

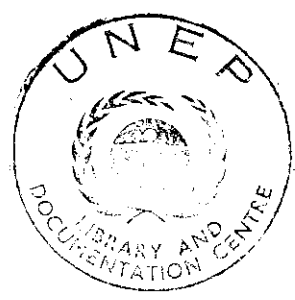
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Environmental Health Criteria 11

PCB COTOXINS

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NOTE TO READERS OF THE CRITERIA DOCUMENTS

While every effort has been made to present information in the criteria documents as accurately as possible without unduly delaying their publication, mistakes might have occurred and are likely to occur in the future. In the interest of all users of the environmental health criteria documents, readers are kindly requested to communicate any errors found to the Division of Environmental Health, World Health Organization, Geneva, Switzerland, in order that they may be included in corrigenda which will appear in subsequent volumes.

In addition, experts in any particular field dealt with in the criteria documents are kindly requested to make available to the WHO Secretariat any important published information that may have inadvertently been omitted and which may change the evaluation of health risks from exposure to the environmental agent under examination, so that the information may be considered in the event of updating and re-evaluation of the conclusions contained in the criteria documents.

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ENVIRONMENTAL HEALTH CRITERIA FOR MYCOTOXINS

Members of the Task Group on Environmental Health Criteria for Mycotoxins met in Geneva from 1 to 7 March 1977 and from 19 to 23 June 1978.

The first meeting was opened on behalf of the Director-General by Dr B. H. Dieterich, Director, Division of Environmental Health, and the second by Dr C. Agthe, Division of Environmental Health.

The first and second draft criteria documents were prepared by Professor C. J. Mirocha. The comments on which the second draft was based were received from the national focal points for the WHO Environmental Health Criteria Programme in Belgium, Czechoslovakia, Federal Republic of Germany, India, New Zealand, Poland, Sweden, Thailand, USSR, and USA, and from the International Agency for Research on Cancer (IARC), Lyons, the United Nations Industrial Development Organization (UNIDO), Vienna, and the Food and Agriculture Organization of the United Nations (FAO), Rome. Comments were also received from the Tropical Products Institute, London.

Dr P. Krogh, Dr D. S. P. Patterson, and Dr P. L. Schuler assisted in the preparation of the third draft criteria document, which was submitted for review to all the members of the Task Group, and to Dr R. Pleština of the Institute for Medical Research and Occupational Health, Yugoslav Academy of Sciences and Arts, Zagreb, Yugoslavia before the second meeting of the Task Group. The final edited draft was kindly reviewed by Dr D. S. P. Patterson. The collaboration of these national institutions, international organizations, WHO collaborating centres, and individual experts is gratefully acknowledged.

The document is based primarily on original publications listed in the reference section. However, several recent publications reviewing the occurrence, health effects, and other aspects of mycotoxins have also been used including monographs prepared by Purchase (1974), Pokrovskij et al. (1977) and Wyllie & Morehouse (1977), and the report on the joint FAO/WHO/UNEP Conference on Mycotoxins in Nairobi 1977 (FAO, 1977). In addition, comprehensive data have been obtained from the proceedings of several symposia and meetings including the Conference on Mycotoxins in Human and Animal Health, held in Maryland, USA, in 1976 (Rodricks et al., 1977).

Details of the WHO Environmental Health Criteria Programme including some of the terms frequently used in the documents, may be found in the general introduction to the Environmental Health Criteria Programme

published together with the environmental health criteria document on mercury (Environmental Health Criteria 1, Mercury, Geneva, World Health Organization, 1976), and now available as a reprint.

I. SUMMARY AND RECOMMENDATIONS FOR FURTHER RESEARCH

1.1 Summary

The ingestion of food containing mycotoxins, the toxic products of microscopic fungi (moulds), may have serious adverse health effects in man. Occasionally, occupational exposure to airborne mycotoxins may also occur.

The occurrence of mycotoxins in foodstuffs depends on their formation by specific strains of fungi and is influenced by environmental factors such as humidity and temperature. Thus, mycotoxin contamination of foodstuffs may vary with geographical conditions, production and storage methods, and also with the type of food, since some food products are more suitable substrates for fungal growth than others.

The present document contains an evaluation of health risks associated with four classes of mycotoxins. Aflatoxins are treated in most detail because more is known about them than about the other mycotoxins and because there is epidemiological evidence associating health effects in man with exposure to aflatoxins.

For the other 3 classes (ochratoxins, zearalenone, and trichothecenes), toxic effects in animals have been established and there is well-documented evidence that human exposure may occur, at least for the first two classes.

1.1.1 Aflatoxins

1.1.1.1 Sources and occurrence

Aflatoxins are produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi are ubiquitous and the potential for contamination of foodstuffs and animal feeds is widespread. The occurrence and magnitude of aflatoxin contamination varies with geographical and seasonal factors, and also with the conditions under which a crop is grown, harvested, and stored. Crops in tropical and subtropical areas are more subject to contamination than those in temperate regions, since optimal conditions for toxin formation are prevalent in areas with high humidity and temperature. Toxin-producing fungi can infect growing crops as a consequence of insect or other damage, and may produce toxins prior to harvest, or during harvesting and storage.

The chemical structures of aflatoxins have been elucidated, and analytical

techniques are available for their identification and determination in foodstuffs and body tissues at the $\mu\text{g}/\text{kg}$ level and lower. Four aflatoxins (B_1 , G_1 , B_2 , G_2), often occurring simultaneously, have been detected in foods of plant origin including maize, groundnuts (peanuts), and tree nuts as well as many other foodstuffs and feeds.

In animals, ingested aflatoxins may be metabolically degraded. Aflatoxin B_1 may be converted into aflatoxin M_1 which may occur in the milk. The concentration of aflatoxin M_1 in the milk of cows is about 300 times lower than the concentration of aflatoxin B_1 consumed in the feed. In certain experimental animals, only small amounts of administered aflatoxins have been found in tissues, 24 h after injection.

In studies on pigs, aflatoxin residues were detected in the liver, kidney, and muscle tissues of animals given aflatoxins in the feed for several months. There do not appear to be any published works on aflatoxin residues in the tissues of slaughtered animals.

The use of resistant varieties of seed and of pesticides, and careful drying and storing procedures can reduce fungal infestation and thus diminish food contamination by aflatoxins. The toxin is not eliminated from foodstuffs or animal feeds by ordinary cooking or processing practices and, since pre- and post-harvest procedures do not ensure total protection from aflatoxin contamination, techniques for decontamination have been developed. The toxin is generally concentrated in a small proportion of seeds that are often different in colour. Segregation of discoloured seeds by sorting can significantly reduce the aflatoxin levels in some crops, such as groundnuts. Visual inspection for mould growth before processing can serve as an initial screening technique but toxin-producing fungi can be present without detectable aflatoxins and vice versa. Because aflatoxin distribution in a contaminated, unprocessed commodity is uneven, adequate sampling is essential for effective monitoring. As aflatoxins can be chemically degraded *in vitro* by several oxidizing agents and alkalis, hydrogen peroxide and ammonia are currently used for the chemical decontamination of animal feeds.

1.1.1.2 *Effects and associated exposures*

Outbreaks of aflatoxicosis in farm animals have been reported from many areas of the world. The liver is mainly affected in such outbreaks and also in experimental studies on animals, including nonhuman primates. The acute liver lesions are characterized by necrosis of the hepatocytes and biliary proliferation, and chronic manifestations may include fibrosis. A feed level of aflatoxin as low as $300 \mu\text{g}/\text{kg}$ can induce chronic aflatoxicosis in pigs within 3–4 months.

Aflatoxin B₁ is a liver carcinogen in at least 8 species including nonhuman primates. Dose-response relationships have been established in studies on rats and rainbow trout, with a 10% tumour incidence estimated to occur at feed levels of aflatoxin B₁ of 1 µg/kg, and 0.1 µg/kg, respectively. In some studies, carcinomas of the colon and kidney have been observed in rats treated with aflatoxins. Aflatoxin B₁ causes chromosomal aberrations and DNA breakage in plant and animal cells, and, after microsomal activation, gene mutations in several bacterial test systems. In high doses, it may be teratogenic.

The acute toxicity and carcinogenicity of aflatoxins are greater in male than in female rats; hormonal involvement may be responsible for this sex-linked difference. Nutritional status in animals, particularly with respect to lipotropes, proteins, vitamin A, and lipids (including cyclopropenoid fatty acids), can modify the expression of acute toxicity or carcinogenicity or both.

There is little information on the association of acute hepatotoxicity in man with exposure to aflatoxins but cases of acute liver damage have been encountered that could possibly be attributed to acute aflatoxicosis. A recent outbreak of acute hepatitis in adjacent districts of two neighbouring states in north-west India, which affected several hundred people, was apparently associated with the ingestion of heavily contaminated maize, some samples of which contained aflatoxin levels in the mg/kg range, the highest reported level being 15 mg/kg.

Liver cancer is more common in some regions of Africa and south-eastern Asia than in other parts of the world and, when local epidemiological information is considered together with experimental animal data, it appears that increased exposure to aflatoxins may increase the risk of primary liver cancer. Pooled data from Kenya, Mozambique, Swaziland, and Thailand, show a positive correlation between daily dietary aflatoxin intake (in the range of 3.5 to 222.4 ng/kg body weight per day) and the crude incidence rate of primary liver cancer (ranging from 1.2 to 13.0 cases per 100 000 people per year). There is also some evidence of a viral involvement in the etiology of the disease.

In view of the evidence concerning the effects, particularly the carcinogenic effects, of aflatoxins in several animal species, and in view of the association between aflatoxin exposure levels and human liver cancer incidence observed in some parts of the world, exposure to aflatoxins should be kept as low as practically achievable. The tolerance levels for food products established in several countries should be understood as management tools intended to facilitate the implementation of aflatoxin control programmes, and not as exposure limits that necessarily ensure health protection.

1.1.2 Other mycotoxins

1.1.2.1 *Ochratoxins*

Ochratoxins are produced by several species of the fungal genera *Aspergillus* and *Penicillium*. These fungi are ubiquitous and the potential for contamination of foodstuffs and animal feed is widespread. Ochratoxin A, the major compound, has been found in more than 10 countries in Europe and the USA. Ochratoxin formation by *Aspergillus* species appears to be limited to conditions of high humidity and temperature, whereas at least some *Penicillium* species may produce ochratoxin at temperatures as low as 5° C.

Analytical techniques have been developed for the identification and quantitative determination of ochratoxin levels in the µg/kg range.

Ochratoxin A has been found in maize, barley, wheat, and oats, as well as in many other food products, but the occurrence of ochratoxin B is rare. Residues of ochratoxin A have been identified in the tissues of pigs in slaughterhouses, and it has been shown, under experimental conditions, that residues can still be detected in pig tissues one month after the termination of exposure.

Field cases of ochratoxicosis in farm animals (pigs, poultry) have been reported from several areas of the world, the primary manifestation being chronic nephropathy. The lesions include tubular atrophy, interstitial fibrosis, and, at later stages, hyalinized glomeruli. Ochratoxin A has been found to be nephrotoxic in all species of animals studied so far, even at the lowest level tested (200 µg/kg feed in rats and pigs). It has also been reported to produce teratogenic effects in mice, rats, and hamsters.

Human endemic nephropathy is a kidney disease of unknown etiology that has so far only been encountered in some areas of the Balkan Peninsula. The renal changes observed with this disease are comparable to those seen in ochratoxin A-associated nephropathy in pigs. High ochratoxin A exposure through diet has been found in some of the areas of the Balkan Peninsula, where endemic nephropathy is prevalent.

1.1.2.2 *Zearalenone*

Zearalenone, a metabolite produced by various species of *Fusarium*, has been observed as a natural contaminant of cereals, in particular maize, in many countries in Africa and Europe, and in the USA.

It has been shown to produce estrogenic effects in animals, and field cases of a specific estrogenic syndrome in pigs and of infertility in cattle have been encountered in association with feed levels of zearalenone of 0.1–6.8 mg/kg and 14 mg/kg, respectively. The compound has also produced congenital malformations in the rat skeleton.

In some countries, zearalenone has been found in samples of cornmeal and cornflakes destined for human consumption, at levels up to 70 µg/kg, corresponding to doses 400–600 times lower than those causing effects in monkeys or mice under experimental conditions. In certain areas of Africa, substantially higher levels have occasionally been found in beer and sour porridge prepared from contaminated maize and sorghum.

No adverse effects due to zearalenone intake have been reported in man, so far, but a possible health hazard connected with the daily intake of zearalenone at levels such as those reported for African fermented preparations needs further attention.

1.1.2.3 *Trichothecenes*

Trichothecene toxins belong to a group of closely related chemical compounds produced by several species of *Fusarium*, *Cephalosporium*, *Myrothecium*, *Trichoderma*, and *Stachybotrys*. Four trichothecenes (T-2 toxin, nivalenol, deoxynivalenol, and diacetoxyscirpenol) have been detected as natural contaminants in a small number of food samples.

