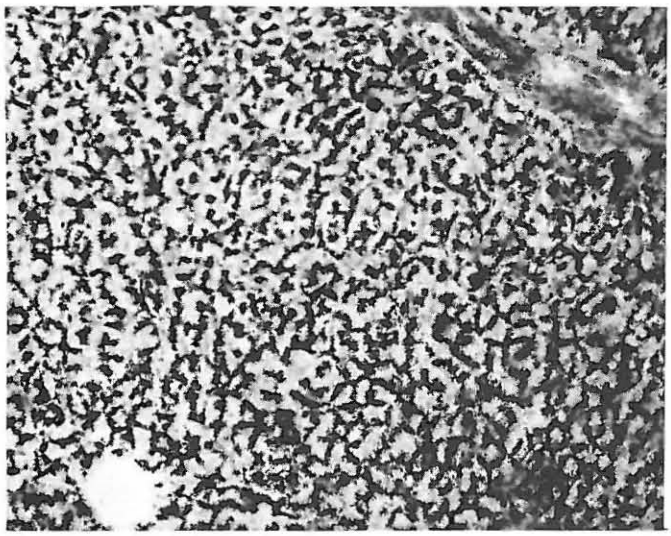


FIGURE 8. Acid phosphatase activity in liver lobule of control animal sixteen hours after administration of wheat germ oil (x200)

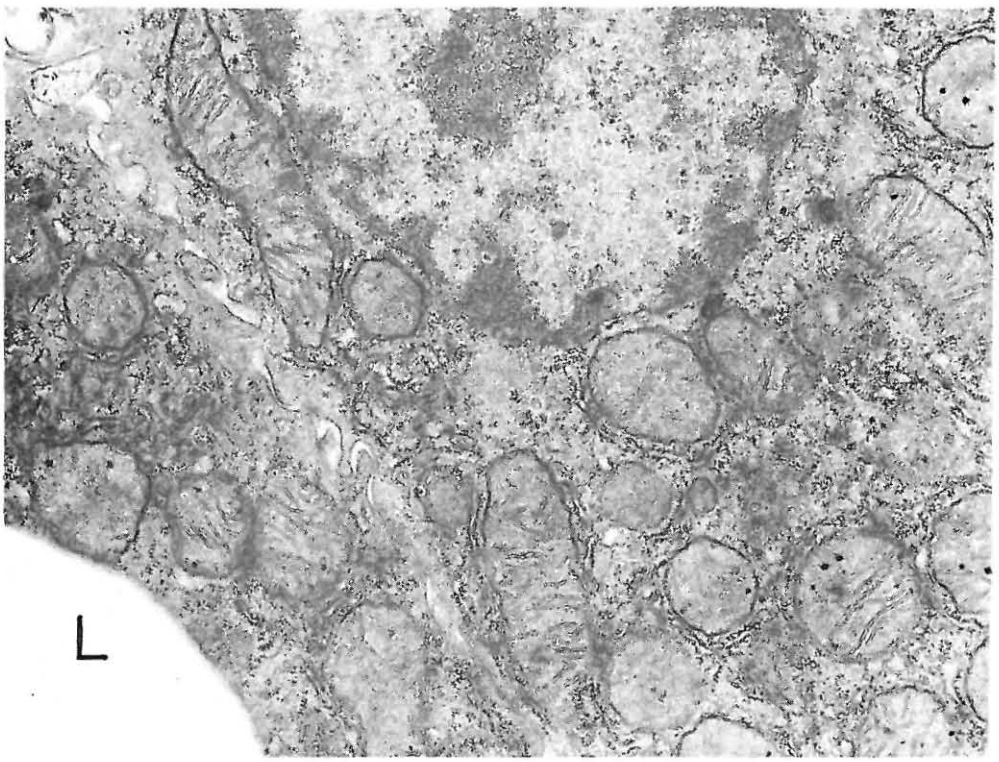


FIGURE 9. Hepatocytes of control animal. Note oval or elongated mitochondria with prominent cristae mitochondriales and moderately dense matrix. The rough ER is seen in the form of flattened cisternae which show a tendency to encircle mitochondria. Moderate numbers of ribosomes are found free in the cytoplasm. L: lipid vacuole (x15,000).

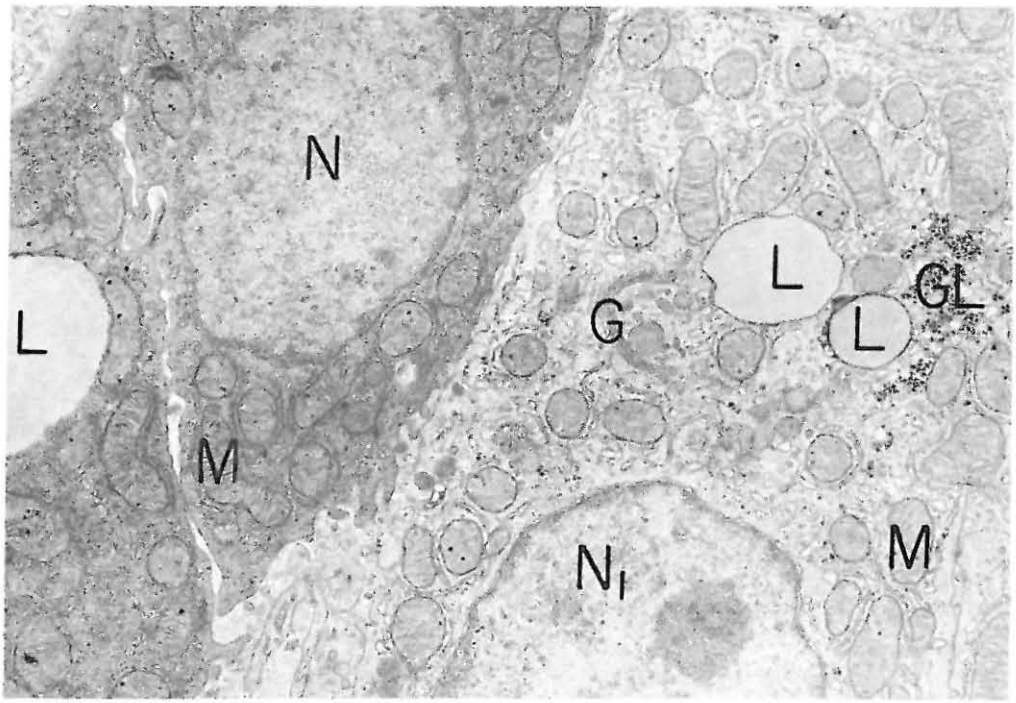


FIGURE 10. Dark and light hepatocytes in liver of experimental animal one hour after aflatoxin administration. The increased density is especially evident in the cytoplasm of the dark cells, while the nucleus (N) of a dark cell retains approximately the same density as the nucleus (N_1) of an adjacent light cell. M: Mitochondria, G: Golgi apparatus, CL: Glycogen, L: Lipid vacuoles (x9,500).

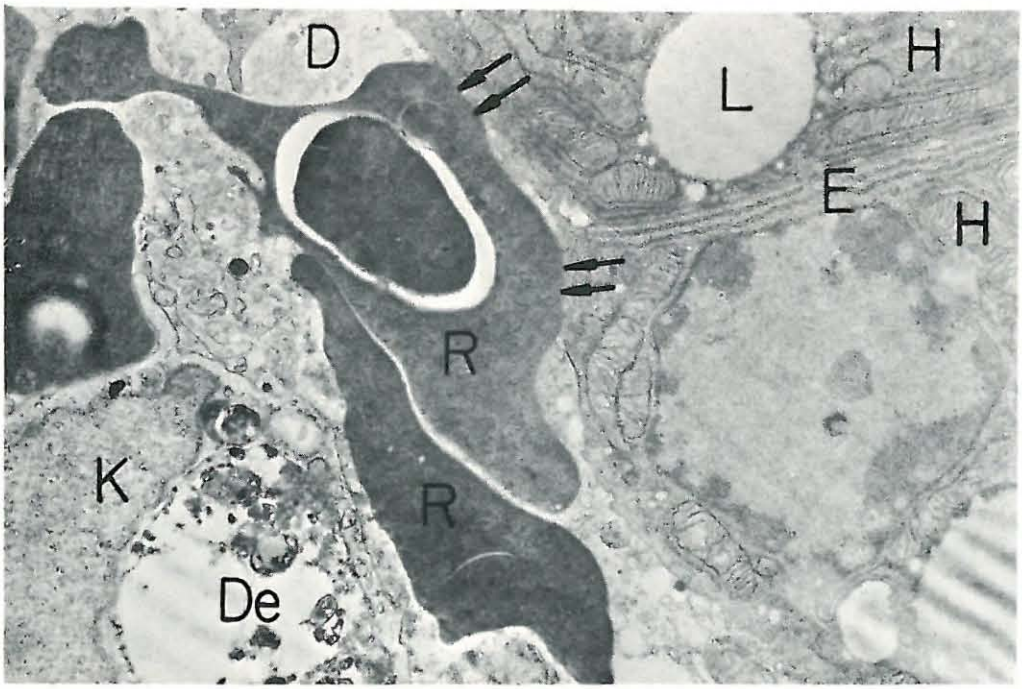


FIGURE 11. One hour after aflatoxin administration: Red blood cells (R) in space of Disse (D). Note close contact (arrows) between red blood cell and sinusoidal surface membrane of dark hepatocytes (H): Kupffer cell (K) shows area of focal cytoplasmic degradation (De). Note extremely elongated parallel cisternae of ER (E) in cytoplasm of the dark hepatocytes (H) L: Lipid vacuole (x9,000).

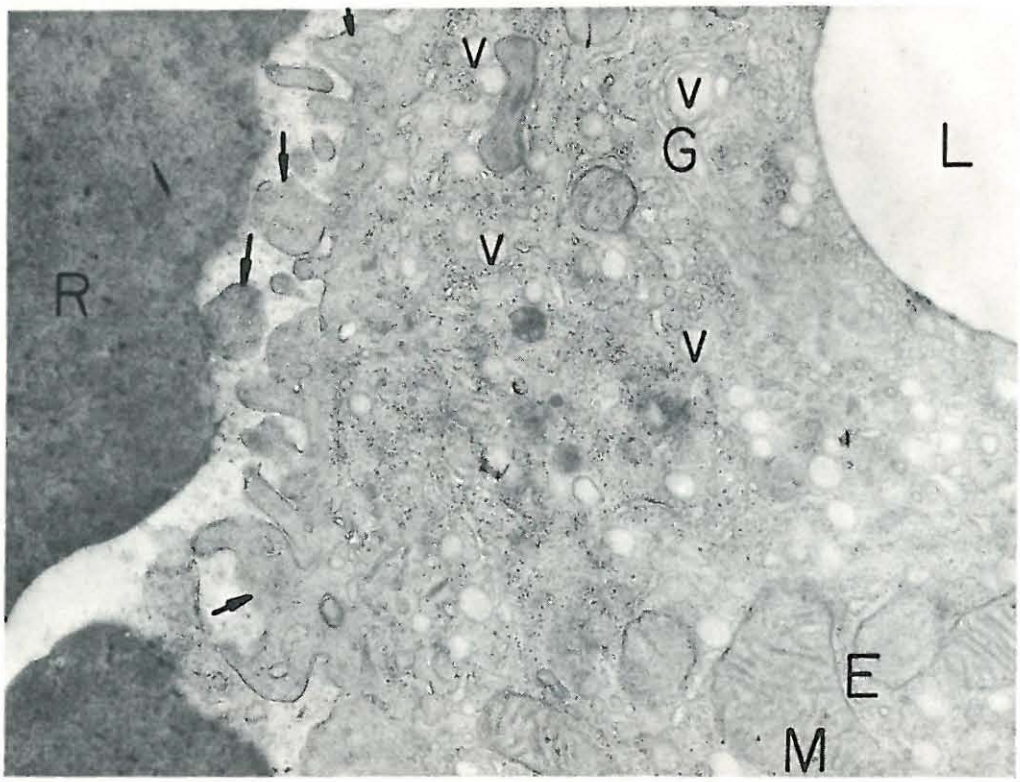


FIGURE 12. One hour after aflatoxin administration: Red blood cell (R) in intimate contact with sinusoidal microvilli of hepatocyte. Some of these microvilli appear swollen and club-shaped (arrows). Large numbers of Golgi vesicles (V) are present in cytoplasm of hepatocyte. The mitochondria (M) and rough ER (E) show no significant changes. G: Golgi apparatus, L: Lipid vacuole (x15,000).

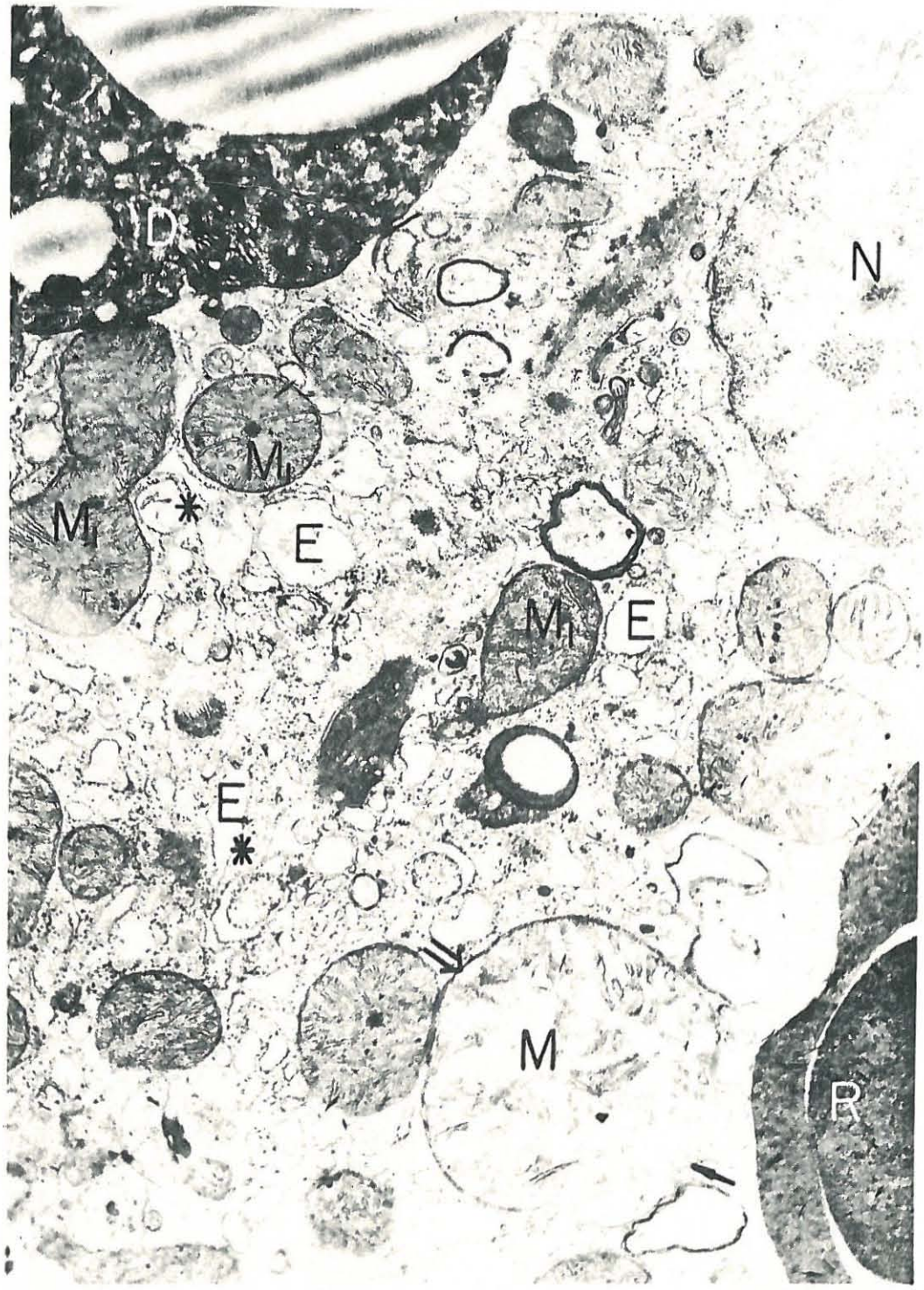


FIGURE 13. Cytoplasm of hepatocyte four hours after aflotoxin administration. A red blood cell (R) is in intimate contact with a mitochondrion (M) which is extremely swollen. The external limiting membranes of the organelle in close contact with the red blood cell appear as a single membrane (arrow) while the usual double limiting membrane (double arrow) can still be seen at the opposite pole of the mitochondrion. Other mitochondrion (M_1) in the same cell are normal in appearance. The cisternae of the ER are dilated (E) and the ER membranes show detachment of ribosomal granules(*). An adjacent dark cell (D) shows dense osmiophilic membranous and granular material in the cytoplasm. N: Nucleus (x15,500).

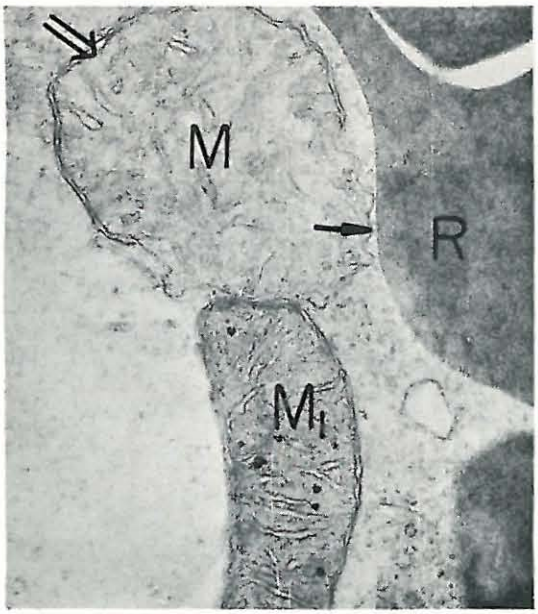


FIGURE 14. Four hours after aflatoxin administration. Swollen mitochondrion (M) in close contact with extravasated red blood cell (R). Note single limiting membrane (arrow) of mitochondrion in areas of intimate contact with red blood cell and double limiting membranes (double arrow) at opposite pole of organelle. Adjacent mitochondrion (M_1) not in close contact with red blood cell is normal in appearance (x26,500).

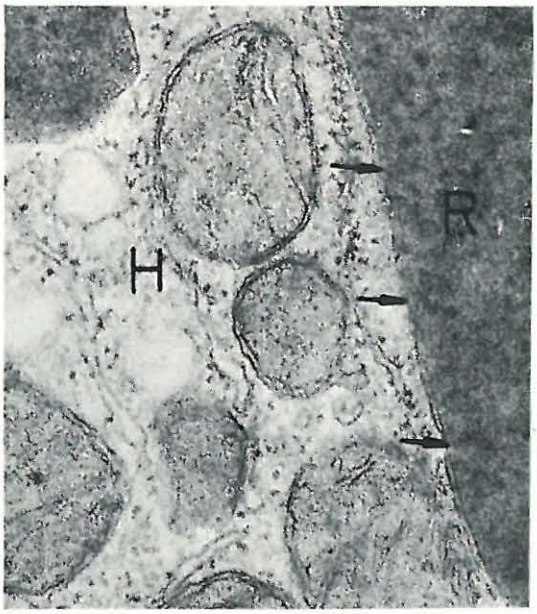


FIGURE 15. Two hours after aflatoxin administration. Red blood cell (R) in close contact with cell membrane of hepatocyte (H). The cell membrane is fragmented (arrows) (x40,000).

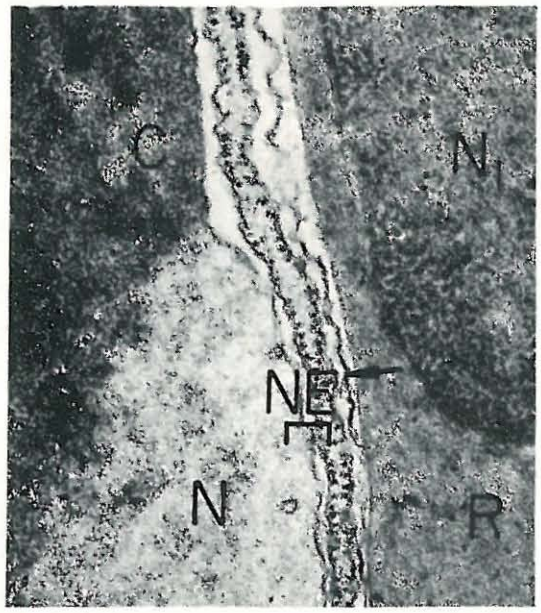


FIGURE 16. Eight hours after aflatoxin administration. A single membrane (arrow) of an ER cisternae in close contact with a red blood cell (R) shows complete loss of ribosomal granules while opposite membrane of the same cisterna has apparently normal numbers of ribosomes. N: Nucleus of liver cell with a dense marginal clump of chromatin (C), NE: Nuclear envelope , N_1: Nucleus of red blood cell (x50,000).

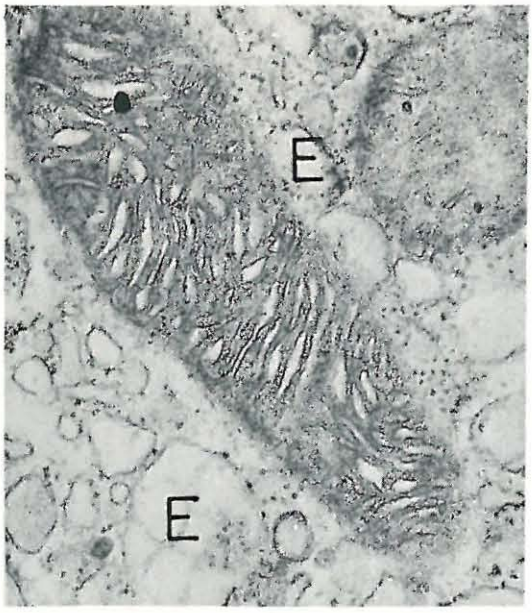


FIGURE 17. Increased density of mitochondrial matrix in hepatocyte of experimantal animal sixteen hours after aflatoxin administration. Note delated cisternae of ER (E) (x40,000).

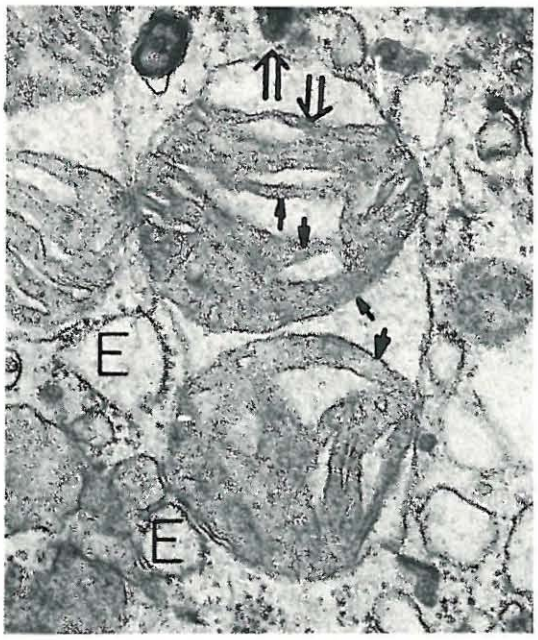


FIGURE 18. Sixteen hours after aflatoxin administration. Dense mitochondrion showing marked swelling between leaflets of individual cristae (single arrows) and in external compartment (double arrows). Dilated cisternae of the ER are shown at E (x60,000).

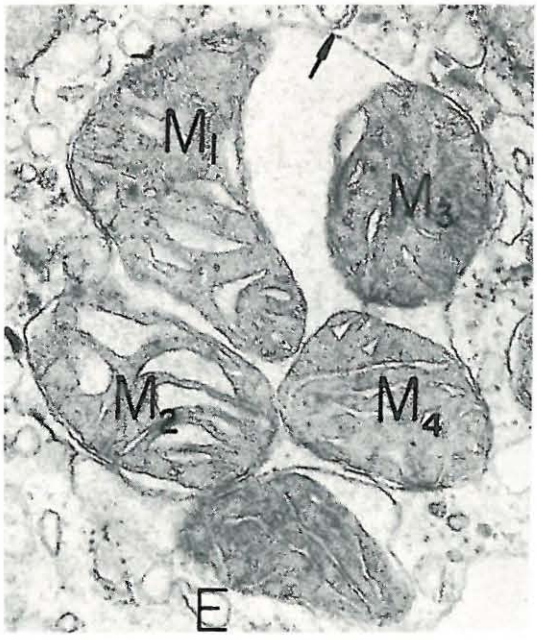


FIGURE 19. Twenty-four hours after aflatoxin administration. Further progression of mitochondrial changes shown in Figure 18 leads to appearance of lobulated mitochondrion enclosed by a single membrane (arrow) and consisting of 3 to 4 "lobules" of dense matrix (M_1 to M_4) separated by electronlucid oedematous areas, E: Dilated cisterna of ER (x45,000).