Alimentary toxic aleukia, a disease diagnosed in man about 40 years ago, was apparently associated with the ingestion of grains invaded by *Fusarium* species. No cases have been reported since the end of the Second World War and the disappearance of the disease is probably due to improved food production and storage conditions. There is no firm evidence connecting the recently identified trichothecenes with alimentary toxic aleukia occurring in the past, or with other human disease.

1.2 Recommendations for Further Studies

1.2.1 General recommendations

- (a) There is a need for more information concerning the occurrence of mycotoxins in various parts of the world and the possible daily intake of mycotoxins by man.
- (b) Further studies should be undertaken on factors affecting fungal growth and mycotoxin formation in foodstuffs, under preharvest, postharvest, and storage conditions.
- (c) The effects of various cooking processes on the levels of mycotoxins in foods should be elucidated.
- (d) Better methods should be developed for the rapid detection and measurement of mycotoxin levels in foodcrops.

- (e) Sampling has proved to be the most difficult step in the surveillance of food commodities. The development of reliable, internationally accepted sampling procedures is strongly recommended.
- (f) Better methods should be developed for the identification and measurement of mycotoxins in human tissues, body fluids, and excreta.
- (g) A network of reference centres should be established to assist Member States in confirming the identity of individual mycotoxins found in human foods and tissues. These reference centres should also provide mycotoxin reference samples, upon request, to reinforce the inter-comparability of analytical results obtained in different parts of the world.
- (h) Better understanding is needed of the role of mycotoxins in human diseases. Where association between exposure to mycotoxins and the incidence of certain diseases is suspected, detailed epidemiological studies should be carried out.
- (i) Improved diagnostic methods for the effects on health of mycotoxins are needed, particularly methods for the detection of early changes that occur before the development of irreversible effects.
- (j) Attempts should be made to monitor exposure levels and to search for effects in workers handling pure mycotoxins or contaminated materials. This could provide important information on the effects of chronic exposure to mycotoxins and also indicate the need for safety measures.

1.2.2 Recommendations for aflatoxins

- (a) The validity of the assumption of a causal relationship between aflatoxin ingestion and primary liver cancer should be examined further by introducing control measures to reduce aflatoxin exposure in areas of high liver cancer incidence and high aflatoxin exposure. This should be followed by the monitoring of liver cancer incidence in these areas and in comparable areas where the aflatoxin exposure has been low.
- (b) The prevalence of hepatitis B antigen should be determined in areas with various levels of aflatoxin exposure and a high incidence of primary liver cancer.
- (c) Suspected outbreaks of acute aflatoxicosis should be studied in detail. Such studies should include measurements of the exposure to aflatoxins through foods and other routes. The presence of aflatoxins and their derivatives in the tissues and excreta of individuals including both those affected and those apparently unaffected by the disease, should be investigated.
- (d) A prolonged, continuous surveillance of the health status of exposed populations is considered essential in localities, where outbreaks of

acute aflatoxicosis have occurred. Such follow-up studies are important to fill the gaps in knowledge on the late effects of short-term exposure to high levels of aflatoxin in man. Recently reported outbreaks of aflatoxin-associated hepatitis in southeastern Asia may provide an ideal opportunity for such studies.

- (e) Reports from the various countries on the presence of aflatoxins in human tissues, body fluids, and excreta should be confirmed using specific assay methods with adequate limits of detection. The frequency of such events should be studied in appropriate samples of the general population of various countries and a search made for the sources of aflatoxin exposure.
- (f) The implication of aflatoxin as a contributing factor in the development of Reye's syndrome should be further investigated using the case-control approach. Data should be obtained on the presence of aflatoxins in tissues, body fluids, and excretion products for each case and its control, and attempts should be made to identify dietary sources of aflatoxins.
- (g) More information is needed on the gastrointestinal absorption of aflatoxins in animals and human subjects, as well as on the rate of disappearance of aflatoxins from farm-animal and human tissues. This is important both for the evaluation of aflatoxin residues in food of animal origin, and for the evaluation of aflatoxin levels in human tissues as a means of assessing exposure.
- (h) The modifying effect of the dietary intake of lipotropes, protein, or vitamin A on aflatoxin-related carcinogenesis should be further studied in experimental animals. This aspect should also be included in epidemiological studies on the association between human liver cancer incidence and aflatoxin intake.

1.2.3 Recommendations for other mycotoxins

- (a) The levels of ochratoxin A and possibly citrinin should be measured in "food-on-the-plate" in areas of the Balkan Peninsula with different incidence rates of Balkan nephropathy.
- (b) Further systematic investigations are needed on the levels of ochratoxin in foodstuffs and animal feeds in different parts of the world, and their association with nephropathy in farm animals. More work is needed in various parts of the world to confirm or exclude the strictly localized occurrence of endemic nephropathy affecting human subjects and considered so far to be confined to certain areas of the Balkan Peninsula.

- (c) Further studies are required on the mechanisms of ochratoxin toxicity, and on possible interactions with other nephrotoxic agents.
- (d) Studies should be made in different countries of the levels of zearalenone in human food and on total daily intake.
- (e) More information is needed on the levels of zearalenone in foods prepared from fermented maize and sorghum, such as those found in certain parts of Africa, and on the possible adverse effects of the daily consumption of these products, particularly in view of the estrogen-like effects of zearalenone observed in animals.

2. MYCOTOXINS AND HUMAN HEALTH

The toxicity of certain mushrooms has been known for a long time. However, the potential human hazard of the toxic products of other fungi was not recognized until the 1850s when an association between the ingestion of rye infected with *Claviceps purpurea* and the clinical features of ergotism was discovered. This was followed by reports of other mycotoxicoses that affected man such as the identification of a syndrome associated with the ingestion of bread infected by *Fusarium graminearum*, recognition of human stachybotryotoxicosis, and studies on the association between alimentary toxic aleukia (ATA) and the ingestion of over-wintered grains infested with *Fusarium poae* and *Fusarium sporotrichioides* (Sarkisov, 1954).

Recognition of the association of ATA with the consumption of food contaminated by moulds and the corresponding preventive measures taken, resulted in the eradication of the disease (Leonov, 1977) showing that, even before the isolation of the first mycotoxins, fungi-related foodborne diseases could be prevented.

The discovery of the hepatotoxic and hepatocarcinogenic properties of *Aspergillus flavus* in the early 1960s quickly followed by the elucidation of the structure of the aflatoxins changed the control strategy in the whole field of mycotoxins. A more quantitative approach is now possible, based primarily upon the chemical determination of the toxins and on studies of their effects in relation to dose.

In spite of increasing knowledge concerning human mycotoxicoses, the majority of data available on mycotoxins and mycotoxicoses have been obtained from veterinary medicine. Field studies, as well as studies on experimental animals indicate that the potential toxicity of mycotoxins is great. Future investigations may well establish a causal role of mycotoxins in other human diseases besides those considered so far.

Almost all plant products can serve as substrates for fungal growth and subsequent mycotoxin formation, thus providing the potential for direct contamination of human food. When farm animals used for food production, ingest feed contaminated with mycotoxins, not only may a direct toxic effect on the animals occur but there may also be a carry-over of the toxins into milk and meat, thus creating a further avenue for human exposure to mycotoxins. Furthermore, occupational exposure may occur through other media such as air.

In this document, the risks of health effects have been considered only for those mycotoxins for which there is evidence of human exposure and of well defined adverse effects, at least in animals. This category includes the

aflatoxins, ochratoxins, and zearalenone. The trichothecenes have also been included, as these have been shown, more recently, to be produced by fungi, that were reported to be associated with outbreaks of human illness several decades ago (ATA).

During recent years many other mycotoxins have been discovered such as: citreoviridin; citrinin; cyclochlorotine; luteoskyrin; maltoryzine; patulin; P R toxin; rubratoxin; rugulosin; sterigmatocystine; and tremorgens.

Some of these toxins, which are not discussed in this document, have been suggested to be related to disease outbreaks in farm animals (Pier et al., 1977). Certain human diseases, suspected of being associated with mycotoxins (van Rensburg, 1977), have not been discussed in this document as the causative agents have not been identified.

Of the four groups of mycotoxins considered, only aflatoxins have been shown to be associated with well recognized human health effects. For this reason, they are treated separately from the other mycotoxins.

3. AFLATOXINS

3.1 Properties and Analytical Methods

3.1.1 Chemical properties

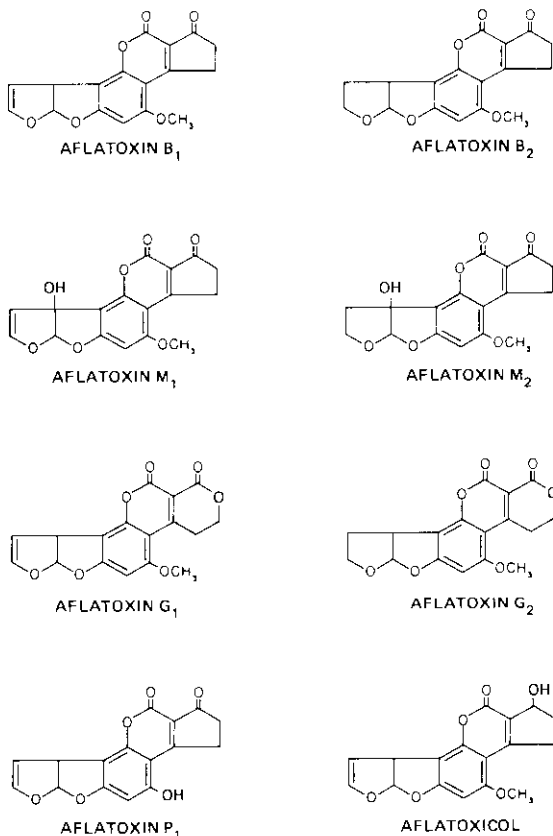
Although 17 compounds, all designated aflatoxins, have been isolated, the term aflatoxins usually refers to 4 compounds of the group of bis-furano-coumarin metabolites produced by *Aspergillus flavus* and *A. parasiticus*, named B₁, B₂, G₁ and G₂, which occur naturally in plant products. The 4 substances are distinguished on the basis of their fluorescent colour, B standing for blue and G for green with subscripts relating to the relative chromatographic mobility. Cows fed rations containing aflatoxin B₁ and B₂ excrete metabolites in the milk called aflatoxin M₁ and aflatoxin M₂ (see section 3.3.4.1); M stands for milk, and again the subscripts relate to the relative chromatographic mobility. (Aflatoxin M₁ is also a fungal metabolite.) Of the 4 major aflatoxins, B₁ is usually found in the highest concentrations, followed by G₁ while B₂ and G₂ occur in lower concentrations.

The structures of a number of aflatoxins and of aflatoxin B₁-related metabolites (see section 3.3.3) are illustrated in Fig. 1. The structure of aflatoxins B₁ and G₁ were determined by Asao et al. (1963, 1965) and that of B₂ by Chang et al. (1963). Aflatoxins B₂ and G₂ are dihydroderivatives of the parent compounds (Hartley et al., 1963). Aflatoxins M₁ and M₂ are the hydroxylated metabolites of B₁ and B₂, respectively (Holzapfel et al., 1966; Masri et al., 1967; Büchi & Weinreb, 1969). Chemical properties of some naturally-occurring aflatoxins and metabolites are summarized in Table 1.

The aflatoxins are intensely fluorescent, when exposed to long-wave ultraviolet (UV) light. This makes it possible to detect these compounds at extremely low levels (ca. 0.5 ng or less per spot on thin-layer chromatograms) and provides the basis for practically all the physicochemical methods for their detection and quantification. A concentration of aflatoxin M₁ of 0.02 µg/litre can be detected in liquid milk (Schuller et al., 1977).

Aflatoxins are freely soluble in moderately polar solvents (e.g., chloroform and methanol) and especially in dimethylsulfoxide (the solvent usually used as a vehicle in the administration of aflatoxins to experimental animals); the solubility of aflatoxins in water ranges from 10–20 mg/litre.

As pure substances, the aflatoxins are very stable at high temperatures, when heated in air. However, they are relatively unstable, when exposed to light, and particularly to UV radiation, and air on a TLC plate and especially when dissolved in highly polar solvents. Chloroform and benzene solutions are stable for years if kept in the dark and cold.



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Fig. 1. The structure of some aflatoxins and their metabolites.

Little or no destruction of aflatoxins occurs under ordinary cooking conditions, and heating for pasteurization. However, roasting groundnuts appreciably reduces the levels of aflatoxins (see section 3.2.3) and they can be totally destroyed by drastic treatment such as autoclaving in the presence of ammonia or by treatment with hypochlorite.

The presence of a lactone ring in the aflatoxin molecule makes them susceptible to alkaline hydrolysis (De Jongh et al., 1962). This characteristic is important in that any food processing involving alkali treatment can decrease the contamination of the products (section 3.2.3) although the presence of protein, the pH, and the duration of treatment may modify the results (Beckwith et al., 1975). However, if the alkaline treatment is mild, acidification will reverse the reaction to reform the original aflatoxin.