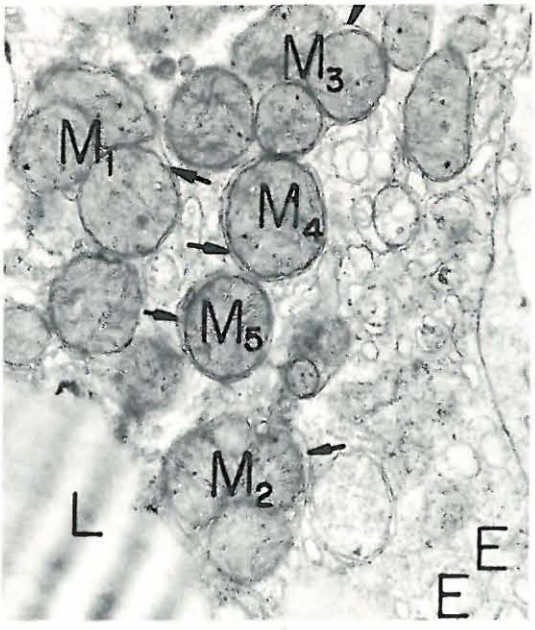


FIGURE 20. Mitochondria in hepatocyte of duckling 72 hours after aflatoxin administration. Apparently structurally normal mitochondria are found in groups of three to four organelles (M_1 to M_3) or as single organelles (M_4 and M_5), each separated from the rest of the cytoplasm by a single membrane (arrows). (See also Text - Figure 2 and Discussion) E: Dilated cisternae of ER, L: Lipid vacuole (x30,000).

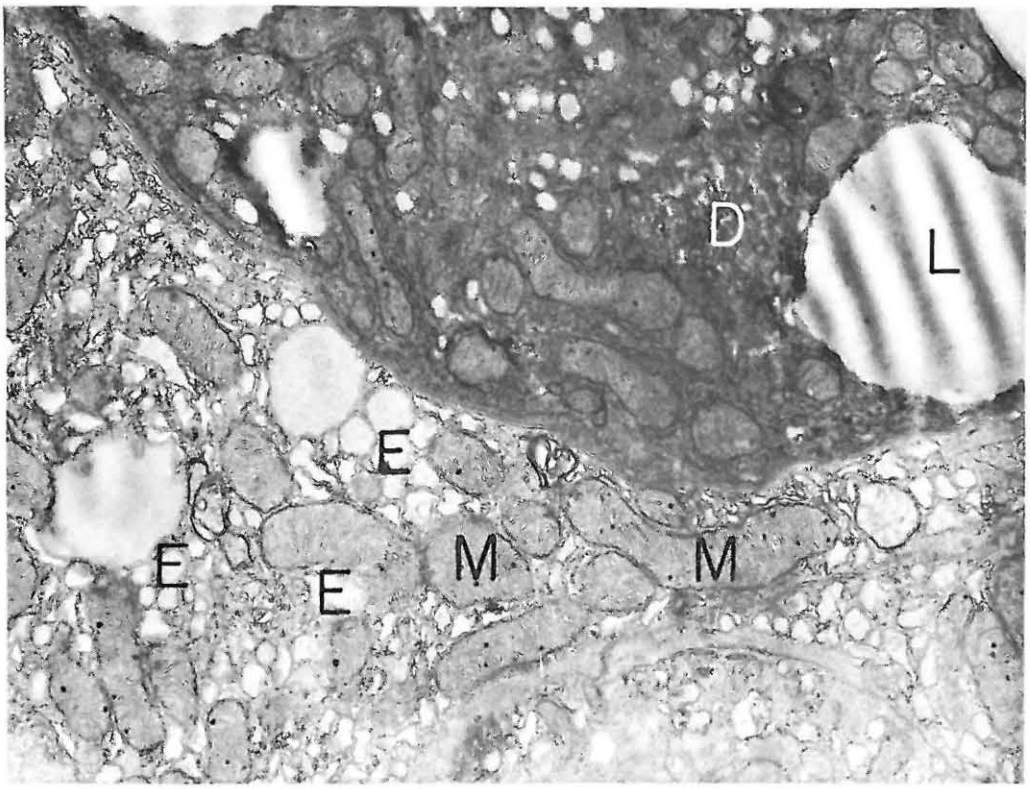


FIGURE 21. Sixteen hours after aflatoxin administration. Light and dark hepatocytes in liver of experimental animal. Dilated cisternae of the ER (E) are prominent in the light cell. The mitochondria (M) of this cell show no striking changes. The dark cell shows area of focal cytoplasmic degradation (D). L: Lipid vacuole (x12,500).

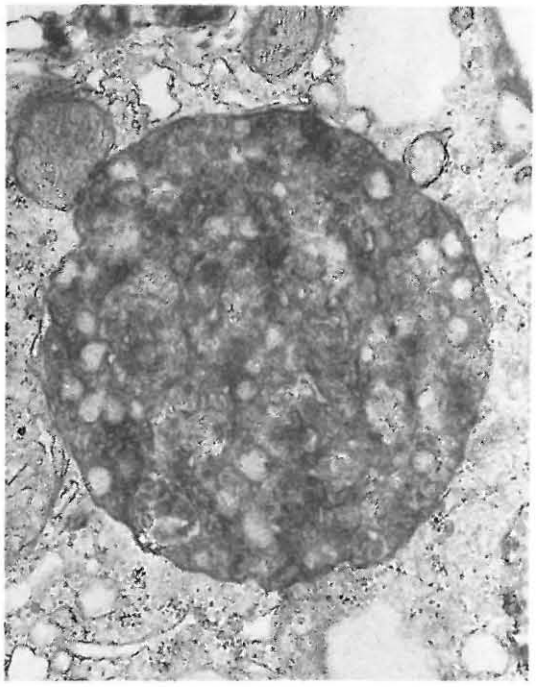


FIGURE 22. Twenty-four hours after aflatoxin administration. Dense osmiophilic organelle limited by a single membrane in the cytoplasm of a light cell (x15,500).

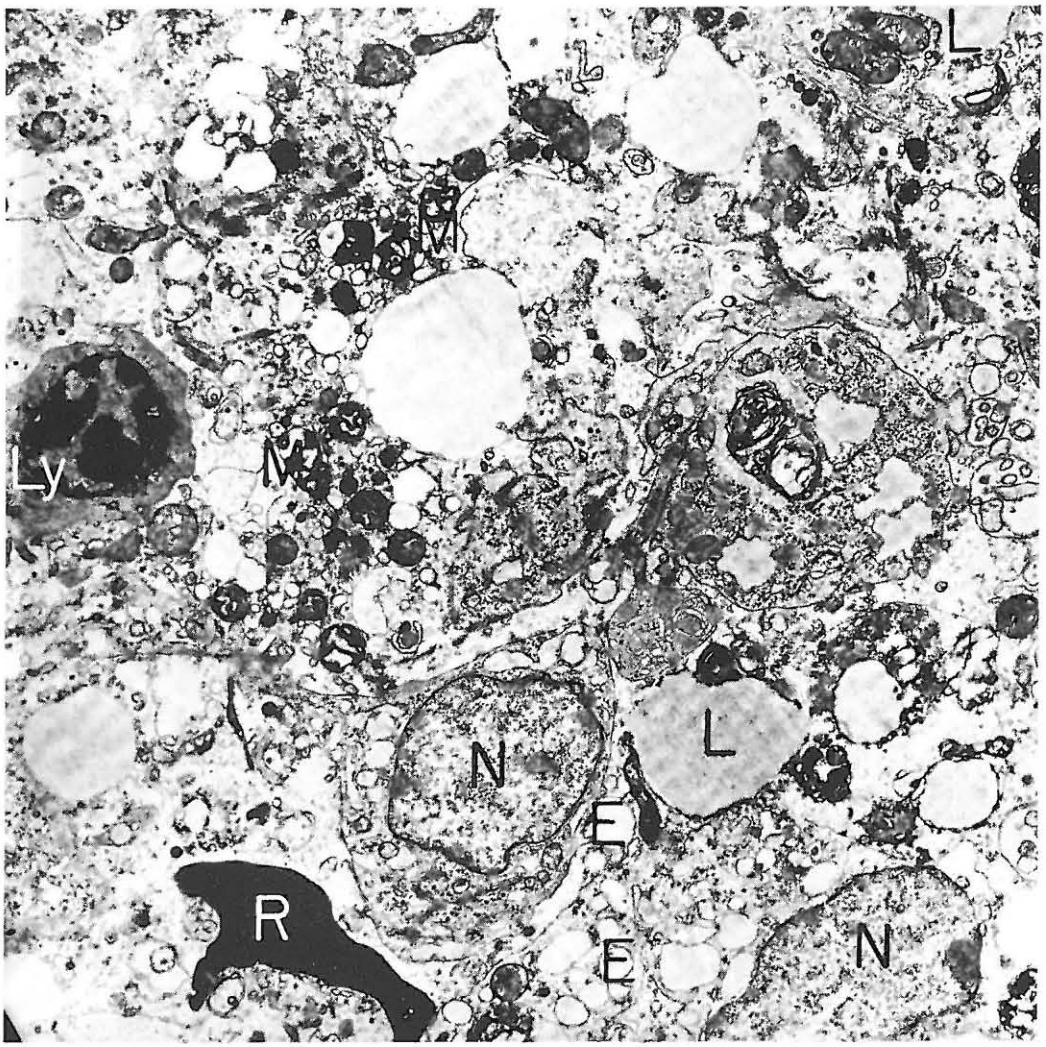


FIGURE 23. Necrotic hepatocytes 24 hours after aflatoxin administration. Note dense mitochondria (M), dilated ER cisternae (E) and lipid droplets (L). R: Red blood cell, Ly: Lymphocyte, N: Nuclei of hepatocytes (x4,600).

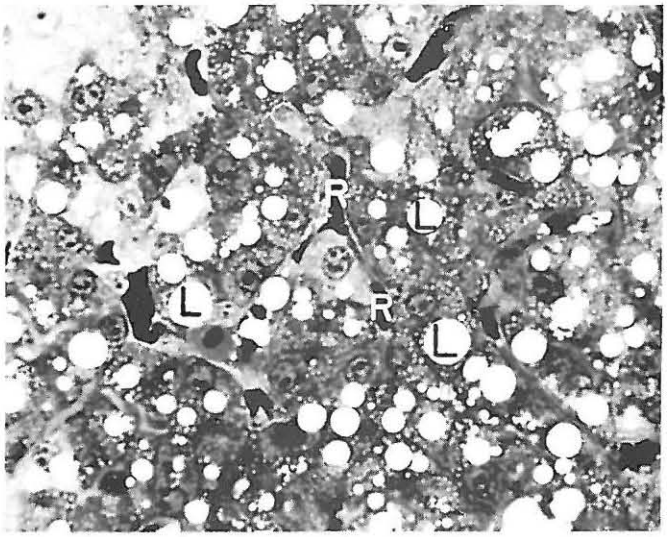


FIGURE 24. Control animal ochratoxin experiment. Moderate numbers of lipid vacuoles (L) are present in the cytoplasm of hepatocytes. The mitochondria of the hepatocytes can be seen as discrete "negative images". R: Red blood cells (Epon, PAS) (x500).