Table 1. Physical and chemical properties of some aflatoxins and their metabolites

Aflatoxin	Molecular formula	Relative molecular mass	Melting point ° C	Ultraviolet absorption (ε) ^c		Fluorescence emission nm	Reference
				265 nm	360-362 nm		
B ₁ ^a	C ₁₇ H ₁₂ O ₆	312	268-269	12 400	21 800	425	Asao et al. (1965)
B ₂ ^a	C ₁₇ H ₁₄ O ₆	314	286-289	12 100	24 000	425	Chang et al. (1963)
G ₁ ^a	C ₁₇ H ₁₂ O ₇	328	244-246	9 600	17 700	450	Asao et al. (1965)
G ₂ ^b	C ₁₇ H ₁₄ O ₇	330 ^d	237-240 ^d	8 200	17 100	450	Hartley et al. (1963)
M ₁ ^a	C ₁₇ H ₁₂ O ₇	328	299	14 150	21 250 (357 nm)	425	Holzappel et al. (1966)
M ₂ ^a	C ₁₇ H ₁₄ O ₇	330	293	12 100 (264 nm)	22 900 (357 nm)	— ^f	Holzappel et al. (1966)
P ₁ ^b	C ₁₆ H ₁₀ O ₆	298	>320	11 200 (267 nm)	15 400 (362 nm)	— ^f	Dalezios et al. (1971) and Büchi et al. (1973)
Q ₁	C ₁₇ H ₁₂ O ₇	328	— ^e	11 450 (267 nm)	17 500 (366 nm)	— ^f	Masri et al. (1974a,b)
Aflatoxicol	C ₁₇ H ₁₄ O ₆	314	230-234 ^d	10 800 (261 nm)	14 100 (325 nm)	425	Detroy & Hesselstine (1970)

^a Molar absorption coefficient for aflatoxins B₁, B₂, G₁, and G₂ obtained from Rodricks et al. (1970) and those for M₁ and M₂ from Stubblefield et al. (1972).

^b P stands for phenolic products of O-demethylation of aflatoxin B₁.

^c Compounds dissolved in methanol except for aflatoxin P₁, which in this case was dissolved in ethanol. Data on molar absorption coefficients for other peaks and on the ultraviolet absorption characteristics of aflatoxins in other solvents can be found in the original papers.

^d Data from Butler (1974).

^e Not available.

^f Violet fluorescence of aflatoxin M₂ and yellow-green fluorescence of aflatoxins P₁ and Q₁, reported in original papers.

The chemistry of the aflatoxins has recently been reviewed by Roberts (1974).

3.1.2 Methods of analysis for aflatoxins in foodstuffs

3.1.2.1 Sampling

Sampling is an integral part of the analytical procedure and the sample drawn should be representative of the lot. The total error made in an analytical procedure consists of the sampling error, the subsampling error, and the error in analysis (Whitaker, 1977). The difficulty in sampling for aflatoxins arises because of the heterogeneity of aflatoxin distribution in contaminated unprocessed commodities. On the basis of a large number of analyses, Whitaker et al. (1974a) were able to calculate the contribution of each error to the total error. The total variance of the analytical procedure is primarily caused by sampling variability, whereas the subsampling variability and the analysis variability are more or less independent of aflatoxin concentration. The coefficient of variation associated with sampling is about 115% at a level of contamination of 20 $\mu\text{g}/\text{kg}$ and about 145% at a level of contamination of 10 $\mu\text{g}/\text{kg}$. Whitaker et al. (1974b) and Whitaker (1977) have summarized a procedure for sampling and have developed a number of sampling plans used in the USA for the control of aflatoxin contamination in shelled groundnuts (peanuts). Other recent publications deal with aflatoxin-testing programmes for maize (Whitaker et al., 1978) and cottonseed (Whitaker & Whitten, 1977).

The sampling of small grains, oilseed cakes, foodstuffs, and feeds is also difficult, although in most cases the aflatoxin distribution within a batch is not likely to be as uneven as in the case of groundnuts. Fluids and well-mixed processed products such as milk and milk products, beer, and cider do not present such a sampling problem.

Peanut butter, flours, and cornmeal do not present the same problems as the original raw materials because a finely divided product is formed during processing from which it is much easier to obtain a representative analytical sample.

Some practical aspects of sampling are dealt with in Chapter 26 of "Official methods of analysis" of the Association of Official Analytical Chemists (Horwitz et al., 1975). For survey purposes, 1–5 kg samples are usually taken and the size of the sample for analysis ranges from 20 to 100 g. A sample of 50 g ensures both a representative sample and solvent economy.

3.1.2.2 Methods of analysis

Biological and chemical procedures have been developed for the detection

and determination of aflatoxins and other mycotoxins. The bioassay techniques that are currently available are not suitable for routine screening purposes and their detection levels are not low enough. The chemical assay techniques, although more accurate and faster, are not always specific. The presence of a certain toxin is usually confirmed by derivative formation and its toxicity verified by bioassay.

Biological methods. In the original biological test (Carnaghan et al., 1963), one-day-old ducklings were used as test animals for determining the presence of aflatoxins in suspect food by measuring the degree of biliary proliferation as a semiquantitative index (see section 3.4.2.1). The lowest dose level of 0.4 $\mu\text{g}/\text{day}$ administered for 5 days represents the minimum intake required to induce a detectable biliary proliferation. The test is also effective for detecting aflatoxin M_1 in both liquid and powdered milk (Purchase, 1967). Little information is available on the sensitivity of this test (and other biological methods) to aflatoxins other than B_1 .

A commonly used method in regulatory actions is the chicken embryo bioassay in which 0.1–0.2 μg of aflatoxin B_1 is applied to the egg membrane and the mortality rate recorded during the 23-day period of hatching (Horwitz, 1975).

Several other biological procedures have been developed, using maize seedlings, zebra fish larvae, brine shrimps, bacteria etc. Detailed descriptions can be found in the reviews by Goldblatt (1969) and Ciegler et al. (1971).

Chemical methods. Although procedures are continually changing, the basic steps remain; extraction, lipid removal, cleanup, separation, and quantification. Since there is considerable overlap in the various methods, most of the published reviews (Jones, 1972; Stoloff, 1972) examine the different procedures by these basic steps. Depending on the nature of the commodity, methods can sometimes be simplified by omitting unnecessary steps. The presence of specific interferences such as theobromine in cacao and gossypol in cottonseed, may require additional steps.

Numerous methods of analysis have been reported for the determination of aflatoxins in human and animal foodstuffs. Many of them are minor modifications of the basic steps adapted to special commodities or problems.

Collaborative studies designed to assess the performances of different laboratories give information on the accuracy, precision, and specificity of the method under consideration, as well as on the occurrence of false negative and false positive results. Only methods that have been subjected to such studies are reported in this document.

Chemical methods have mainly been developed for such commodities as groundnuts. In the first method for the analysis of groundnuts, the aflatoxins

were extracted from the contaminated sample using methanol; this was later replaced by chloroform. An improvement was made by Lee (1965) who showed that the addition of water to hydrophilic plant tissues during extraction with chloroform resulted in more effective removal of aflatoxin. The combination of liquid-liquid extraction techniques and partition chromatography led to a method, which is now one of the most widely used, known as the Contamination Branch (CB) method (Eppley, 1966). The sample is extracted with water and the water extracted with chloroform, the lipids and aflatoxins are transferred to a silica-gel column where the lipids are selectively eluted with hexane and the pigments and other interfering material eluted with absolute diethylether; finally the aflatoxins are eluted from the column with 3% methanol in chloroform.

Because the CB method is time-consuming, attempts have been made to simplify it. Walkling et al. (1968) drew attention to the fact that a separation funnel was simpler and faster for liquid-liquid partition than the silica-gel column, and that centrifuging was a faster method of separating a solid than filtration. Thus the Best Foods (BF) method was developed, which is faster and more economical in terms of the amounts of solvents used but provides a poorer cleanup. The sample is extracted and defatted with a two-phase aqueous methanol-hexane system, the aflatoxins are then partitioned from the aqueous phase into chloroform, leaving lipids and pigments in the hexane and aqueous methanol.

In both the CB and BF methods, the aflatoxins are concentrated by evaporation of the chloroform, and then separated by thin-layer chromatography (TLC). Aflatoxins are intensely fluorescent when exposed to long-wave ultraviolet radiation, which makes it possible to determine these compounds at extremely low levels. An analyst experienced in this field can detect 0.5 ng aflatoxin B₁ on a TLC plate. In most methods, the intensity of fluorescence of the sample is compared with that of a standard. Under ideal conditions this technique has a coefficient of variation of about 20% which can be reduced to 5%–9% by the use of a fluorodensitometer.

It should be pointed out that quantification and confirmation of identity can only be obtained if pure authentic standards are available for reference. Current sources of aflatoxin standards and methods for the determination of mass concentration and purity can be found in Chapter 26 of the AOAC "Official methods of analysis" (Horwitz et al., 1975).

When the CB and BF methods were compared in a collaborative study (Walkling, 1970), the methods were found to be equivalent in accuracy and precision with a recovery of about 70% of added aflatoxin and an overall coefficient of variation of about 35% for total aflatoxin levels down to about 20 µg/kg. This result has been confirmed by the latest International Aflatoxin Check Sample Study (Coon et al., 1972) in which 129

laboratories participated. However, the coefficient of variation was about twice as high as in the original collaborative studies. This illustrates the inadequacies of many laboratories.

Another collaborative study was conducted (Stack, 1974) in which the CB and BF methods were compared at levels down to the 2–10 $\mu\text{g}/\text{kg}$ range. Again both methods proved to be equally accurate (about 80% recovery) for total aflatoxins at the 5–10 $\mu\text{g}/\text{kg}$ levels. The CB method, however, was as precise (coefficient of variation = 30%) at the lowest level of 2 $\mu\text{g}/\text{kg}$ as at the highest levels. The BF method lost precision at these low levels and the coefficient of variation was of the order of 100%.

In spite of cleanup and separation procedures, there might still be problems with compounds that have fluorescent and chromatographic properties similar to those of the aflatoxins. Thus, the presence of a spot on a TLC plate is only presumptive evidence of identity and additional confirmatory tests are necessary. Probably the first step in confirming the presence of aflatoxins is to use additional solvent systems in the TLC. The developed TLC plate can be sprayed with 25% sulfuric acid (Schuller et al., 1967), which changes the fluorescence colour of the aflatoxin spots to yellow. This test, if negative, would rule out the presence of aflatoxins but does not provide confirmatory evidence. The formation of chemical derivatives was first described for aflatoxins B_1 and G_1 (Andrellos & Reid, 1964). The reagents used were formic acid–thionyl chloride, acetic acid–thionyl chloride, and trifluoroacetic acid, the acid-catalysed addition products formed were a dimeric acetate and an addition product with water, respectively. The characteristic mobilities and fluorescent properties on thin-layer chromatograms can be compared with those of standard derivatives. Pohland et al. (1970) simplified the preparation of the derivatives by using a mixture of hydrochloric acid and acetic anhydride, and hydrochloric acid alone. Further improvement, by elimination of the preparative chromatography step in these procedures, has been achieved by Przybylski (1975). The water adduct is formed directly on a TLC plate from as little as 0.5 ng of aflatoxin B_1 or G_2 .

In addition to the procedures for groundnuts, methods of analysis have been developed for cottonseed, copra, maize, various tree nuts (pistachio, walnut, Brazil nuts, etc.) and for animal feeds. Many of these methods are modifications of the CB and BF methods. Milk and dairy products require a far greater sensitivity for the determination of M_1 and M_2 because these animal metabolites are usually only found at sub $\mu\text{g}/\text{kg}$ levels; additional cleanup to eliminate interferences and sometimes two-dimensional TLC techniques (Schuller et al., 1973) are necessary to attain satisfactory performance. These methods are described in Chapter 26 of the AOAC "Official methods of analysis" (Horwitz et al., 1975).

Several analytical methods for the detection of aflatoxin residues in animal tissues have been developed, and their detection limits evaluated (Jemmali & Murphy, 1976).

Column detection methods are being used for control purposes in the field because of their simplicity. The method of Romer (1975) is of particular value because it combines column detection, TLC quantification, and TLC plate chemical derivative confirmation in a method that has a wide application for a number of foodstuffs and feeds including mixed feeds.

It appears that methods using high-pressure liquid chromatography will become the methods of choice for mycotoxin analyses in the near future because of their sensitivity and improved accuracy, and because they can be applied to a number of mycotoxins including aflatoxins B₁, B₂, G₁, and G₂ (Panalaks & Scott, 1977).

3.2 Sources and Occurrence

3.2.1 Formation by fungi

The ability to produce aflatoxins seems to be confined to strains of the two species *Aspergillus flavus* Link and *A. parasiticus* Speare, both members of the *A. flavus* group.

Aflatoxin-producing strains of *A. flavus* are common and widespread, and have been isolated from a host of different materials. As indicated in Table 2, a high proportion (from 20% to 98%) of isolated strains of *A. flavus* is able to produce aflatoxins.

Table 2. Aflatoxin-producing strains of *A. flavus* isolated from four field crops^a

Source	Isolates tested (No.)	Isolates producing aflatoxin (%)	Maximum yield of aflatoxin B ₁ (µg/flask)
groundnut	100	98	3300
cottonseed	59	81	3200
rice	127	20	1100
sorghum	63	24	3300

^a Data from Schroeder & Boller quoted by Hesseltine (1976) in "Mycotoxins and other fungal related food problems".