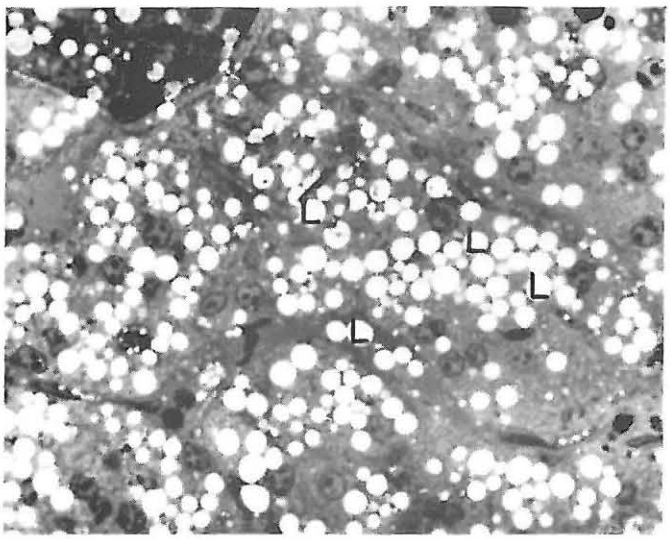


FIGURE 25. Increased numbers of lipid vacuoles (L) are found in the hepatocytes, 16 hours after ochratoxin administration. The mitochondria of the liver cells appear "blurred" (Compare with Figure 24) (Epon, PAS) (x500).

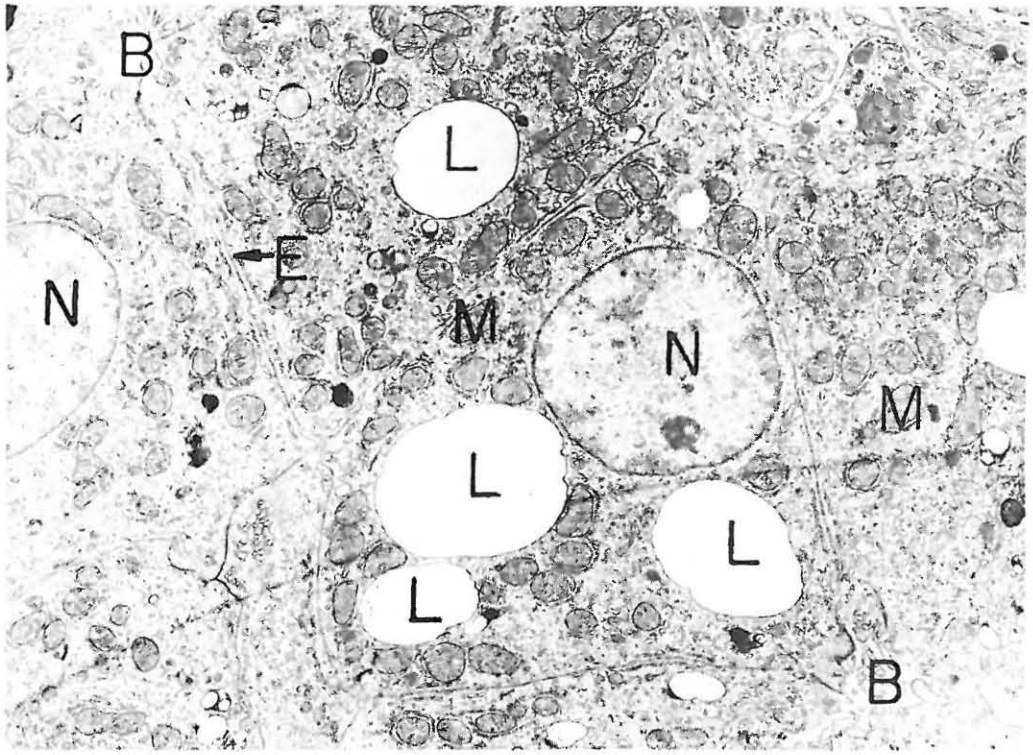


FIGURE 26. Liver cells of control animals, ochratoxin experiment. Note moderate numbers of lipid vacuoles (L): N: Nucleus, B: Bile canaliculus, M: Mitochondria, E: Endoplasmic reticulum (x4,600).

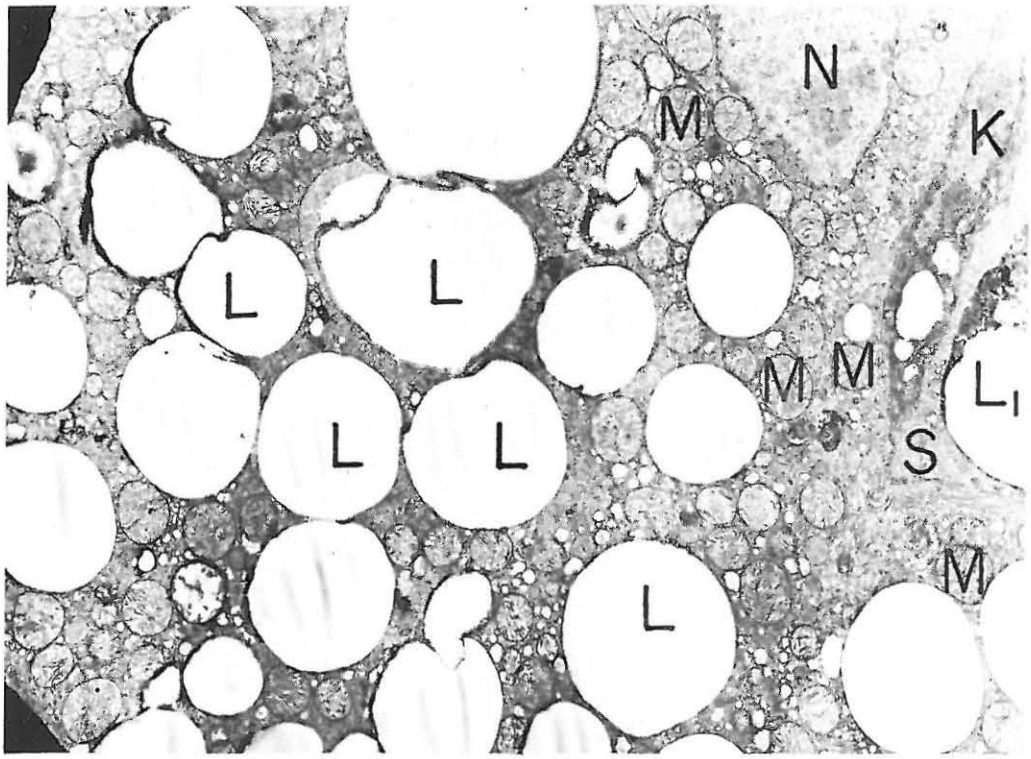


FIGURE 27. Sixteen hours after ochratoxin administration. There is a marked increase in the number of lipid droplets (L) in the cytoplasm of a hepatocyte. (Compare with Figure 26). A lipid vacuole (L_1) is present in a sinusoid (S). K: Kupffer cell, N: Nucleus of hepatocyte, M: round, swollen mitochondria (x4,600).

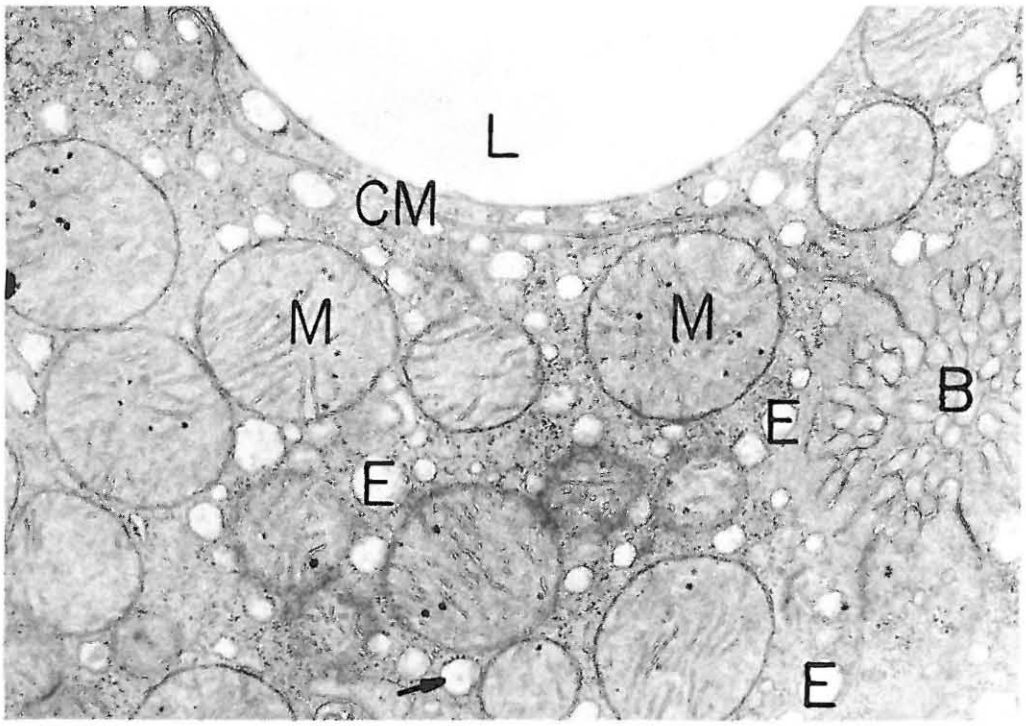


FIGURE 28. Round swollen mitochondria (M) are seen in the cytoplasm of a hepatocyte 4 hours after ochratoxin administration. The ER is seen in the form of isolated dilated vesicles (E). Some of these vesicles show focal loss of ribosomes (arrow). Increased numbers of ribosomes are found free in the cytoplasm. L: lipid vacuole, CM: Intercellular membranes, B: Bile canaliculus (x25,000).

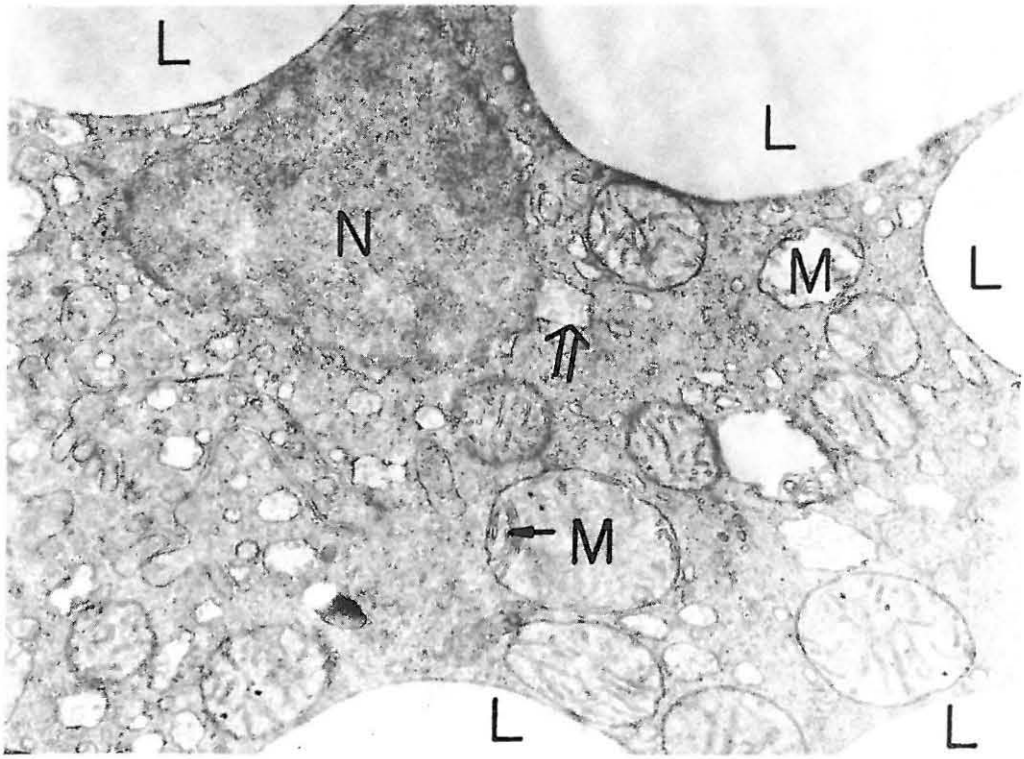


FIGURE 29: Eight hours after ochratoxin administration. The matrix of some mitochondria (M) is coarsely granular, while the cristae are compressed against the external limiting membranes of the organelle (arrow). The nuclear envelope shows focal dilated areas (double arrow). M: Nucleus, L: Lipid vacuoles (x24,000).

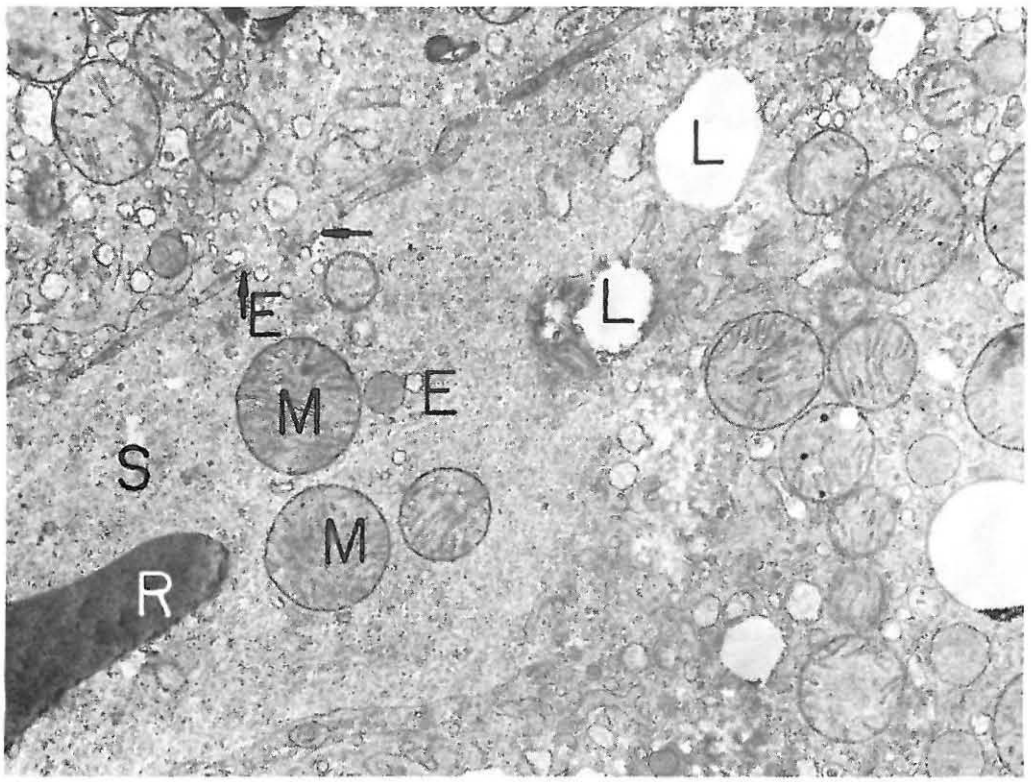


FIGURE 30. Liver cell mitochondria (M), ER vesicles (E) and lipid droplets (L) are found free in a sinusoid (S), in a duckling sixteen hours after ochratoxin administration. The arrows delimit gaps in the sinusoidal endothelium through which the liver cell organelles appear to be discharged into the sinusoid. R: Red blood cell (x11,000).

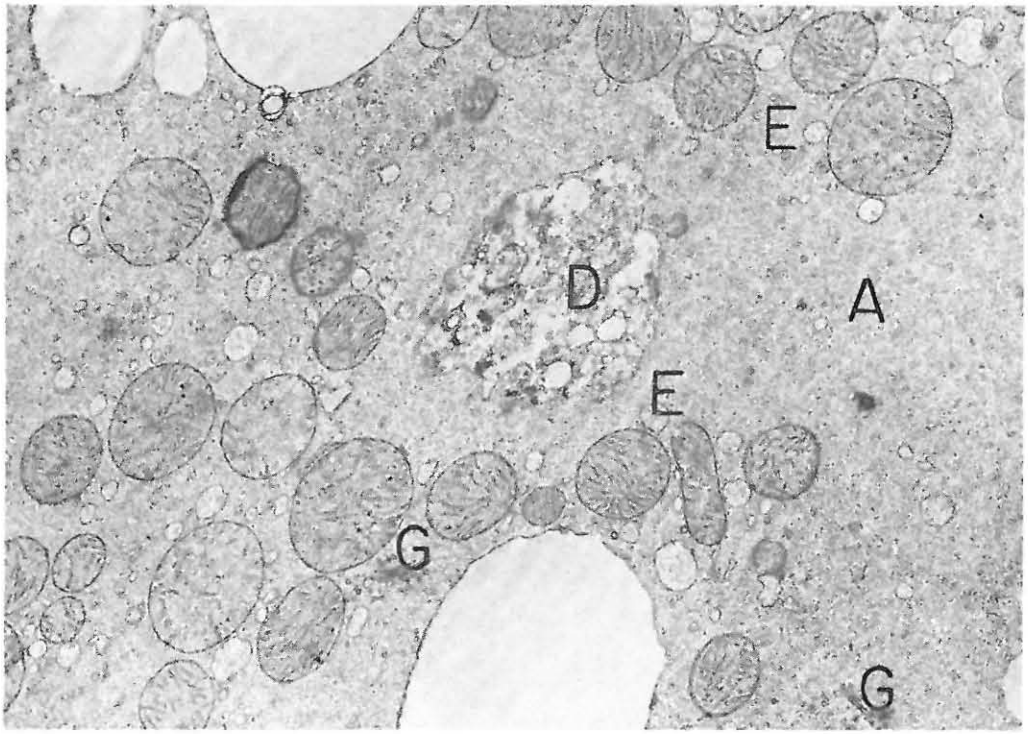


FIGURE 31. Twenty-four hours after ochratoxin administration. Note "empty" appearance of cytoplasm at A, with marked decrease in number of ribosomal granules. E: ER vesicles, G: Golgi lamellae, D: Area of focal cytoplasmic degradation (x12,000).

MYCOTOXINS AS POSSIBLE CAUSES OF PRIMARY CARCINOMA OF THE LIVER IN MAN

A.G. Oettlé

Cancer Research Unit of the National Cancer Association of South Africa,
South African Institute for Medical Research, Johannesburg.

SYNOPSIS

Although the evidence is still inadequate, mycotoxins offer a plausible explanation of endemic liver cancer in Africa and comparable regions.

Mycotoxins include the most potent liver carcinogens of experimental animals. As humidity-dependent substances, their presumptive distribution is consistent with the known epidemiology of liver cancer.

Previous suggestions, such as malnutrition, Senecio poisoning, infectious hepatitis and bilharziasis, fail to explain the low rates in Egypt, or the remarkable variations in intensity between neighbouring regions. Chronic alcoholism, which is known to be a factor in the West, has been excluded as a cause of liver cancer in certain African groups.

SAMEVATTING

Hoewel genoegsame bewyse nog ontbreek, bestaan daar n sterk moontlikheid dat endemiese lewerkanker in Afrika en vergelykbare streke aan mikotoksiene toegeskryf kan word.

Die sterkste verwekkers van lewerkanker by proefdiere ressorteer onder die mikotoksiene. As humiditeitafhanklike stowwe stem hulle vermoedelike verspreiding ooreen met die bekende epidemiologie van lewerkanker.

Ondervoeding, Senecio-vergiftiging, besmetlike lewerontsteking en bilharziasis, wat almal reeds as moontlike oorsake beskou is, verklaar nie die lae voorkomssyfer in Egipte of die merkwaardige verskil in intensiteit tussen buurstreke nie. Chroniese dranksug, wat in die Weste bekend is as n oorsaaklike faktor, kan by sekere groepe in Afrika buite rekening gelaat word as oorsaak van lewerkanker.

The recent discovery that potent hepatotoxins are produced by fungi on spoiled foods has provided a plausible explanation of the occurrence and distribution of human liver cancer in Africa and similar regions. (Oettlé, 1964). The evidence, however, still falls short of what is regarded as adequate proof, and this suggestion should be regarded for the time being as a working hypothesis and no more. This paper summarises the information on the epidemiology of liver cancer, examines the alternative hypotheses and indicates how mycotoxins fit the facts better than the earlier suggestions.

EPIDEMIOLOGY OF LIVER CANCER IN AFRICA

Primary carcinoma of the liver is a rare disease in most parts of the world, but since the beginning of this century it has been recognised to be unusually common in the Cape Coloured and Bantu of South Africa (Oettlé, 1955). A high incidence of this cancer has come to be accepted as a feature of the indigenous races of sub-Saharan Africa. The pattern of incidence is not uniform, however, and the following outline indicates the major facts which must be taken into account in any hypothesis.

1. ETHNIC GROUP AND TRIBE

The high incidence affects the negriform race chiefly and to a lesser extent the mixed group. It does not appear to affect whites or Asians living alongside them, nor does it occur in animals. The risk is not uniform within the Bantu and West African negro groups. Among South African Bantu gold miners, for example, those from Portuguese East Africa have an incidence which is six times that of the South African recruits (Berman, 1951). In some regions this cancer accounts for more than one third of all cancers recorded in both sexes, e.g. in Mozambique, Congo Republic (Brazzaville), Senegal, Gambia, Portuguese Guinea, Mali, Niger and Togoland. The disease is relatively rare in Egypt, Tunisia and Morocco.

2. SEX

Males are affected four or five times as often as females, in regions of high as well as of low incidence. An exceptionally high male-to-female ratio was reported from Madagascar where Brygoo (1961) recorded 24 male to only 1 female case, but this would need to be confirmed.

3. AGE

Initially this was thought to be a disease of young men, but that was because mining was an occupation of young men. In general, the incidence rises with age. This is evident in Johannesburg (Higginson and Oettlé, 1960) but in Lourenco Marques and Kampala the curve tends to flatten or decline slightly after 45 and 50 respectively. This might be explained on failure to register the older age-groups adequately, but it seems wiser to withhold judgement on this question until more evidence is available.

In regions of high incidence, cases occur in the first decade, but in regions of intermediate incidence the youngest cases tend to occur after puberty. This is consistent with a relatively short latent period after exposure to the causal agent.

4. HISTOLOGIC TYPE

The increased incidence involves one histologic type of carcinoma only, viz. the hepatocellular hepatomas, including those with an adenoid pattern. Intrahepatic cholangiocellular carcinomas have not been shown to be more common in Africa than they are in the west, and there is no increase in carcinomas of the extrahepatic biliary ducts, which suggests that the agent is not excreted in the bile, and probably affects the liver cell directly.

5. CIRRHOSIS

Cirrhosis of the liver is a common but not a necessary precursor of liver cancer, and where the incidence of liver cancer is high, this is also seen as an increased rate of malignant change in the non-cirrhotic livers as well. The type of cirrhosis is important. In cases with post-necrotic cirrhosis seen at post-mortem in Johannesburg malignant degeneration had occurred in over 50 per cent, whereas it was much less common in septal cirrhosis (Isaacson, Seftel, Keeley and Bothwell, 1961).

The proportion of cirrhotics undergoing malignant change appears to be higher in areas of high incidence. Thus, in Lourenco Marques the figure is 61.5 per cent in males and 51.4 per cent in females (Prates and Torres, to be published), whereas in Johannesburg the figures are 47 and 12 per cent respectively (Higginson and Steiner, 1961).

Both in Lourenco Marques and in Johannesburg the proportion of elderly

cirrhotics undergoing malignant degeneration is less than that in the younger group. It is possible that this is an artifact, e.g. it may be that elderly patients with a relatively acute disease such as liver cancer may be less likely to present themselves for treatment by comparison with those with a more prolonged illness. On the other hand, this trend may reflect a lessened intensity of exposure in the older group, and merits investigation.

6. ACUTENESS OF THE DISEASE

In the west, primary carcinoma of the liver following upon cirrhosis tends to develop at the end of a long illness, and may be discovered unexpectedly at post-mortem as a complication of the underlying chronic disease. In Africa, however, it is noteworthy that many cases are of acute onset in people who previously appeared to be completely healthy, and, in the case of Gold miners, may have successfully passed three separate medical examinations a few months previously. In some the first evidence of an abnormality is the terminal intraperitoneal haemorrhage. Others on the other hand, run a chronic course similar to that familiar in the west, and all intermediate gradations are known.

7. FLUCTUATIONS IN INCIDENCE

In 1953 and 1954 in Johannesburg, a seasonal fluctuation was observed in registrations with liver cancer, more admissions being noted from December to May than from June to November (Higginson and Oettlé, 1957). Confirmation of this tendency is being sought at present (Oettlé and Macedo, unpublished results, 1965).

In 1948 an extraordinary drop in primary cancer of the liver was apparent in figures from French West Africa published by Denoix, Schlumberger, Laurent and Maujol (1957). This affected both sexes, the proportion of all cancers assigned to the liver falling from a level of 30 per cent in 1947 to 9 per cent in 1948, returning to 34 per cent in 1949.

Such findings, inadequate as they are, suggest a very rapidly acting influence.

PATHOGENESIS

At this point it may be legitimate to speculate on the pathogenesis of liver carcinoma, as observed in Africa.