3.2.1.1 Moisture content and temperature

The moisture content of the substrate and temperature are the main factors regulating fungal growth and mycotoxin formation.

Koehler (1938) established that a moisture content of 18.3% on a wet weight basis, was the lower limit for the growth of *A. flavus* in shelled corn. Extensive studies under precisely controlled conditions (Sanders et al., 1968; Diener & Davis, 1969; Davis & Diener, 1970) established a moisture

content in equilibrium with a relative humidity of 85% (or water activity (a_w) = 0.85) as the lower limit for growth of *A. flavus* and for the production of aflatoxins. In starchy, cereal grain such as wheat, oats, barley, rice, sorghum, and maize, the lower limit is a moisture content of 18.3%–18.5% on a wet weight basis and in groundnuts, Brazil nuts, other nuts, copra, and sunflower and safflower seeds, all of which have a high oil content, it is a moisture content of 9%–10%.

The minimum, optimum, and maximum temperatures for aflatoxin production are 12° C, 27° C, and 40–42° C, respectively (Davis & Diener, 1970). Northolt et al. (1976) studied the effect of water activity and temperature on the growth and aflatoxin production of *A. parasiticus* and came to the conclusion that no detectable quantities of aflatoxin B₁ were formed at an a_w value below 0.83 and at temperatures below 10° C.

3.2.1.2 Invasion of field crops by *A. flavus*

Groundnut seeds may be invaded by *A. flavus* before harvest but are more likely to be invaded very rapidly after the plants have been pulled and piled for preliminary drying before the nuts are removed. This postharvest period is the “high hazard” time for aflatoxin production. On the other hand, studies of aflatoxin contamination in North Carolina (USA) (Dickens & Satterwhite, 1973) under conditions of drought, suggest that drought after groundnuts are formed but before they are dug is conducive to their infection with *A. flavus*. Damage caused by the lesser cornstalk borer (LCB) might also aid in the infection process because insects may carry fungal spores, although many drought area fields infested with LCB did not produce groundnuts with a high aflatoxin content. Drought alone does not result in high levels of aflatoxin contamination. It must coincide with, or promote infestation by insects which in turn infect the groundnut. The LCB may act as a vector for *A. flavus*.

Pettit et al. (1971) reported that groundnuts grown under dry land conditions (drought stress) accumulated more aflatoxin before digging than those grown under irrigation. Dry land fresh-dug kernels contained a maximum aflatoxin level of 35 800 µg/kg while a maximum of 50 µg/kg was detected in kernels from irrigated plots. Apparently, the higher kernel moisture content occurring under irrigated conditions reduced the aflatoxin production potential, whereas a moisture content of about 31% under drought conditions was near optimum. Similar observations have been reported from West Africa.

In some irrigated regions with moist weather at harvest time, cottonseed may be invaded by *A. flavus* while still on the plant and after the bolls open and may contain large amounts of aflatoxins (Marsh et al., 1973). Stephenson & Russell (1974) related the high aflatoxin contamination in the

field (in USA) to invasion by insects that provided a site of injury and served as vectors for *A. flavus*.

Insect injury in ears of maize in the field may also be accompanied or followed by infection with *A. flavus* and by aflatoxin formation before harvest (Lillehoj et al., 1976). To what extent this constitutes a contamination problem in many regions of the world, where maize is an important crop, is not known. Aflatoxins have also been reported in heads of sorghum heavily infected with mould in India (Tripathi, 1973). Pistachio nuts can become contaminated with aflatoxins prior to harvest but the cause of infection with aflatoxin-producing strains of fungi has not yet been found. The contamination of almonds and of walnuts has been traced to specific types of insect damage in the orchard (Stoloff, 1977).

3.2.2 Occurrence in foodstuffs

This subject has been reviewed recently by Stoloff (1976). Of the four major aflatoxins (B_1 , B_2 , G_1 , G_2) B_1 is usually found in the greatest concentrations. Measurements of toxin concentration are based on the wet weight of the commodity in question. The four toxins may occur together, although they need not, and their concentrations in relation to each other and their occurrence may vary depending on the fungal strain and substrate. For example, Hesseltine et al. (1970) found that most fungal strains that produced aflatoxin G_1 also produced aflatoxin B_1 , but that not all strains that produced aflatoxin B_1 produced aflatoxin G_1 . One strain of *A. flavus* from black pepper produced only aflatoxin B_2 on 2 natural substrates tested (Schroeder & Carlton, 1973).

Although aflatoxins have been found in a variety of foodstuffs, the most pronounced contamination has been encountered in groundnuts and other oilseeds including cottonseed and maize. The most frequently contaminated tree nuts are Brazil nuts and pistachios.

3.2.2.1 Maize

Surveys in the USA of more than 1500 samples of maize collected in crop years 1964–67, mainly from commercial channels, revealed that 2%–3% of the samples contained aflatoxins (total aflatoxin B_1 and G_1) in the range of 3–37 $\mu\text{g}/\text{kg}$ (Shotwell et al., 1969a, 1970). In a subsequent survey of 293 samples, 8 samples (2.7%) contained aflatoxin B_1 levels in the range of 6–25 $\mu\text{g}/\text{kg}$, one of the samples containing aflatoxin G_1 (25 $\mu\text{g}/\text{kg}$) as well as aflatoxin B_1 (Shotwell et al., 1971). Aflatoxin B_2 was also detected in some of the samples. In a further study of 60 samples from south-east USA (Shotwell et al., 1973), aflatoxin B_1 was found in 21 samples (35%) at levels

ranging from 6–308 $\mu\text{g}/\text{kg}$ in the 1969–70 period. Aflatoxin B_2 at levels ranging from a trace to 40 $\mu\text{g}/\text{kg}$ was found in 15 of these samples and aflatoxin G_1 was found in 5 samples at levels of a trace to 10 $\mu\text{g}/\text{kg}$. In 2 samples, aflatoxin G_2 (1 and <2 $\mu\text{g}/\text{kg}$) was detected in addition to aflatoxin B_1 . In some of the southeastern states of the USA a high frequency of aflatoxin contamination was also found in maize in the field (Anderson et al., 1975; Lillehoj et al., 1976). In some of the field samples, the levels of aflatoxin B_1 ranged up to several thousand $\mu\text{g}/\text{kg}$ or more. Most of the field contamination was associated with damage caused by insects such as the European corn borer, corn ear worms, or weevils, and it seems likely that wherever such damage is prevalent, field contamination with aflatoxins will occur. In Thailand, 35% of maize samples contained aflatoxin B_1 (average level 400 $\mu\text{g}/\text{kg}$) while 40% contamination by aflatoxin B_1 (average level 133 $\mu\text{g}/\text{kg}$) was found in Uganda (Stoloff, 1976), and 97% in the Philippine island of Sebu (average 213 $\mu\text{g}/\text{kg}$) (Alpert et al., 1971; Campbell & Salamat, 1971; Shank et al., 1972a; Campbell & Stoloff, 1974; Stoloff, 1976).

Aflatoxin levels found in household maize samples in connexion with the outbreak of acute toxic hepatitis in north-west India (Krishnamachari et al., 1975a,b; Tandon et al., 1977) are discussed in section 3.5.1.2.

2.2.2 *Wheat, barley, oats, rye, rice, and sorghum*

Shotwell et al. (1968b) reported the presence of aflatoxins at levels of less than 19 $\mu\text{g}/\text{kg}$ in 9/1368 samples of wheat, sorghum, and oats in the USA. Shotwell et al. (1976b) did not detect any aflatoxin B_1 (detection limit 1–3 $\mu\text{g}/\text{kg}$) in 848 samples of wheat from various districts of the USA. The presence of aflatoxins B_1 , B_2 , G_1 , and G_2 was reported by Tripathi (1973) in heads of sorghum heavily infected with mould, in field samples in India, but he did not apply any confirmatory tests. Aflatoxins were also found in sorghum in Uganda (Alpert et al., 1971) and, in a survey in the USA, aflatoxins were detected in 2/66 samples of sorghum grain (13 and 50 $\mu\text{g}/\text{kg}$) (Stoloff, 1976). Aflatoxins have been detected in less than 2% of more than 400 samples of rice from markets in Africa, the Philippines, and Thailand (Alpert et al., 1971; Campbell & Salamat, 1971; Shank et al., 1972a). However, Lucas et al. (1970–71) reported that out of 139 samples of rice obtained from the Ho Chi Minh (Saigon) area in Viet Nam, 31% were found positive for aflatoxins, no confirmatory tests were included in this study. In surveys of wheat and other cereals in the USSR, Lvova et al. (1976) found aflatoxin B_1 at a level of 100 $\mu\text{g}/\text{kg}$ in 1/169 samples (0.6%) in the 1972 crop. Aflatoxins (aflatoxin B_1 at levels ranging from 20 to 444 $\mu\text{g}/\text{kg}$ and aflatoxin G_1 at levels of 10–333 $\mu\text{g}/\text{kg}$) were found in 24/138 samples (17.4%) in the 1973 crop year. In this year, the samples to be

analysed were specially selected from those that were mouldy or had undergone heating or both. In a survey of wheat in southern USSR (Kazakstan), Bučarbaeva & Nikov (1977) found aflatoxin B₁ in 2/50 samples (4%) from one district and 3/50 samples (6%) from another (levels ranging from 5 to 10 µg/kg).

3.2.2.3 *Groundnuts (peanuts)*

In the 1973 survey in the USA of shelled consumer groundnuts, 15% of 361 samples contained aflatoxins in the range of trace to 50 µg/kg (Stoloff, 1976). Krogh & Hald (1969) found aflatoxins in 86.5% of 52 samples of groundnut products imported into Denmark for feed, one sample contained 3465 µg/kg. Aflatoxins were found in 41% of 173 samples of groundnuts in the Sudan, 16% of the samples containing more than 250 µg/kg, and 9%, more than 1000 µg/kg (Habish et al., 1971). In the Philippines, all the samples of peanut butter, tested in 1967-69, contained aflatoxins with a median value of 155 µg/kg and a mean value of 500 µg/kg. The highest level detected was 8600 µg/kg (Campbell & Salamat, 1971). In Thailand, 49% of market samples contained an average level of aflatoxins of 1530 µg/kg (Shank et al., 1972a).

3.2.2.4 *Soybeans and common beans*

No significant degree of aflatoxin contamination has been found in soybeans or common beans in commerce in the USA (Stoloff, 1976), although aflatoxin contamination sufficient to be of public health concern has been found in various types of edible beans in Thailand (Shank et al., 1972a) and in Africa (Alpert et al., 1971).

3.2.2.5 *Tree nuts*

Aflatoxin has been found occasionally in Brazil nuts, almonds, walnuts, pistachio nuts, pecans, and filberts. In some of these, contamination occurs when the nuts are still on the tree and is usually associated with damage of one sort or another. However, apparently sound, undamaged pecans may contain aflatoxins (Stoloff, 1976). Yndestad & Underdal (1975) in Norway found 66% of Brazil nuts contaminated with aflatoxin B₁ and Nilsson et al. (1974) found that all of 23 batches of Brazil nuts intended for importation to Sweden were contaminated. Fourteen percent of 74 samples of California almonds were contaminated with aflatoxin B₁ with levels of less than 20 µg/kg in 90% of the contaminated samples (Schade et al., 1975).

3.2.2.6 *Copra*

Aflatoxins were found in 88% of 72 samples of copra and copra meal (Stoloff, 1976) in amounts ranging from a trace to 30 µg/kg, and similar

contamination was found by Krogh et al. (1970) in copra imported into Finland.

3.2.2.7 *Cottonseed*

In 3 successive crop years (1964–67), aflatoxin B₁ was detected in 6.5%–8.8% of more than 3000 cottonseed samples and in 12.8%–21.5% of more than 3000 samples of cottonseed meal (Stoloff, 1976). In contrast, aflatoxin was not detected in cottonseed hulls (Whitten, 1969). Relatively high levels of aflatoxin contamination were found in an area in southern California. Aflatoxin levels increased from 1735 µg/kg in some samples of seed harvested in November to 2578 µg/kg in some samples going into storage in late January. The amount present in the stored seeds did not increase with time, even though fungi, including *A. flavus*, could be seen growing on some of the seeds. Marsh et al. (1973) tested cottonseeds from 13 locations across the USA cotton belt in 1969 and from 11 locations in 1970. Aflatoxins B₁ and B₂ were found in one or more samples from 3 regions in areas where boll rot caused by *A. flavus* had been repeatedly observed in previous years. Seeds from individual lots contained aflatoxin B₁ levels ranging from 200 000 to 300 000 µg/kg indicating the high potential hazard that might occur from cottonseed.

3.2.2.8 *Spices and condiments*

Scott & Kennedy (1973) did not find any aflatoxins in 24 samples of ground black or white pepper. Low concentrations (up to 8 µg/kg) were found in 10/33 samples of cayenne pepper and 6/6 samples of Indian chili powder, mainly as trace amounts.

3.2.2.9 *Animal feeds*

In studies by Strzelecki & Gasiorowska (1974), aflatoxins occurred in 12.7% of 306 samples of animal feed and feed components in Poland, 4.2% of the samples containing more than 100 µg/kg and 2.6% of the samples containing more than 1000 µg/kg. Feed components, mainly groundnut meals, were contaminated by aflatoxins more frequently and with higher levels. On the other hand, aflatoxin was detected in only one sample (2.7%) of cattle and sheep feeds (300 µg/kg) and in one sample (1.7%) of poultry feeds (30 µg/kg). Swine feeds contained aflatoxins in 11.4% of samples, with 6 samples (5.7%) exceeding 250 µg/kg. Two recent surveys of mixed feeds in the Federal Republic of Germany revealed that 1 in 60 samples contained aflatoxin B₁ levels exceeding 20 µg/kg (Seibold & Ruch, 1977); 45 out of another 105 samples contained levels of between 7 and 300 µg/kg (Kiermeier et al., 1977). Similar results were obtained in the United Kingdom (Patterson, personal communication) where 95/172 samples of

dairy feed were contaminated with aflatoxin B₁ levels of 1–350 µg/kg, and 92.4% contained no more than 30 µg/kg.

3.2.2.10 *Animal products*

Surveys in several countries have shown that aflatoxin M₁ may be present in liquid or dried milk (Table 3) and in milk products (Kiermeier, 1977). In addition, highly exceptional aflatoxin levels in the range of 50–500 µg/litre were reported by Suzanger et al. (1976) in half of the samples of cow's milk collected in villages around Isfahan, Iran (15/30 samples collected in 1973 and 21/37 samples in 1974). In contrast, no aflatoxins were detected in 8 samples of milk obtained from large-scale producers in the same area in 1974 and only 10% of such samples (2/20) contained aflatoxin M₁ (in the range 8–10 µg/litre) in 1973. Aflatoxin M₁ was identified in all the positive samples. Eight of the 36 village samples containing aflatoxin M₁ also contained aflatoxin M₂ and 2 samples contained aflatoxin B₁. Considerable differences in the handling and storage of animal feeds were thought by the authors to be responsible for the differences in the aflatoxin M₁ contents of milk samples from villages and large-scale producers in this area. However, levels of aflatoxins in animal feeds were not reported in this paper, but they must have been exceptionally high.

Aflatoxin residues have been found in animal tissues, eggs, and poultry following the experimental ingestion of aflatoxin-contaminated feed and this subject has been reviewed by Rodricks & Stoloff (1977). However, the toxins have not yet been found in these products on the market.

3.2.3 **Fate of aflatoxins during the handling and processing of food**

Aflatoxins are affected by some ordinary food processing procedures. In the roasting of groundnuts, approximately 50% of the aflatoxins are altered to such an extent that they can no longer be detected (Lee et al., 1969b; Walkling, 1971). The chemical nature of the alteration products has not been fully elucidated.

The usual methods of processing groundnuts to make peanut butter and some nuts for confections may appreciably reduce aflatoxin contamination. The removal of undersized nuts (shrivels and pegs); the removal of nuts that resist splitting and blanching; and the removal of discoloured nuts by hand or electronic sorting are effective means of reducing contamination (Rodricks et al., 1977).

In the removal of oil from oilseeds, most of the aflatoxins are found in the oilseed meal. Small amounts remaining in the crude vegetable oil are mainly taken out in the soap stock, the byproduct from the alkali refining step. The remaining traces of aflatoxins are removed in the bleaching refining steps to give aflatoxin-free refined oil (Parker & Melnick, 1966).

Table 3. Selected surveys of aflatoxin M₁ in cow's milk

Milk samples	Country	Total no. of samples analysed	No. containing aflatoxin M ₁	Range of concentrations in the positive samples (µg/litre or µg/kg)	Reference
<i>Liquid</i>	Belgium	68	42	0.02-0.2	Van Pee et al. (1977)
	German Democratic Republic	36	4 ^a	1.7-6.5 ^c	Fritz et al. (1977)
	Germany, Federal Republic of	61	28	0.01-0.25	Kiermeier (1973)
	Germany, Federal Republic of	419	79	trace-0.54	Kiermeier et al. (1977)
	Germany, Federal Republic of	260	118 ^a	0.05-0.33	Polzhofer (1977)
	India	21	3	up to 13.3	Paul et al. (1976)
	Netherlands	95	74	0.09-0.5	Schuller et al. (1977)
	United Kingdom	278	85 ^a	0.03-0.52 ^d	Patterson et al. (in press)
	German Democratic Republic	18	0 ^f	—	Fritz et al. (1977)
	Germany, Federal Republic of	166	8	0.67-2.0	Neumann-Kleinpaul & Terplan (1972)
<i>Dried</i>	Germany, Federal Republic of	52	35	trace-4.0	Hanssen & Jung (1972)
	Germany, Federal Republic of	120	7 ^a	0.05-0.13	Jung & Hanssen (1974)
	South Africa	56	0	—	Lück et al. (1977)
	USA	320	24	0.1-0.4	FDA (1977) 1973 survey
	USA	302	192	trace-3.9 ^e	FDA (1977) 1977 survey

^a Seasonal effect observed, i.e., concentration obviously dependent upon level of concentrate feeding.

^b Samples collected in retail outlets in 4 southeast States of the USA; it has been estimated that approximately two-thirds of the crops in these areas contained aflatoxin concentrations exceeding 20 µg/kg because of unusual drought, insect damage, and high temperature conditions that occurred in 1977.

^c Values for 4 positive samples collected in winter; aflatoxin was not detected in the other 32 winter milk samples as well as in 12 milk samples collected in summer (detection limit = 0.1 µg/kg).

^d 92.5% samples contained aflatoxin M₁ concentrations of less than 0.1 µg/litre.

^e Levels ranging from 0.1 to 0.4 µg/kg reported in 158 samples; levels exceeding 0.5 µg/kg reported in 19 samples.

^f Aflatoxin B₁ contamination detected in one sample.

The normal alkali processing of maize to produce tortilla-type foods, a common practice in some areas of the world including some Latin American countries, effectively reduces the levels of aflatoxins in contaminated maize (Ulloa-Sosa & Schroeder, 1969). Although the mechanism of this reduction has not been clarified, some of the aflatoxin is most likely washed out by the initial soaking of the maize in lye water and some is undoubtedly chemically changed by the alkali; although this process is reported to produce a substantial reduction in aflatoxin contamination, it is not enough to give a safe product, when highly contaminated maize is being processed.

Jemmali & Lafont (1972) reported only partial destruction of aflatoxins during bread making, indicating the importance of the contamination of wheat with *A. flavus*.

The above treatments are normal steps in the processing of particular foods. In addition to such steps, procedures have been developed specifically for the destruction or removal of aflatoxins from grains, nuts, oilseeds, and oilseed meals (cake) (FAO, 1977). Treatments with ammonia or hydrogen peroxide (H_2O_2) have been the most effective procedures developed to date for the detoxification of foodstuffs and animal feeds (Goldblatt & Dollear, 1977). The treatments with ammonia, developed for the industrial decontamination of aflatoxin-contaminated groundnut and cottonseed meal (cake) and maize, are limited to the production of animal feeds. These procedures have recently been discussed in detail at the joint FAO/WHO/UNEP conference on mycotoxins in Nairobi (FAO, 1977). The process of treating groundnut protein isolate with hydrogen peroxide to obtain a product suitable for use as a human food supplement was also discussed. This process has been developed in India and is reported to be operating on a small commercial scale.

The feasibility of methods combining the physical separation of contaminated portions of produce (for detailed descriptions of segregation techniques see, for example, Rodricks et al., 1977) with chemical decontamination, was considered at the same conference (FAO, 1977).

3.2.4 Pathways and levels of exposure

From the previous discussion, it can be seen that a range of commodities may become contaminated with trace amounts of aflatoxins. In vegetable foods, this contamination results directly from fungal spoilage, maize and nuts being particularly susceptible. On the other hand, milk, and possibly meat and eggs can become indirectly contaminated through the absorption by farm animals of aflatoxins from contaminated feed resulting in residues of the parent toxin or its metabolites in body fluids or tissues.

Thus, the level of man's exposure to dietary aflatoxins depends upon the food available and on eating habits and will vary from country to country according to the local conditions, including the traditions of different ethnic groups, and amongst individuals. Where contaminated groundnuts or maize make a significant contribution to the diet, the level of exposure will be relatively higher than where less commonly contaminated commodities take their place as the staple food or when milk is the sole aflatoxin-containing constituent of the diet.

In this connexion, the Task Group felt it was important to identify the infant as being potentially at risk because: (a) baby food products may be made from dried milk or even maize, commodities known to be prone to contamination by aflatoxins; and (b) in terms of larger amounts of food consumed per kg body weight, any level of aflatoxin contamination is more significant for the child than for the adult.

Attempts to quantify dietary exposure to aflatoxins are discussed in detail in section 3.6.1.1.

Occupational exposure (section 3.5.2) to aflatoxins with its attendant high risks, concerns two groups of individuals: those who handle grain, animal feedstuffs, groundnuts, groundnut meal etc. (where exposure could occur largely through inhalation of contaminated dust), and those who work with toxins in experiments or with pure toxins as analytical standards.

In one paper (van Nieuwenhuize et al., 1973) available to the Task Group, an attempt was made to quantify occupational exposure to airborne aflatoxins in an oil-mill crushing groundnuts and other oil-seeds. Based on airborne dust determinations (mean aflatoxin concentrations of 250 and 410 $\mu\text{g}/\text{kg}$ airborne dust), the estimated airborne aflatoxin levels ranged from 0.87 to 72 ng/m^3 of air.

3.3 Metabolism

3.3.1 Absorption

Although quantitative data on absorption are not available at present, there is no doubt that most of the field cases of aflatoxin-induced diseases in animals and man have been associated with ingestion of aflatoxin-contaminated foodstuffs and thus with the absorption of aflatoxins in the alimentary tract. In spite of reports of respiratory exposure (sections 3.2.4 and 3.5.2), there is no quantitative information available on aflatoxin re-sorption from the respiratory tract or on percutaneous absorption.

3.3.2 Tissue distribution

3.3.2.1 Animal studies

Experiments with ^{14}C -ring-labelled aflatoxin B_1 have shown that rats retain about 20% of the ^{14}C activity 24 h after a single intraperitoneal dose

of 0.07 mg/kg body weight (Wogan et al., 1967). The highest concentration was found in the liver, which contained amounts of radioactivity equivalent to the entire remainder of the carcass (about 5%–8% of the total ^{14}C recovered).

When poultry were fed rations containing aflatoxins at concentrations ranging from 25 to 15 000 $\mu\text{g}/\text{kg}$ for 8 weeks, residues of aflatoxin B_1 were found in the liver and in muscle tissue (Mintzclaff et al., 1974). The liver contained the highest concentration, with a mean value of 15 $\mu\text{g}/\text{kg}$ at the highest exposure level. Similarly the highest concentration of aflatoxin B_1 was found in the livers of pigs (range: trace–137 $\mu\text{g}/\text{kg}$) fed rations containing aflatoxins (both aflatoxin B_1 and B_2) at levels of 300 and 500 $\mu\text{g}/\text{kg}$ for 4 months (Krogh et al., 1973a). Aflatoxin residues were also detected in kidneys, and muscle and adipose tissue.

3.3.2.2 *Studies in man*

Levels of aflatoxins in the tissues of children with Reye's syndrome are discussed in section 3.6.2.2. In a liver biopsy from a subject with carcinoma of the rectum and liver in the USA, Phillips et al. (1976) found 520 $\mu\text{g}/\text{kg}$ of aflatoxin B_1 . In France, Richir et al. (1976) found aflatoxin B_1 in liver biopsies in 6 out of 100 subjects suffering from various diseases. Concentrations observed ranged from 1.6–8 $\mu\text{g}/\text{kg}$.

3.3.3 **Metabolic transformation and activation**

With one exception, all primary biotransformations of aflatoxin B_1 involve its conversion to hydroxylated metabolites but only one such derivative, aflatoxin M_1 has appreciable oral toxicity (Holzapfel et al., 1966). Even so, this metabolite may be detoxified by conjugation with taurocholic and glucuronic acids prior to excretion in the bile or urine (Bassir & Osiyemi, 1967). In this respect, two recently discovered metabolites, P_1 (Dalezios et al., 1971; Büchi et al., 1973) and Q_1 (Masri et al., 1974a,b) are similar in that they also undergo this type of detoxification (Dalezios et al., 1971; Dalezios & Wogan, 1972).

The conversion in the liver (Fig. 2) of aflatoxin B_1 to aflatoxicol (Patterson & Roberts, 1971) and to aflatoxicol H_1 via aflatoxin Q_1 (Salhab & Hsieh, 1975) is unusual in that, unlike other biotransformations that are catalysed by liver microsomal enzymes, a cytoplasmic NADH-dependent dehydrogenase is involved. Furthermore, the formation of aflatoxicol can be inhibited by 17-ketosteroid sex hormones (Patterson & Roberts, 1972a) and this is the only metabolic transformation of aflatoxin *in vitro* known to be sensitive to hormones.