The striking association with postnecrotic cirrhosis suggests that cancer follows exposure to an agent producing liver necrosis and reactive hyperplasia. By analogy with the phenomenon of promotion in epidermal carcinogenesis, such hyperplasia might become the precursor of malignant change in a large proportion of individuals. This mechanism is consistent with the acute onset already mentioned. The occurrence of cases without cirrhosis demonstrates that this agent may have a direct influence without producing scarring of the liver.

If a two-stage mechanism of carcinogenesis is involved, prior exposure to initiating agents will also have to be postulated.

Predisposing factors must also be considered. Animal experiments have shown that mitosis in the liver is induced by a fall in blood albumin, however produced, this substance apparently being the specific inhibitor of cell division in the liver. A low level of serum albumin is commonly found in the Bantu, the albumin-globulin ratio being reversed in 69 per cent of adult Bantu males in Johannesburg (Bersohn, Wayburne, Hirsch and Sussman, 1954), which may account for a greater susceptibility to hepatotoxic influences. In addition, abnormalities in liver function tests are common, being detectable within the first two years of life, (Annual Report, 1953). This remains pure speculation, however, and we have no evidence that the risk of liver cancer is greater in persons showing these abnormalities than it is in those who do not.

REQUIREMENTS FOR PROOF OF A CAUSAL RELATIONSHIP IN HUMAN CANCER

Since experimental testing of carcinogens is out of the question in man, proof of causation of human cancer must inevitably come short of the reliability of experimental proof. Medicine is dependent on decisions of lesser stringency than those attainable in a perfect experiment, where the risk of error is limited to truly random variations. Despite the possibility of hidden bias, the success of scientific medicine is proof that valid inferences can be drawn. In bacteriology, Koch's postulates were accepted as proof of a causal association between an organism and a disease. Similar postulates exist for the association between a cancer and a carcinogenic factor, viz.

I. In the presence of the factor there should be an increased risk.
II. The risk should vary with dosage (allowing for the appropriate latent period.)
III. The site affected should have been exposed to the factor.
IV. The factor should be capable of inducing cancer of the same or of a com-

parable site in susceptible experimental animals.

The case is strengthened if a chemical carcinogen can be identified, and its nature and dosage is compatible with current knowledge of carcinogenesis.

The complete web of causation is infinitely complex. In medicine we are concerned with effective action. This requires no more than the identification of the relevant proximate influences, which, if removed, would lead to a substantial reduction in risk of the disease. On this empirical basis, a causal relation would be diagnosed with predisposing, carcinogenic (initiating) and co-carcinogenic (promoting) factors. The factors involved might be several degrees away from those immediately responsible for carcinogenesis, but their discovery will indicate the chains of causality whose precise links must be discovered by subsequent intensive research.

EARLIER HYPOTHESES

These illustrate the fact that a smattering of information is the condition most productive of a luxuriant crop of hypotheses. Correlation, however, is not necessarily causation.

1. ETHNIC GROUP

This disease was once regarded as a feature of pigmented races. As with so many other examples of racial predisposition, it is now accepted that this association with race is an expression of differential environmental exposure. The profound differences in incidence seen within the Bantu in South Africa, e.g. between the Southern and Northern Lowveld (Oettlé, unpublished material, 1965) cannot be explained on genetics.

2. MALNUTRITION

This has been another favourite, conveniently vague. It has been suggested that malnutrition in infancy renders the liver more susceptible to later hepatic stresses, but a high incidence of kwashiorkor is not necessarily followed by a high risk of liver cancer e.g. in Egypt, Greece and South America. There is no evidence of quantitative differences in malnutrition between areas of high and of intermediate liver cancer incidence. The association may be coincidental, indirectly correlated or causal.

3. ALCOHOL

There seems little doubt that in white populations chronic alcoholism is an important factor in the development of cirrhosis and subsequent malignant change. Questioning of Bantu in Johannesburg, however, showed no association with alcohol, and this is confirmed by the lack of an association with haemosiderosis at post-mortem. (As a result of brewing in iron receptacles, the South African Bantu drinker has a high iron intake, since the acid fermentation may dissolve up to 85 mgm. of iron per 100 ml. (Walker and Arvidsson, 1953).

4. BILHARZIASIS

The high incidence of bilharziasis in Lourenco Marques led Prates to consider it to be causal. This association could not be confirmed in Johannesburg in a series of cases of liver cancer matched with controls at post-mortem (Higginson and de Meillon, 1955). Furthermore bilharziasis is rare in French West Africa where liver cancer is frequent, while liver cancer is rare in Egypt where bilharziasis is frequent. Recent studies in Lourenco Marques have shown that the previous association was an artifact of diagnostic stringency, for more cases with liver cancer came to post-mortem. When post-mortem cases were matched, no correlation was discernable (Prates and Torres, to be published).

5. INFECTIOUS HEPATITIS

The association with post-necrotic cirrhosis led Higginson and Steiner (1961) to suspect infectious hepatitis, but there is no evidence that this disease is excessively common in the Bantu. In Egypt, where infectious hepatitis is endemic, liver cancer is rare. In the absence of a satisfactory test for previous exposure, this theory remains speculation.

6. SENECIO POISONING

Schoental and Magee (1959) have cast suspicion on the hepatotoxic alkaloids of this plant genus, but there is no apparent association between liver cancer and Seneciosis in Africa, either from case histories or from the geographical distribution of these two diseases.

7. TANNIC ACID

Tannic acid is hepatotoxic, and can cause carcinoma of the liver experimentally (Korpassy, 1960) but there is no reason to regard it as relevant in Africa.

THE MYCOTOXIN HYPOTHESIS

The hypothesis which best fits the epidemiologic evidence at present is that the high incidence of liver cancer in Africa is due to the ingestion of food contaminated with mycotoxins such as Aflatoxin.

The evidence still falls short of what is acceptable as proof, but it does explain many of the features of primary carcinoma of the liver in Africa.

1. HUMIDITY

The growth of Aspergillus flavus requires a relative humidity above 80 per cent : it is noteworthy that all regions of high liver cancer incidence in Africa are regions of high relative humidity. The low rate in Egypt can thus be attributed to its dry climate, despite the presence of factors likely to cause liver damage, e.g. malnutrition, bilharziasis and infectious hepatitis.

2. DEFECTIVE STORAGE OF FOOD

Apart from fungal contamination during harvesting, primitive methods of food storage will add to further production of mycotoxins. Fungal contamination of stored grain is well known in Africa, where underground storage may be traditional.

3. ETHNIC GROUPS

The different risks of various groups can be explained on their harvesting and storage practices, the greater dependence of the poorer strata on grain products, as well as the possibility that previous liver damage (of which there is abundant evidence) may have left them more susceptible to mycotoxins, quite apart from their greater likelihood of exposure.

4. FLUCTUATIONS IN INCIDENCE

This may be explained on the need during periods of scarcity to eat mouldy grain that would otherwise have been left untouched or discarded. Seasonal

fluctuations in mould contamination are also common : the recent contamination of the South African groundnut crop in 1963 was explicable on unseasonable humidity.

The hypothesis has certain obvious deficiencies. In the first place, it is at present too elastic. By postulating an accidental contamination with a toxigenic strain of a common mould, one can make this hypothesis fit any facts, unless the toxic materials are demonstrated. Secondly, the sex ratio of liver cancer is not easily explained, although it may be attributed to the wellknown greater susceptibility of the male liver to damage. Thirdly, the rising age incidence of liver cancer is not easily accounted for on a single acute exposure, although it would be compatible with a summation of subliminal hepatic lesions.

Other anomalies may spring to mind. Thus Brazil, which was an exporter of toxic groundnuts, has not been reported to have a high liver cancer incidence. I am told, however, that their groundnut crop is not consumed by the local population.

As regards the postulates quoted earlier, the evidence provides grounds for suspicion, but falls short of proof.

I. Nigeria and South Africa are examples of areas where Aflatoxin is known to be present and where there is an increased risk of liver cancer. Far more evidence is needed.

II. There is as yet no evidence that the risk increases in proportion to the dose of Aflatoxin, although this is consistent with the relative humidity conditions in regions of varying incidence. Unfortunately Aflatoxin is very difficult to detect in the liver e.g. of a rat 6 hours after feeding 1 mg. experimentally, as well as after continued feeding of 3-4 p.p.m. for 6 wks. (Butler, personal communication). A study of secular changes in the incidence of liver cancer may prove useful, for a reduction in mould contamination of foodstuffs can be expected to be brought about in a number of countries. On the basis of this hypothesis, a fall in liver cancer should be observable after the appropriate latent period. The importance of assays of mycotoxins in food products in many strategic areas will be apparent.

III. The site is certainly exposed to the factor.

IV. Experimental confirmation is abundantly available. Aflatoxin is by far the most active hepatocarcinogen known. Short term administration of as little

as 10 µg per day is sufficient to give nearly 100 per cent incidence of liver tumours in rats (Butler, personal communication). This agent is 1000 times more potent than the azo dyes commonly used in experimental hepatic carcinogenesis.

RECOMMENDATIONS

The most acute present need is for quantitative measurement of exposure and of risk of liver cancer, in order to meet the requirements of the first and second postulates.

Some might ask if it be necessary to obtain further proof. Would it not be reasonable simply to improve the quality of food storage processes immediately? While such hygienic measures are justifiable on many grounds, e.g. to eliminate a toxic substance from the human diet and from animal feeds, proof that this would eliminate liver cancer is still wanting. The following discussion refers only to preventive action against liver cancer.

One of the principles of epidemiology is that it is wasteful of money and effort to introduce widescale sanitary measures in dealing with a single specific agent. The rifle is more accurate than the shotgun, and specific agents require specific action, akin to species sanitation in malariology.

A second objection stems from the size of the problem. If the mycotoxin hypothesis be accepted it will probably involve astronomical sums of money, and will affect the economic stability of many countries, some of which are dependent, like Gambia, on a single crop (groundnuts). It will involve the condemnation of vast quantities of food, of which the populations are already short, and considerable investment in food-storage machinery.

The problem affects nearly all the countries of sub-Saharan Africa, as well as China, Japan and Indonesia. The United States may also be involved, for the incidence of primary carcinoma of the liver in both whites and non-whites in the United States is many times higher than that of Denmark, though still well below that of the South African Bantu.

Further investigation is warranted. This is no justification of laisser-faire, of which there is enough, nor of deliberate attempts at suppression of evidence, of which there are hints. The opposite error, beloved of politicians is equally unjustified - that of instituting sweeping reforms whose effectiveness has not been demonstrated nor practicability confirmed by a pilot scheme. This, it may be

remembered, was the fallacy of the well-meant scheme for fortification of bread.

In the field of the mycotoxicoses there is sufficient ignorance as well as sufficient evidence to justify a substantial investment in research to obtain the information necessary for an informed decision. If this hypothesis be confirmed it will involve considerable expenditure, but also a tremendous reward - the prevention of one of the commonest cancers in Africa.

REFERENCES

ANNUAL REPORT. 1953. The South African Institute for Medical Research. p. 21.

BERMAN, C. 1951. Primary Carcinoma of the Liver. London, H.K. Lewis. 166pp.

BERSOHN, I., WAYBURNE, S., HIRSCH, H., and SUSSMAN, C.D. 1954. S.Afr.J.Lab.clin. Med. 5 : 35-44.

BRYGOO, E.R. 1961. Acta Un.Int.Cancr. 17 : 705-710

BUTLER, W.H. 1964 (Personal communication).

DENOIX, P.F., SCHLUMBERGER, J.R., LAURENT, C., and MAUJOL, L. 1957. Le cancer chez le noir en Afrique Francaise. Monographie de l'Institut National d'Hygiene, No. 12. Ministere de la Sante Publique. 179 pp.

HIGGINSON, J. and DE MEILLON, B. 1955. Arch.Path. (Chicago) 60 : 341-346.

HIGGINSON, J. and OETTLÉ, A.G. 1957. Acta Un.Int.Cancr. 13 : 601-604.

HIGGINSON, J. and OETTLÉ, A.G. 1960. J.nat.Cancer Inst. 24 : 589-671.

HIGGINSON, J. and STEINER, P.E. 1961. Acta Un.Int.Cancr. 17 : 654-666.

ISAACSON, C., SEFTEL, H.C., KEELEY, K.J. and BOTHWELL, T.H. 1961. J.Lab.clin.Med. 58 : 845-853.