Liver homogenates of certain avian and rodent species are particularly active in converting aflatoxins B_1 and G_1 to their 2-hydroxy, 2,3-dihydro derivatives or hemiacetals called also aflatoxins B_{2a} and G_{2a} (Patterson &

Roberts, 1970). These metabolites bind strongly to protein and are probably sufficiently reactive, when formed *in vivo*, to cause many of the acute effects of aflatoxin poisoning (Patterson & Roberts, 1972b; Patterson, 1973, 1977).

At present, there is only indirect evidence for the formation of the epoxides of aflatoxins B₁ and G₁ but this is probably the more important form of metabolic activation. When either of the parent toxins is incubated

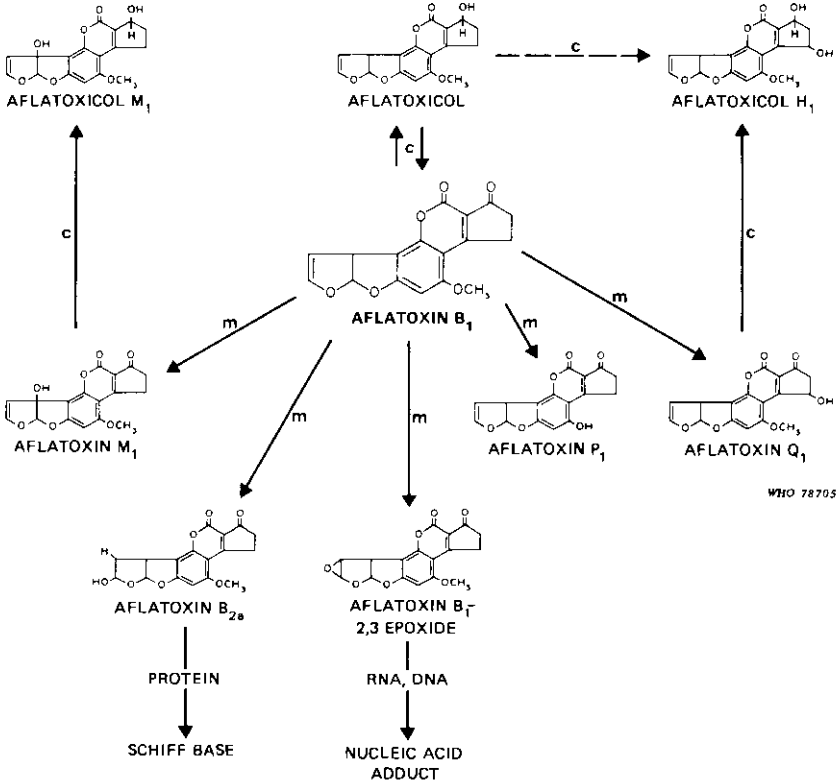


Fig. 2. Aflatoxin B₁ metabolism in the liver.

with microsomes prepared from the livers of many animal species including man, a metabolite is formed which appears to have only a transient existence, is highly reactive, binds covalently to DNA, and induces mutation in a bacterial *in vitro* test system (Garner et al., 1971, 1972; Ames et al., 1973). The metabolite of B₁ has not been isolated but the 2,3-dihydrodiol has been recovered following mild acid hydrolysis of an adduct formed when the microsomal metabolite was generated in the presence of added DNA or RNA (Swenson et al., 1974) and, more recently, after *in vivo* intraperitoneal injection of aflatoxin B₁ (Swenson et al., 1977). This has

been assumed to be indirect evidence of the formation of the 2,3-epoxide and, in view of the interaction with DNA, it is now generally accepted that the epoxide of aflatoxin B₁ is the bacterial mutagen and the proximal carcinogen.

Certain of these biotransformations are better developed in some animal species than others (Patterson, 1977) and attempts have been made to correlate liver metabolism of aflatoxins with toxicity. In the first such attempt (Patterson, 1973), it was proposed that rapid *in vitro* formation of aflatoxin hemiacetal was correlated with susceptibility to acute aflatoxin poisoning. More recently (Hsieh et al., 1977), it has been suggested that the reversible formation of aflatoxicol, which is thought to provide a "metabolic reservoir" of aflatoxin (Patterson & Roberts, 1972b), is correlated with susceptibility to liver tumour induction. On the basis of this, it has been tentatively suggested (Hsieh, 1977; Salhab & Edwards, 1977) that the human liver might be relatively more resistant to aflatoxin carcinogenesis than that of some other species, particularly the rat.

3.3.4 Excretion

3.3.4.1 *Animal studies*

Excretory pathways. Using aflatoxin B₁, ring-labelled or methoxy-labelled with ¹⁴C, Wogan et al. (1967) have shown that rats excrete 70%–80% of a single intraperitoneal dose within 24 h. A major excretory route of the ring-labelled toxin was through biliary excretion into the faeces, accounting for about 60% of the administered dose; approximately 20% of administered radioactivity was excreted in the urine, and only negligible amounts in expired air in the form of ¹⁴CO₂. In contrast, approximately 25% of radioactivity from methoxy-labelled material appeared in expired air as ¹⁴CO₂ with a concomitant decrease in the faeces, indicating that *O*-demethylation is a significant metabolic pathway for aflatoxin B₁ in the rat.

Excretion in the milk of farm animals. Several reviews deal with the excretion of aflatoxins in the milk of farm animals (Allcroft, 1969; Kiermeier, 1973, 1977; Patterson, 1977; Rodricks & Stoloff, 1977). When cattle (Allcroft et al., 1968), sheep (Nabney et al., 1967) or goats (Vesely et al., 1978) are given feed contaminated with aflatoxin B₁ their milk contains aflatoxin M₁. In the cow, there is a linear relationship between the amount of aflatoxin B₁ ingested daily and the level of aflatoxin M₁ in the milk (Allcroft & Roberts, 1968; Purchase, 1972; Patterson, 1977; see Fig. 3), indicating that about 1.5% of aflatoxin B₁ is excreted as the metabolite M₁ (Kiermeier, 1973), and that the concentration of aflatoxin B₁ in milk is approximately 1/300 of the concentration of aflatoxin B₁ in the dairy ration (Rodricks & Stoloff, 1977). Smaller quantities of unmetabolized aflatoxin

B₁ have been found in cow's and sheep's milk (Nabney et al., 1967; Allcroft et al., 1968; Wogan, 1969).

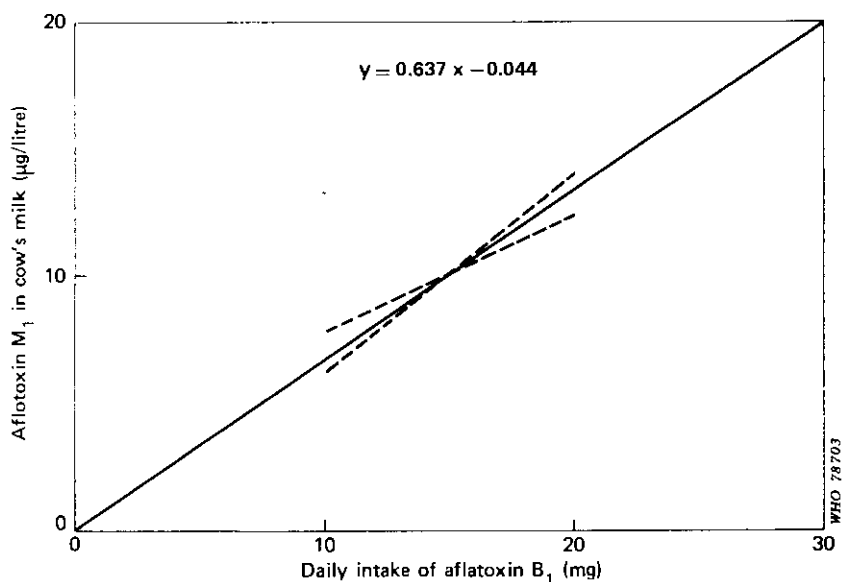


Fig. 3. The relationship between the daily intake of aflatoxin B₁ and the resulting concentration of aflatoxin M₁ in cow's milk based on published experimental data. The 95% confidence limits for the slope of the calculated line are represented by dotted lines (From: Patterson (1977) *Mycotoxic fungi, mycotoxins, mycotoxicoses*, pp. 131-136 and 144-189, by courtesy of Marcel Dekker, Inc.).

3.3.4.2 Studies in man

In the Philippines, aflatoxin M₁ has been found (not measured) in the urine of human subjects known to have ingested aflatoxin-contaminated peanut butter (Campbell et al., 1970).

Claims concerning an aflatoxin involvement in the etiology of juvenile cirrhosis in India (section 3.6.3.2) based on a blue-fluorescent B₁ spot in the breast milk of mothers and the urine of children with the disease (Robinson, 1967) are largely discounted by the later studies of Yadgiri et al. (1970) who produced spectrophotometric evidence that, although such a spot could be identified in the urine of children with the overt disease, this was not aflatoxin B₁. For other reports see section 3.5.1.

3.4 Effects in Animals

The effects of aflatoxins in animals have been reviewed by Allcroft (1969), Newberne & Butler (1969) and Butler (1974).

3.4.1 Field observations

When foodstuffs are affected by microbial deterioration, man normally eats the less affected parts, whereas domestic animals may be exposed to more contaminated rations. This explains why the discovery of several mycotoxins has been based on field observations in domestic animals.

The first observation of a disease in animals subsequently associated with aflatoxins was an acute outbreak of a lethal disease in turkey poults in England in 1960 causing an estimated loss of at least 100 000 birds. Extensive research eventually revealed that the disease was caused by aflatoxins contained in a batch of Brazilian groundnut meal. The concentration of aflatoxin B₁ in the original groundnut meal was later estimated to be about 10 mg/kg. The disease was characterized by rapid deterioration in the condition of the birds, subcutaneous haemorrhages, and death. At postmortem, the livers of the birds were pale, fatty, and showed extensive necrosis and biliary proliferation (Butler, 1974). A similar case of acute disease was observed in day-old ducklings fed "toxic" groundnut meal (Asplin & Carnaghan, 1961), where the liver changes described were followed by cirrhosis. Outbreaks of liver disease in chickens have also been associated with aflatoxin-contaminated feed (Asplin & Carnaghan, 1961).

Loosmore & Harding (1961) noted outbreaks in pigs fed groundnut meal in which the toxic factor was later identified as aflatoxins. The lesions in the pigs included haemorrhages, and liver damage characterized by dissecting fibrosis and biliary proliferation. Calves fed rations containing 15% toxic groundnut meal also developed liver lesions characterized by fibrosis and biliary proliferation (Loosmore & Markson, 1961). Outbreaks associated with "toxic" groundnut meal and characterized by similar liver lesions have been reported in older cattle even though they are more resistant (Clegg & Bryston, 1962); there was also a drop in milk production.

A liver disease "hepatitis X" has been reported in dogs in southeastern USA (Seibold & Bailey, 1952; Newberne et al., 1955). Icterus and in some cases ascites were observed and the liver lesions included fatty changes with centrilobular parenchymal necrosis and biliary proliferation. Commercial dog food thought to be the cause of toxicity was later found to contain aflatoxin B₁ (up to 1.75 mg/kg) (Newberne et al., 1966a). Similar lesions have been reproduced in dogs by peroral administration of aflatoxins, and it has been suggested that the "hepatitis X" in dogs could be causally associated with aflatoxins in the diet (Newberne et al., 1966a). During an outbreak of toxic hepatitis affecting several hundred people in north-west India and considered to be possibly associated with the consumption of maize heavily contaminated by aflatoxins (section 3.5.2 and 3.6.2.1), dogs fed food remnants from households in affected villages manifested a disease

characterized by jaundice, ascites, and frequently death (Krisnamachari et al., 1975a,b; Tandon et al., 1977). A nonportal type of micronodular cirrhosis, with less conspicuous parenchymal and cholangiolar changes was found on histological examination of the livers of two dogs (Tandon et al., 1977).

3.4.2 Experimental studies

3.4.2.1 Acute and chronic effects: hepatotoxicity

Different species vary in their susceptibility to acute poisoning by aflatoxins, with LD₅₀ values ranging from 0.3 to 17.9 mg/kg body weight (Table 4). In all the animals studied, the liver was the principal target organ (see for example Butler, 1974).

Table 4. Acute toxicity of aflatoxin B₁^a

Species	LD ₅₀ (mg/kg body weight)	Zone of liver lesion
chick embryo	0.025 ^d	
rabbit	0.3	midzonal
duckling	0.335	periportal
cat	0.55	periportal
pig	0.62	centrilobular
dog	0.5-1.0	centrilobular
sheep	1.0	centrilobular
guineapig	1.4	centrilobular
baboon ^b	2.0	centrilobular
rat (male)	7.2	periportal
macaque female ^c	7.8	centrilobular
mouse	9.0	
hamster	10.2	
rat (female)	17.9	periportal

^a Adapted from: Newberne & Butler (1969) and Butler (1974).

^b From: Peers & Linsell (1976).

^c From: Shank et al. (1971b).

^d µg/embryo.

The lesions observed in field cases (section 3.4.1) in poultry, pigs, cattle, and dogs have all been reproduced in the same animal species by feeding experiments during periods of time ranging from a few weeks to a few months, using diets containing aflatoxins, or pure aflatoxins ranging from 0.3 to several mg/kg (Newberne & Butler, 1969).

In the study by Carnaghan et al. (1966), chickens were fed a diet containing aflatoxin B₁ at a level of 1.5 mg/kg. Groups of 3 control and 3 test chicks were killed after 3½ days, 7 days, and then at weekly intervals for 8 weeks. After 4 weeks, the liver lesions included fatty change, biliary proliferation, and fibrosis.

In 20 pigs, aflatoxins (aflatoxins B₁ and B₂) at a feed level as low as 300 µg/kg resulted in the development of centrilobular necrosis and fibrosis of

the liver as well as growth depression, during a normal feeding period of 3–4 months (Krogh et al., 1973a). In cattle, the liver lesions (centrilobular degeneration, fibrosis, biliary proliferation) occurred in all 4 animals after 4 months on a feed containing an aflatoxin level of 2 mg/kg (Allcroft & Lewis, 1963). The hepatic lesions induced in the duckling by the aflatoxins formed the basis of a bioassay originally described by Sargeant et al. (1961). At sublethal doses of aflatoxins, the bioassay depends upon an assessment of the degree of biliary proliferation. Liver lesions similar to those observed in farm animals have been experimentally induced by the administration of aflatoxins in a number of laboratory animals, including the rat, cat, guinea-pig, and rabbit (Newberne & Butler, 1969).

In a study of Madhavan et al. (1965b), 2 rhesus monkeys (*Macaca mulatta*) were given daily oral doses of aflatoxins at 500 µg/animal for 18 days (corresponding approximately to 250 µg/kg body weight per day) and then 1 mg/animal per day (corresponding approximately to 500 µg/kg body weight per day) until death occurred after 32 and 34 days. Three rhesus monkeys were given 1 mg each, daily, until death occurred after 19, 20, and 27 days respectively. The liver lesions included fatty infiltration, biliary proliferation, and portal fibrosis. Death or similar lesions were not observed in the 2 control monkeys.

Deo et al. (1970) studied the effect on male rhesus monkeys of repeated administration by gastric tube of 3 different levels of aflatoxins ($B_1 + G_1$). At the highest dose level (1 mg/kg body weight daily for 3 weeks), 35/35 animals died within 22 days with extensive haemorrhagic necrosis of the liver. A dose level of 0.25 mg/kg body weight, twice a week, for 5 months induced various degrees of liver changes in 24/24 animals characterized by biliary proliferation and focal appearance of the liver cells with multiple nuclei and giant-sized liver cells with enlarged hyperchromatic nuclei. At the lowest dose, 5 animals were given 62 µg/kg body weight once a week for periods ranging from a few days to 2 years. Liver changes were similar to the changes seen in the second group but in a milder form.

Cynomolgus monkeys (*Macaca fascicularis* = *M. irus*) fed a dietary level of aflatoxin B_1 of 5 mg/kg rapidly developed liver damage with biliary proliferation and all 6 animals died within 2 months. When fed aflatoxin B_1 at a dietary level of 1.8 mg/kg, 5 animals died within 3 months showing liver damage characterized by centrilobular necrosis, biliary proliferation, and fibrosis. Two animals survived and were killed after 3 years; the liver of one animal had the appearance of nodular cirrhosis. Two groups of 4 animals each were fed lower levels of aflatoxin B_1 (0.07 and 0.36 mg/kg, respectively) for 3 years without showing any signs of liver lesions (Cuthbertson et al., 1967).

The relationship between the chemical structure of different aflatoxins

and their biological activity (discussed also in section 3.4.2.3) was investigated in a small number of experiments; more extensive studies were not possible because of the limited quantities available of some of the pure aflatoxins. Carnaghan et al. (1963) compared 6-day mortality following single doses of different aflatoxins, administered by intubation to one-day-old Khaki Cambell ducklings, and concluded that both aflatoxins B_2 and G_2 were less toxic than aflatoxins B_1 and G_1 , the ratio of LD_{50} values being 1:4.7 for B_1 : B_2 and 1:4.4 for G_1 : G_2 . Aflatoxins G_1 and G_2 were less toxic than the corresponding aflatoxins B_1 and B_2 , the ratio of the LD_{50} values being 1:2.15 for B_1 : G_1 and 1:2.03 for B_2 : G_2 . The corresponding LD_{50} values for aflatoxins B_1 , B_2 , G_1 , and G_2 were 0.36, 1.70, 0.78, and 3.45 mg/kg, respectively. Comparable results were obtained by Wogan et al. (1971) who recorded 14 day mortality after intubation of male Pekin ducklings and reported LD_{50} values of 0.73, 1.76, 1.18 and 2.83 mg/kg for aflatoxins B_1 , B_2 , G_1 , and G_2 , respectively. In the same paper, a study was reported on the 14-day mortality of male Fischer rats after a single (intra-peritoneal) dose of aflatoxin. The LD_{50} value for aflatoxin B_1 was 1.16 mg/kg body weight (95% confidence interval 0.91 to 1.48 mg/kg) whereas the LD_{50} for aflatoxin G_1 was between 1.5 and 2.0 mg/kg body weight. On the other hand, no deaths occurred in 20 rats given 12–200 mg of aflatoxin B_2 per kg body weight, and all 4 rats given 170–200 mg of aflatoxin G_2 per kg body weight survived. A similar difference in the toxicity of aflatoxins was observed when male Fischer rats were given repeated doses of aflatoxins by stomach tube over a 4-week period. The 4-week mortality in rats given a total dose of 1 mg of aflatoxin B_1 per rat was 8/10 whereas all 10 rats given the same dose of aflatoxin G_1 survived and only 4/10 animals given double this dose of aflatoxin G_1 died. In another trial, all 11 rats survived intragastric administration of 3.75 mg of aflatoxin B_2 per rat repeated every second day for 4 weeks to give a total dose of 52.5 mg per rat.

Holzapfel et al. (1966) and Purchase (1967) reported 7-day mortality after oral dosing of one-day-old Pekin ducklings with aflatoxins B_1 , M_1 , and M_2 . Five groups of 2–3 ducklings (body weight 40–50 g) were used for each of the aflatoxins tested, and the following LD_{50} s were calculated (with 95% confidence limits given in brackets): aflatoxin B_1 , 12 (3.9–37.2) μ g per duckling, aflatoxin M_1 , 16 (5.4–51.5) μ g per duckling, and aflatoxin M_2 , 61.4 (37–100) μ g per duckling. Ducklings receiving aflatoxin M_1 showed characteristic liver lesions indistinguishable from those observed after a similar dose of aflatoxin B_1 . Higher doses of aflatoxin M_2 produced similar effects (Purchase, 1967). A study comparing the acute toxicity of synthetic (racemic) aflatoxins B_1 and M_1 and the natural optical isomer of aflatoxin B_1 was reported by Pong & Wogan (1971), suggesting that only one isomer of each synthesized racemic mixture was biologically active. Fourteen-day

mortality rates observed after a single intraperitoneal dose of 1.5 mg/kg body weight of synthetic aflatoxin B₁ and synthetic aflatoxin M₁ were 1/1 and 1/2, respectively. However, no deaths occurred in groups of rats (each consisting of 4 animals) given these synthetic aflatoxins at doses of 1, 0.8, 0.6, or 0.4 mg/kg body weight. With the natural aflatoxin B₁, the observed mortalities at these dose levels were 4/4, 2/4, 2/4, and 0/4 respectively.

For information on the toxicity of certain other aflatoxin metabolites or derivatives, see Wogan et al. (1971) and Patterson (1976).

3.4.2.2 *Hepatotoxicity connected with extrahepatic effects*

Many other organs besides the liver are more or less severely affected in acute experiments with high doses of aflatoxins (Butler, 1964): in male and female rats, a single dose of aflatoxin B₁ proved lethal in half of the animals (7.2 mg/kg body weight in the male and 17.9 mg/kg body weight in the female, by gavage). Frequent bilateral adrenal haemorrhages, petechial haemorrhages in many organs, particularly in the congested lungs, and occasionally patchy necroses in the myocardium and in other organs (kidney, spleen) were observed during the first few days following administration. These changes were not detected in male or female rats given aflatoxin B₁ at 3.5 mg/kg body weight. With higher doses, the haemorrhages seen in the lungs, kidneys, and adrenals were more extensive. Animals dying within the first few days often had altered blood in the whole of the small intestine and in the colon. Ascites and oedema of the omentum were observed in some of the animals a week or more (but not one month) after aflatoxin administration. After a month, with the exception of the liver damage, all the other organs appeared normal in surviving animals. Histologically, certain renal changes were detected in the loops of Henle at this stage, consisting of a few cells with large irregular hyperchromatic nuclei, very similar to those seen in the liver (Butler, 1964).

Congested lungs with small petechial haemorrhages, haemorrhagic necroses in the adrenals (localized in the inner zone of the reticularis) and patchy necroses in the kidneys, pancreas, and spleen were observed in guineapigs 2–3 days after a single intraperitoneal injection of aflatoxin B₁ at 1.4 mg/kg body weight (lethal in half of the males and females). Even at this dose, the small intestine was frequently filled with altered blood. At higher doses, the haemorrhagic disease was more marked, with pleural, pericardial, and peritoneal haemorrhages. The only change seen in the heart of the guineapig, 2–3 days after aflatoxin administration, was an occasional small area of fatty degeneration of the myocardium. Many animals showed marked ascites and oedema of the omentum and subcutaneous tissue during the first week after injection (Butler, 1966).

Bourgeois et al. (1971) reported a special syndrome induced by oral

administration of aflatoxin B₁ in the macaque (*Macaca fascicularis*). In 2 groups of 4 young females, each receiving a single oral dose of aflatoxin B₁ at 13.5 or 40.5 mg/kg body weight, all animals died within 149 h. Death occurred in 1 out of 4 other animals receiving a dose of 4.5 mg/kg body weight. Doses of toxin of 1.5 mg/kg or 0.5 mg/kg (4 animals in each group) did not result in death or unusual clinical signs. Cough, vomiting, diarrhoea, and coma were characteristic clinical findings in animals exposed to toxic doses. Analysis of blood serum revealed a dose-dependent decrease in serum levels of phospholipids within 24 h of administration of the aflatoxin. A dose-dependent decrease in serum levels of glucose and an increase in nonesterified fatty acids occurred within 72 h of aflatoxin administration. The liver lesions included centrilobular necrosis, some biliary proliferation, and massive fatty degeneration which was also observed in the heart and kidneys. Cerebral oedema with neuronal degeneration was seen. Some of these findings resemble those associated with Reye's syndrome in children (see section 3.5.1.2).

3.4.2.3 Carcinogenesis

The carcinogenesis of aflatoxins has been reviewed by Wogan (1973, 1977) and re-evaluated by IARC (1976).

Hepatic and renal tumours. Orally administered aflatoxins, mainly B₁, have been hepatocarcinogenic in all species of test animals studied so far (including nonhuman primates), with the exception of the mouse, in which carcinogenic effects have been demonstrated only following intraperitoneal administration of aflatoxin B₁ to neonates (Tables 5 and 6). These studies were concerned with repeated or long-term exposure to aflatoxins. In a study by Carnaghan (1967), 2 groups consisting of 16 and 18 weanling female Wistar rats, respectively, were given single oral doses of crystalline aflatoxin B₁ or a mixture of aflatoxins containing about 40% aflatoxin B₁ and 60% aflatoxin G₁, at the rate of 0.5 mg/rat in 0.1 ml dimethylformamide. These doses corresponded to averages of 7.65 mg aflatoxin B₁/kg body weight and 2.7 mg aflatoxin B₁ plus 4.0 mg aflatoxin G₁/kg body weight, respectively. Within 21–32 months, 7 rats out of each group developed hepatic tumours with metastases in half the cases. Hepatic tumours were not observed in 19 control rats given the solvent only. No hepatocellular carcinomas were found in 22 male Fischer rats killed successively 16 weeks (3 rats), 25 weeks (5 rats), 38 weeks (5 rats), 55 weeks (4 rats), and 69 weeks (5 rats) after a single dose of aflatoxin B₁ at 5.0 mg/kg body weight, administered by gavage (Wogan & Newberne, 1967).

A linear dose-response relationship was observed by Wogan et al. (1974) for the development of liver-cell carcinomas in male Fischer rats fed dietary concentrations of aflatoxin B₁ ranging from 1–100 µg/kg (Table 7). At 1

Table 5. Hepatocarcinogenicity of aflatoxin B₁ in rodents^a

Species	Dosing regimen	Duration of treatment	Period of observation	Liver tumour incidence	Reference
rat, Fischer	1.0 mg/kg diet	33 weeks	52 weeks	3/6	Svoboda et al. (1966)
rat, Fischer	1.0 mg/kg diet	41-64 weeks	41-64 weeks	18/21	Wogan & Newberne (1967)
rat, Porton	1.0 mg/kg diet	20 weeks	90 weeks	19/30	Butler (1969)
rat, Wistar	1.0 mg/kg diet	21 weeks	87 weeks	12/14	Epstein et al. (1969)
mouse, Swiss	150 mg/kg diet ^b	80 weeks	80 weeks	0/60	Wogan (1973)
mouse, C57Bl/6NB	1.0 mg/kg diet	80 weeks	80 weeks	0/30	Wogan (1973)
mouse, C3HfB/HEN	1.0 mg/kg diet	80 weeks	80 weeks	0/30	Wogan (1973)
mouse, hybrid F ₁ , 4, days old	6.0 µg/g body weight	3 doses (i.p.)	80 weeks	16/16	Vesselinovitich et al. (1972)

^aFrom: Wogan (1977).