KORPASSY, B. 1960. Abhandlungen der Deutschen Akademie der Wissenschaften zu Berlin. Klasse für Medizin No. 3. 18-23.

OETTLÉ, A.G. 1956. J.nat.Cancer Inst. 17 : 249-280.

OETTLÉ, A.G. 1964. J.nat.Cancer Inst. 33 : 383-439.

PRATES, M. and TORRES, (to be published) 1965.

SCHOENTAL, R. and MAGEE, P.N. 1959. J.Path.Bact. 78 : 471-482.

WALKER, A.R.P. and ARVIDSSON, U.B. 1953. Trans.Roy.Soc.Trop.Med.Hyg. 47 : 536-548.

MYCOTOXINS IN VETERINARY MEDICINE

L. Abrams

B. V. Sc (Pretoria)

Head, Poultry Research, Veterinary Research Institute, Onderstepoort.

SYNOPSIS

Mycotoxins have been recognised as important aetiological factors in veterinary medicine producing symptoms and mortality in most domestic and laboratory animals and poultry.

Mycotoxins occur in feedstuffs e.g. groundnut cake meal (aflatoxin) the litter of poultry houses and pastures which effect mainly sheep and cattle.

i) Hepatotoxin (aflatoxin)
ii) Those responsible for Haemorrhagic Disease (Poultry)
iii) Photosensitivity associated with liver lesions.

The incidence, symptoms, pathology and implications of these conditions are discussed in this paper.

---ooOoo---

SAMEVATTING

Mikotoksiene (swamgifstowwe) word vandag erken as belangrike aetiologiese faktore in die Veeartsenykunde. Hulle veroorsaak simptome en mortaliteit by feitlik alle huis- en laboratorium diere asook pluimvee.

Mikotoksiene kom in veevoedselbestanddele soos byvoorbeeld grondbonekoekmeel (aflatoksien) voor en word ook gevind in die skropgoed in pluimveehuise en op weidings waar skape en beeste hoofsaaklik aangetas word.

Mikotoksiene veroorsaak drie siekte toestande by diere:-

i) Hepatotoksiene (aflatoksien)
ii) Verantwoordelik vir Haemorrhagiese siekte (Pluimvee)
iii) Fotosensitiwiteit wat gepaard gaan met lewer letsels.

Die voorkoms, simptome, patologie en implikasies van hierdie drie toestande word in hierdie referaat bespreek.

In recent years mycotoxins have been recognised as major aetiological factors in diseases of livestock and poultry. Intensification of the poultry industry was been followed by a fairly new complex of diseases associated with managerial deficiencies, some of which create ideal environmental conditions for fungal proliferation. The use of an important ingredient (which contained mycotoxins) in the rations of livestock led to extensive mortality which, if not recognised, could have had disasterous results, particularly for chickens, ducklings and dogs.

In Veterinary Medicine the mycotoxins cause three major disease syndromes.

(1) The hepatoxins which affect only the liver e.g. aflatoxin.

(2) Those producing a haemorrhagic syndrome, e.g. haemorrhagic disease of fowls.

(3) Those producing liver lesions and photosensitivity e.g. facial eczema in sheep.

I. AFLATOXICOSES

Aflatoxin is a hepatotoxin which affects ducklings, poults, pheasants[1,2,3] and New Hampshire[4] chickens up to the age of ten weeks; the most susceptible age being one to four weeks; dogs of any age, piglets under the age of eight weeks and calves under the age of three months. Sheep are not susceptible to amounts of toxin normally encountered in rations, but are affected when given two ounces of groundnut cake meal containing upwards of 60 parts per million twice a week for four to six weeks. Chickens of the Cornish Game, White Rock, White Leghorn and Rhode Island Red breeds are unaffected when given rations containing 0.75 p.p.m. toxin. It is strange that the Rhode Island Red should be refractory since the New Hampshire breed is derived from the Rhode Island Red. Cornish Game, New Hampshire Crosses are also refractory.

There is a suspicion, yet to be confirmed, that chinchillas are also susceptible, Of the laboratory animals generally used ferrets, white rats and guinea pigs are susceptible, while white mice are refractory. Trout have also been found to be susceptible to very small amounts of the toxin.

In India Rhesus monkeys were found to develop acute aflatoxicosis when given high doses of the pure toxin.[5] All the afore-mentioned susceptible animals can develop acute aflatoxicosis, the degree of which

depends on the toxic level of the feed, save in rats[6,7,8] which develop hepatomas after feeding continuously for nine months or longer on meal containing highly toxic ground nut cake meal. At present monkeys are being maintained on low levels of toxin to determine their susceptibility which might give an indication of human susceptibility. If these animals are found to be susceptible it will not in any way be conclusive, as it must be remembered that there is a difference in species susceptibility and even in breeds of fowls.

An attempt was made to determine the reason for the susceptibility of New Hampshire chickens and the non-susceptibility of other breeds, but unfortunately to date no answer to this intriguing question can be given. However, during the course of these studies we have obtained many interesting facts about the biochemical mode of action of this toxin.

Certain liver enzymes of the respiratory chain were studied and it was found that the succinic Dehydrogenase, Cytochrome C and others were markedly affected. (Details of this work are being published elsewhere)[9]. We have also found that there is a marked reduction of the total plasma proteins in all affected birds. The reduction of plasma albumins accounts for the impairment in growth in birds that survive. The drop in globulins probably renders them susceptible to bacterial and viral diseases. An example of this is that from four groups of ducklings and two groups of Hampshire chickens, Salmonella with somatic factors 6 and 7 were isolated. A similar finding was reported in turkeys when the disease was first encountered in England[10]. Biochemical changes can be observed prior to any structural changes taking place in the liver. Within a few days of removing the toxic feeds from ducks and chickens normal enzyme values are once again found. Within a week of replacing these birds on toxic rations, they were again affected. The practical application of these findings is that if birds or animals are erroneously given toxic groundnut cake meal for short periods they will recover when placed on toxin-free rations. Naturally this will be detrimental to the weight gains of broiler chickens, or ducklings raised as table birds. The degree of damage will depend on the amount of toxin present in the ration and the length of time that they are maintained on this diet.

In all studies of mycotoxicoses, irrespective of the fungi involved in poultry, we have found that the selenium content of the liver was markedly raised to between 10-30 p.p.m, whereas the normal values of fowl livers is 0,50 to 3 p.p.m, when fed rations containing

a maximum of 3 p.p.m Se. What the significance of this finding is, is not quite clear at the moment, but it may be that selenium accumulates following liver damage that is found in mycotoxicosis. This interesting facet of the disease is still being investigated.

Aflatoxin has not been found to be present in the eggs of hens maintained on rations containing 75 p.p.m. toxin, nor has it any effect on the fertility and hatchability of eggs. This is in agreement with the findings of Allcroft and Carnaghan[11]. The toxin has not been found to be present in muscle from birds which had chronic aflatoxicosis. Eggs and muscles were fed to ducklings and New Hampshire chickens continuously for periods of up to eight weeks without any detrimental affect on their weight gain, or production of biochemical or histological changes. Cows maintained on rations containing 20% groundnut cake meal which had 8 p.p.m. aflatoxin B1, and given 16 pounds of this mash per day for six months, did not at any stage produce milk containing any toxin or metabolite of the toxin. This milk was fed to ducklings continuously for 8 weeks at different periods during the 6 months test period. Milk powder made from the milk of another group of cows receiving 5 lbs. of groundnut cake meal daily containing 8 p.p.m. Aflatoxin was fed to ducklings without any effect. A methanol and chloroform extract made from this powdered milk was fed to another group of ducklings and once again no biochemical changes or liver lesions could be found. In England[11] and Holland[12] extracts made from milk obtained from cows fed 20% highly toxic groundnut cake meal, produced lesions typical of aflatoxicosis when fed to ducklings.

It must be emphasized, however, that these cows were fed abnormally high amounts of toxin, and that the extracts made from the milk represented three litres of milk fed to each duckling, over a period of three days. The amount of toxin found in milk[13] from cows fed 2 Kilos of groundnut cake meal containing 4 p.p.m. per day would require a child of ten pounds to consume 27 litres of milk per day to have the same affect as on young ducklings, provided that the human is as susceptible as a duckling. The same British Workers found no evidence of the toxin in bulk milk samples, so there should be no possibility of milk containing toxin reaching the public. There is one possible reason for the overseas findings differing from ours, and that is a breed susceptibility to the toxin.

Recently cows fed groundnut cake meal containing a minimum of 60 p.p.m. Aflatoxin produced milk which, when fed to ducklings, produced lesions of Aflatoxicoses. No cow in practice could consume

that mount of toxin and remain alive. These animals fed this type of groundnut (they were actually dosed) became ill within a week of eating this food and would most certainly have succumbed had the ration not been changed. The milk production was also reduced to minimal quantities. All food products for human consumption obtained from birds and animals fed small amounts of toxin are thus free from toxin or metabolites of the toxin.

Aflatoxin at first causes marked fatty degeneration of the liver followed by proliferation of the bile ducts, cirrhosis and necrosis. The lesions vary somewhat in different species. It has been reported that some rats maintained on a diet in which toxic groundnut cake meal was present, produced hepatomas after ten to twelve months continuous feeding[6,7,8]. Whether aflatoxin is a Carcinogen has yet to be definitely established. Only when purified aflatoxin is available, in larger amounts, can this claim be verified. Certainly in ducks no tumours are found. From our biochemical studies it is doubtful whether aflatoxin is carcinogenic. There is no evidence that the Hepatomas found in rats can maintain themselves when the toxin is withdrawn. We are presently feeding a large number of rats with rations containing different levels of toxic groundnut cake meal and hope to be able to report more fully on our findings at a later stage.

Marked changes in mitochondria were found in the livers of ducks and chickens with aflatoxicosis[4]. These changes are consistent with the biochemical changes mentioned earlier. Electron microscope studies of the mitochondria are at present being undertaken.

It must be pointed out that seneciosis is easily confused with aflatoxicosis in pigs and cattle. It is, therefore, essential that livers are examined histiologically and a thorough investigation of the food consumed be made. In dogs the condition can be confused with phosphorus poisoning. One must be careful not to confuse the liver of the young healthy duckling with that of a duckling suffering from early aflatoxicosis.

Control:

As far as rations are concerned, we have made the following recommendations to the balanced feed industry:

All rations for ducklings, chickens and poults up to the age of twelve weeks, dogs of any age, piglets under the age of eight weeks, calves under three months, chinchillas and laboratory animals, must be free

from toxin. Adult poultry, that is, older than twelve weeks, pigs and non dairy cattle can be given rations that contain a maximum of 0.15 p.p.m. aflatoxin. The amount of toxin if any to be allowed in dairy rations is still under review. From experiments to species it would seem that .15 p.p.m. toxin recommended for other sources will be permissable. Thus groundnut cake meal containing 3 p.p.m. toxin cannot be used in excess of 5% in a ration, ground cake meal with 2 p.p.m. toxin cannot be used in excess of 7% in a ration and groundnut cake meal with 1 p.p.m. not to exceed 10% in a ration.

No groundnut cake meal containing more than 3 p.p.m. toxin should be kept on the premises of a feed compounder.

If these recommendations are followed, no cases of aflatoxicosis will occur in our livestock fed compounded feeds. Some cases might occur in areas where stock farmers grow their own groundnuts and when affected nuts, usually following hand sorting, are fed to animal.

II HAEMORRHAGIC DISEASE SYNDROME IN POULTRY, PIGS, HORSES, CATTLE AND MAN.

In poultry this syndrome is caused by the ingestion of toxins produced by a number of fungi which proliferate on feed litter and water into which feed has spilled. The fungi involved are Aspergillus clavatus, A. fumigatus, Penicillium citrinium, P. purpugenum, P. rubrum, Alternaria and Fusarium and probably others[4, 14]. This condition is usually seen in the hot and humid periods of the year and in closed environment houses where the ventilation system is not very efficient, when water condenses on the litter during Winter. Most outbreaks are associated with faulty watering devices which result in water spillage. The damp litter and spilled food are suitable media for supporting fungal growth. In many cases only a portion of the wet litter is removed, and with this moisture and constant temperature that is maintained in this type of house, optimal conditions for fungal growth are provided. The clinical signs of the disease usually occur about 14days after the wetting of the litter. This toxicosis has been confused in the past with what was though to be sulpha-quinoxoline poisoning. We have consistently found this condition in the absence of any coccidostat being fed. In addition we have also given birds four times the recommended dose of Sulpha-quinoxoline for long periods (four weeks) without ever producing any haemorrhages or any lesions for that matter, except a mild

nephrosis, in some birds. While not claiming that all cases of haemorrhagic disease are caused by mycotoxins certainly very few are caused by drugs normally used in treatment and prevention of coccidiosis. It must be remembered that the conditions required for outbreaks of coccidiosis are quite similar to those for fungal proliferation. Birds housed in these conditions will develop coccidiosis prior to mycotoxicoses. The end of drug treatment for coccidiosis will coincide with the clinical signs of mycotoxicoses and this has probably resulted in the confusion in most cases.

Haemorrhagic disease is usually encountered in birds of four to eight weeks of age, although it has been seen in younger and slightly older birds. The severity of the disease depends on the amount of toxin present in the litter or feed. We have encountered the disease in isolated houses or widely distributed on farms where the hygiene has deteriorated. The first signs of the syndrome is paleness of the combs, depression and mortality which can be very high. In outbreaks where mortality is low, the weight gains of birds is very much reduced. Many birds bleed very easily, particularly under the wings, and the feathers are very easily removed. At autopsy the birds are anaemic with haemorrhages in the breast and thigh muscles, the myocard, proventiculus, the gizzard when its lining is removed, intestine, kidneys and the liver. The kidneys are usually very pale.

The bone marrow is very pale and in severe cases is almost yellow in colour. (Aplastic anaemia). The total red cell count is reduced to 750,000 erythrocytes per cubic mm, that of normal fowls being 3 to $3\frac{1}{2}$ million. Leucopaenia and thrombocytopaenia are always found. The agranulocytosis in these birds renders them susceptible to bacterial infection, as was seen on a farm where the owner regularly cut the toenails of all broilers. As mentioned earlier, high selenium values were a constant finding, and this can be used as a diagnostic aid. The control of haemorrhagic disease is dependent on good hygiene, adequate ventilation and litter management. Feed hoppers and water troughs must be regularly cleaned and automatic waterers must be adjusted so that no over-spillage can occur. The litter must be turned regularly and kept dry.

Pigs and cattle:

A haemorrhagic disease syndrome is encountered in pigs[15] and cattle[16] that have eaten maize or grains which contain toxins produced by A. flavus, A. chevalieri, P. rubrum and Fusarium. The

condition frequently occurs in animals which are allowed to graze on maize lands, during autumn or wet winters where cobs, mainly those lying on the ground, are contaminated with these fungi. Grains kept under bad storage conditions, which do not prevent moisture seepage are also excellent media for fungal proliferation.

Rations in which such grains are incorporated are a source of danger to livestock.

In both cattle and pigs the disease is either acute or chronic, depending on the amount of toxin ingested.

The acute form is characterized by malaise, anorexia, salivation, lachrymation, anaemia, diarrhoea, wasting and death.

In cattle the more chronic form of the disease is characterized by thickening of the skin (hyperkeratosis) of the neck and body. Hyperkeratosis in cattle can also be caused by Napthelene poisoning[17]. In pigs hyperkeratosis is sometimes encountered in chronic cases, which must not be confused with zinc deficiency and sarcoptic mange.

At autopsy of acute cases of cattle, haemorrhages are seen in the trachae, muscles, myocard, abomasum, small intestine, kidneys and lymph nodes and sometimes a haemothorax and haemopericard is seen. In pigs the haemorrhages are seen in the gastro-intestinal tract, kidneys, heart and muscles. Fatty changes are seen in the liver in the acute cases, with necrosis in the more chronic cases.

Horses:

In Russia a condition caused by the toxin of Stachybothrys atra occurs in horses[15]. Two forms occur. The typical which is more chronic and the shocking form which is more acute. A post mortem haemorrhages in most tissues and necrosis of the liver are seen.

Alimentary Toxic Aleucia (A.T.A.):[15]

In humans in Russia a condition caused by the toxins of Fusarium sporotrichoides growing in cereal grains but mainly millet which has been allowed to over-winter under the snow is encountered. The main changes seen in these patients is anaemia with changes in the bone marrow, increased clotting time, a progressive leucopaenia and also minute haemorrhages in the skin, nose, mouth, gastro-intestinal tract and kidneys.

Patients are very prone to secondary bacterial infection which are often fatal.

Thus there is a very similar pattern in all species with haemorrhagic syndrome, such as anaemia, leucopaenia, bone marrow changes, haemorrhages and moderate liver changes.

III CONDITIONS ASSOCIATED WITH PHOTOSENSITIVITY.[18]

Facial eczema:[19, 20, 21.]

This condition occurs in sheep in New Zealand and in cattle, and is caused by Sporodesmin, a toxin produced by Pithomyces chartatum which grows on clover and perennial rye grass (lolium species) pastures.

A similar disease occurs in cattle in the United States which is caused by the toxin of the fungus Periconia minutissima which grows on Bermuda grass (Cynodem dactylon)[18]. The incidence of facial eczema varies from year to year and is seasonal. The climatic conditions at the time of these outbreaks is important. Outbreaks occur in Autumn, which are preceded by dry summers, followed by warm humid weather. Factors for ideal conditions for facial eczema to develop are periods of moderate rainfall accompanied by two or more consecutive nights when the minimum temperature of the grass is above 54°F, or a single prolonged period of high minimum temperatures of the grass, accompanied by continuous rains. Most outbreaks occur on perennial rye grass pastures that have been heavily grazed. For the disease to develop in an animal exposure to sunlight is essential.

The disease associated with Bermuda grass occurs chiefly in Autumn when heavy frost is followed by warm rain, and the dead grass becomes infected with fungus, or when rains follow a severe drought which kills the grass. The disease usually occurs three to eight weeks after the rains.

In sheep the disease is characterized by hyper-irritability, lachrymation and nasal discharge, shaking of the head and signs of itching on the face and avoidance of the sun. This is followed by oedema of the ears, eyelids, face lips and frequently of the vulva and coronets. Unpigmented, and areas unprotected by wool exhibit lesions of burning, followed by serum exudation and encrustation, and then necrosis and sloughing. In the termial stage of the disease icterus develops although this is not seen in all animals. Cachexia is followed by death. Ewes with permanent liver damage succumb to the stress of gestation, and

consequently mortality is often high at lambing.[15]

In cattle a drop in milk production is followed by the teats and udders becoming itchy, hyperaemia, oedema and serum discharge of the udder. In some animals these changes are seen around the muzzle, the lips and the eyes. A skin reaction similar to the lesions in sheep on the lightly pigmented areas is seen followed by icterus and septic mastitis.

In Bermuda grass toxicosis typical signs of photosensitisation is seen on the nostrils, eyelids, muzzle, teats, ears and lightly pigmented areas of the body, with sloughing of the skin and icterus.

At autopsy of sheep the main lesions found are in the liver (cirrhosis) and icterus. Generally, the liver lesions in cattle are the same as found in sheep.

It must be mentioned that photosensitisation and liver damage is also found in animals that have eaten the following plants: Lantana camara, L., Lippia spp., Asaemia axillaris (Vuursiektebossie), Trifolium hybridum, Trifolium pratense, Lupins, Tribulus sp., Panicum sp., and Brachiaria sp.[22] Animals develop Dikkop without icterus after eating plants of the Hypericum sp. and Fagopyrum sp. (Buck wheat). Toxic agents of some of these plants are known viz. Lippia, Lantana, and Hypericum, whereas the toxic factors of Tribulus, Panicum and Brachiaria species are unknown. These could well be mycotoxins.

From the diagnostic point of view, any animal or bird, with severe anaemia, liver lesions, e.g. fatty changes necrosis and cirrhosis, icterus and haemorrhages throughout the body, or photosensitivity, must be regarded as highly suspicious of Mycotoxicosis, more especially if the outbreaks are associated with recent rains and humidity.

Many toxic fungi still have to be identified and a great deal of work still has to be done on the chemistry and modes of action of these toxins. At the same time we must be careful not to incriminate an unnecessarily large number of fungi as being harmful, merely on the evidence that when grown on artificial media, ducklings and laboratory test animals are affected with toxins thus produced.

REFERENCES

1. ASPLIN, F.D. and GARNAGHAN, R.B.A.: The toxicity of certain groudnut meals for poultry with special reference to their effect on ducklings and chickens. Vet. Rec. 73, 1215, 1961.
2. ALLCROFT, R., CARNAGHAN, R.B.A., SARGEANT, K., and O'KELLY, J.: A toxic factor in Brazilian groundnut meal. Vet. Rec. 73, 428, 1961.
3. GARNAGHAN, R.B.A. and SARGEANT, K.: The toxicity of certain groundnut meals to poultry. Vet. Rec., 73, 726-727, 1961.
4. ABRAMS, L. J.S.A.V.M.A. in press, 1965.
5. TULPULE, P.G., MADHAVAN, T.V. and COPALAN, C.: Effect of feeding aflatoxin to young monkeys. Lancet, No. 7340, Vol. 1, 962-963, 1964.
6. LANCASTER, M.C., JENKINS, F.P., Mch. and PHILIP, J.: Toxicity associated with certain samples of groundnuts. Nature, 192, 1095 - 1096, 1961.
7. SCHOENTAL, R.: Liver changes and primary liver tumours in rats given toxic guinea pig diet. (M.R.C. diet 18). Brit. J. Cancer, 15, 812-815, 1961.
8. NEWBERNE, P.M., CARLTON, W.W. and WOGAN, G.N.: Hepatomas in rats and Hepatorenal injury in ducklings fed peanut meal or Aspergillus flavus extract. Path. Vet. 1, 105-132, 1964.
9. BROWN, J.M.M. & ABRAMS, L.: In press. Onderstepoort Journal, 1965.
10. SILLER, W.G. and OSTLER, P.C.: Histopathology of an enterohepatic syndrome of turkey poults. Vet. Rec., 73, 134, 1961.
11. ALLCROFT, R. and CARNAGHAN, R.B.A.: Groundnut toxicity: An examination for toxin in human food products from animals fed toxic groundnut meal. Vet. Rec., 75, 259-263, 1963.
12. DE IONGH, H., VLES, R.Q. and VAN PELT, J.G.: Milk of mammals fed on aflatoxin - containing diet. Nature, 202, 466-467, 1964.
13. VAN DER LINDE, J.A., FRENS, A.M., DE IONGH, H. en VLES, R.O.: Onderzoek van melk afkomstig van koeien gevoed met aflatoxinehoudend grondnoten meel. Tijdschrift voor Diergeneeskund, Deel 89, 1082-1088, 1964.
14. FORGACS, J., KOCH, H., CARL, W.T. and WHITE-STEVENS, R.H.: Relationship of toxic fungi to mouldy-feed toxicosis in

poultry.
Avian Diseases 6, 363-380, 1962.

15. FORGACS, J. and CARLL, W.T.:
Advances in Vet. Sci., 7, 273, 1962.

16. ALBRIGHT, J.L., AUST, S.D., BYERS, J.H., FRITZ, T.E., BROODI, B.O., OLSEN, R.E., LINK, R.P., SIMON, J., RHODES, H.E. and BREWER, R.L.: Mouldy corn toxicosis in cattle. J.A.V.M.A., 144, 1013-1019, 1964.

17. VLAHOS, K., McENTEE, K., OLAFSON, P. and HANSEL, W.: Destruction and restoration of spermatogenesis in a bull, experimentally poisened with highly chlorinated naphthelene. Cornell Vet., 45, 198-209, 1954.

18. CLARE, N.T.: Photosensitization in animals. Advances in Vet. Sci., 2, 182-211, 1955.

19. PERCIVAL, J.C.: Photosensitivity diseases in New Zealand. XVIII. The association of Sporidesmium bakeri with facial eczema. New Zealand, J. Agr. Res. 2, 1041-1056, 1959.

20. THORNTON, R.H. and ROSS, D.J.: The isolation and cultivation of some fungi from soils and pastures associated with facial eczema disease of sheep. New Zealand J. Agr. Res., 2, 1002-1016, 1959.

21. THORNTON, R.H. and SINCLAIRE, D.P.: Sporidesmium bakeri and facial eczema of sheep in the field. Nature, 184, 1327-1328, 1959.

22. STEYN, D.G.: Vergifting van mens en dier. Van Schaik Bep., Pretoria, Bls. 88, 1949.