^bA mixture of aflatoxins B₁ and G₁ was used in this experiment.

Table 6. Hepatocarcinogenicity of aflatoxin B₁ in nonrodent species^a

Species	Dosing regimen	Duration of treatment	Period of observation	Liver tumour incidence	Reference
monkey, rhesus (M)	1.655 g total ^b	5.5 years	8.0 years	1/1	Gopalan et al. (1972)
monkey, rhesus (F)	1.655 g total	5.5 years	10.75 years	1/1	Tilak (1975)
monkey, rhesus (F)	0.504 g total	6.0 years	8.0 years	1/1	Adanson et al. (1973)
marmoset	3.0 mg total	50–55 weeks	50–55 weeks	1/3	Lin et al. (1974)
	5.04–5.84 mg total ^c	87–94 weeks	87–94 weeks	2/3	Lin et al. (1974)
tree shrew (M & F)	2.4–6.6 mg total	74–172 weeks	74–172 weeks	9/12	Reddy et al. (1976)
ferret	0.3–2.0 µg/kg	28–37 months	28–37 months	7/9	Butler (1969)
duck	30 µg/kg	14 months	14 months	8/11	Carneghan (1965)
rainbow trout	4 µg/kg in diet	12 months	12 months	15%	Sinnhuber et al. (1968b)
	8 µg/kg in diet	12 months	12 months	40%	Sinnhuber et al. (1968b)
rainbow trout embryos	0.5 mg/kg in water	1 h	296–321 days	38%	Sinnhuber & Wales (1974)
salmon	12 µg/kg in diet ^d	20 months	20 months	50%	Wales & Sinnhuber (1972)
guppy	6 mg/kg in diet	11 months	11 months	7/11	Sato et al. (1973)

^a Modified from: Wogan (1977).

^b A mixture of aflatoxins B₁ and G₁ was used in this experiment.

^c These animals were infected simultaneously with hepatitis virus.

^d This diet also contained 50 mg/kg cyclopropanoid fatty acids.

Table 7. Dose-response characteristics of aflatoxin B₁ carcinogenesis in male Fischer strain rats^a

Dietary aflatoxin level (µg/kg)	Duration of feeding (week)	Liver carcinoma incidence ^b	Time of appearance of earliest tumour (week)
0	74-109	0/18 ^c	—
1	78-105	2/22	104
5	65-93	1/22	93
15	69-96	4/21	96
50	71-97	20/25 ^d	82
100	54-88	28/28 ^e	54

^a From: Wogan et al. (1974).

^b In animals at risk (surviving longer than 50 weeks).

^c Animals surviving for maximum period.

^d Two animals had pulmonary metastases.

^e Four animals had pulmonary metastases.

µg/kg, a 10% tumour incidence was found, compared with no tumours in the control group and at 100 µg/kg the tumour incidence was 100%. A linear log (dose)-response relationship has been demonstrated in trout fed dietary levels of aflatoxin B₁ ranging from 0.5 to 20.0 µg/kg, for 20 months. Extrapolating this relationship to lower exposure levels, an incidence of approximately 10% would be expected with a dietary concentration of 0.1 µg/kg.

In a recent study (FDA, 1978), an attempt was made to calculate the life-time liver cancer risk in rats, which could be connected with aflatoxin feed levels lower than those directly tested in animal experiments. Estimates of life-time liver cancer incidence rates corresponding to aflatoxin feed levels of 0.1 and 0.3 µg/kg were derived and compared for several selected rat studies using the mathematical procedure developed by Mantel & Bryan (1961) and modified by Mantel et al. (1975). A more detailed description of this procedure can be found in other publications including WHO (1978) and Hoel et al. (1975). Thus, the estimated life-time liver cancer risk derived from the experimental results reported by Wogan et al. (1974) (see Table 7) corresponded to life-time liver cancer incidence rates of 70 (600) per 10⁵ rats for an aflatoxin dietary level of 0.1 µg/kg and 360 (2300) for a level of 0.3 µg/kg. (Numbers in parentheses are upper 99% confidence limits.) Estimates derived from different rat studies varied considerably. The life-time incidence rates calculated for the combined studies were 240 (470) and 1100 (1900) per 10⁵ rats for aflatoxin feed levels of 0.1 and 0.3 µg/kg, respectively (FDA, 1978).

The studies in primates are included in Table 6. No attempts have been made to establish dose-response relationships in primates, but they are susceptible to aflatoxin hepatocarcinogenesis.

The carcinogenic effects of different purified aflatoxins have been compared in a limited number of studies. The results of a study by Butler et

al. (1969) in which 8–9 week old, male (M) and female (F) MRC rats were given aflatoxins B₁, B₂, or G₁ in drinking water for 10 or 20 weeks, are shown in Table 8. The earliest renal neoplasm was seen 54 weeks after the discontinuation of aflatoxin treatment. Renal tumours were detected only in males. The 2 rats treated with aflatoxin B₁ that developed renal tumours did not have hepatic carcinomas, but 5 of the 11 rats receiving aflatoxin G₁ developed both renal and hepatic carcinomas.

Wogan et al. (1971) studied the relationship between the chemical structures of aflatoxins and their hepatocarcinogenicity in male Fischer rats, and concluded that aflatoxin B₁ was apparently more carcinogenic than aflatoxin G₁ and that both were much more active than aflatoxin B₂. A total intraperitoneal dose of aflatoxin B₂ of 150 mg per rat given in 40 equal doses over 8 weeks induced hepatocellular carcinomas in 3/9 rats. A similar regimen, containing a total dose of aflatoxin B₁ of 1.3 mg, induced liver tumours in 9/9 animals. In other experiments to compare the carcinogenicity of aflatoxins G₁ and B₁ given by stomach tube to rats, aflatoxin G₁ in a total dose of 1.4 mg per animal (divided into 14 equal doses over 2.5 weeks) induced hepatocellular carcinoma in 3/5 rats within 68 weeks. Hepatocellular carcinomas were observed in all 18 animals given a total dose of 2 mg of aflatoxin G₁ (divided in 40 equal doses over 8 weeks) and killed within 45–64 weeks. Six of these rats had pulmonary metastases.

Hepatocellular carcinomas were also found in 7/7 rats given aflatoxin B₁ in a total dose of 0.5 mg per animal (divided in 20 equal doses over 4 weeks) and sacrificed within 74 weeks, in 18/18 rats given 1 mg per animal (divided into 40 equal doses over 8 weeks) and killed within 42–58 weeks, and in 17/17 given 1.5 mg per animal (divided into 40 equal doses over 8 weeks) and killed within 42–46 weeks. With the 2 higher doses, 2 and 7 animals, respectively had pulmonary metastases. Renal adenocarcinomas were found in 4/26 rats given aflatoxin G₁.

Synthetic racemic aflatoxin M₁ induced hepatocarcinomas at 100 weeks in 1/29 male Fischer rats given 1 mg of the compound intragastrically in divided doses over a period of 8 weeks. The incidence of hepatocarcinomas in 9 rats given the same dose of natural aflatoxin B₁ was 100%, 1 year after treatment (Wogan & Paglialunga, 1974). Natural aflatoxin M₁ was less effective than natural aflatoxin B₁ in inducing tumours in trout, particularly in the males. Only 14% of male trout, fed natural aflatoxin M₁ for a year at a dietary level of 4 µg/kg, developed liver tumours compared with 68% of those receiving a similar feed level of aflatoxin B₁ (the experiment was terminated 8 months after the discontinuation of aflatoxin feeding). In females, the difference was less pronounced, liver tumours occurring in 48% of aflatoxin M₁-treated trout and 78% of aflatoxin B₁-treated trout (see Table 9) (Sinnhuber et al., 1974). Haemorrhages within the cancerous liver

Table 8. Carcinogenesis in rats due to ingestion of aflatoxin B₁, G₁ or B₂ in drinking water^a

Compound	Concentration (µg/ml)	Daily dose (µg)	Duration weeks	Total dose (mg)	No. and sex of animals treated	No. of animals with tumours		No. of animals with other neoplasms
						liver	kidney	
aflatoxin B ₁	1	20	20	2	15 M	B	2	4
					15 F	11	0	1
aflatoxin B ₁	1	20	10	1	10 M	3	0	2
aflatoxin G ₁	3	60	20	6	11 M	9	6	5
					15 F	12	0	3
aflatoxin G ₁	1	20	20	2	15 M	2	5	2
					15 F	1	0	7
aflatoxin G ₁	1	20	10	1	10 M	1	0	1
aflatoxin B ₂	1	20	10	1	10 M	0	0	2
					15 M	0	0	6
controls	0	0	0	0	15 M	0	0	0
					15 F	0	0	0

^a From: Butler et al. (1969).

Table 9. Aflatoxin M₁ liver carcinogenesis in rainbow trout (*Salmo gairdneri*)^a

Aflatoxin in the diet	Liver tumour incidence	
	males	females
M ₁ , 4 µg/kg	4/28 (14%)	13/27 (48%)
M ₁ , 16 µg/kg	22/27 (81%)	11/14 (79%)
M ₁ , 32 µg/kg	24/25 (96%)	13/14 (93%)
M ₁ , 64 µg/kg	21/24 (88%)	9/10 (90%)
B ₁ , 4 µg/kg	15/22 (68%)	18/23 (78%)

^a From: Sinnhuber et al. (1974).

resulting in death, which were observed in most females receiving dietary levels of aflatoxin M₁ of 16–64 µg/kg, were not observed in similarly treated males.

A probable effect of rat strain on aflatoxin carcinogenesis was seen in the high incidence of renal epithelial neoplasms reported in male Wistar strain rats fed diets containing aflatoxin B₁, for 147 days, and then maintained on a basal diet until death (Epstein et al., 1969). Renal tumours developed in 57% (8/14), 28% (5/18), and 23% (3/13) of male rats exposed to diets containing aflatoxin B₁ levels of 1.0, 0.5, and 0.25 mg/kg feed, respectively. The incidences of malignant hepatomas in corresponding groups were 86%, 72%, and 62% respectively. No renal tumours or malignant hepatomas were detected in a control group (24 animals). Approximately one-third of the aflatoxin-exposed rats with renal tumours did not have hepatomas. The first malignant hepatoma and the first renal tumour were detected 463 and 468 days after initiation of the experiment, respectively; both these tumours occurred in the group with the highest exposure. Approximately half of the renal tumours were bilateral.

Early hepatic lesions possibly related to carcinogenesis. Aflatoxin B₁ given to rats in repeated doses of 15–25 µg/day, for 3.5 weeks, making a total dose of 375 µg, elicited increased DNA synthesis and mitosis in clusters of liver cells that could be distinguished from the surrounding cells by their histological appearance and biochemical activity (Newberne & Wogan, 1968a; Rogers & Newberne, 1969). Development of the abnormal foci was more prominent in rats fed a high-fat, lipotrope-deficient diet, which enhances aflatoxin carcinogenesis (section 3.4.2.7), than in rats fed a nutritionally adequate diet. The abnormal foci were already present in deficient rats at the end of carcinogen administration. Similar foci are found in the livers of rats exposed to other hepatic carcinogens and may be useful in studying pathogenesis or metabolic aspects of liver cancer, since they are thought to be the possible precursors of tumours. However, many of the foci disappear with time after treatment, and progression is not inevitable.

Other tumours. Carcinoma of the colon have occasionally been reported following aflatoxin exposure (Newberne & Butler, 1969). Increased incidence of tumours in the distal half of the colon was observed in vitamin A-deficient rats fed aflatoxin B₁ (section 3.4.2.7) (Newberne & Rogers, 1973; Newberne & Suphakarn, 1977). Tumours of the colon were also found in more than 20% (12/53) of F344 rats (NIH) exposed, either from conception (7/34), or from 6–7 weeks of age (5/19), to a diet containing an aflatoxin B₁ level of 2 mg/kg and an unspecified amount of vitamin A (Ward et al., 1975). The tumours developed in both males and females at 42–64 weeks of age and were primarily polyploid neoplasms in the ascending colon, although 2 rats had tumours in the descending colon. No colon tumours were found in 18 control rats.

Carcinomas of the glandular stomach were observed in 1/6 young rats given a diet of groundnut meal containing an aflatoxin level of 3–4 mg/kg for 3 weeks and in 1/16 animals given the same diet at the age of one year (Butler & Barnes, 1966). Two definite and one probable adenocarcinomas of the stomach had previously been observed in rats fed a diet prepared from the same batch of groundnut meal (Butler & Barnes, 1963).

Other extrahepatic tumours have occasionally been reported after oral aflatoxin exposure, e.g., tumours of the lacrimal glands (Dickens et al., 1966; Goodall & Butler, 1969; Butler et al., 1969), squamous cell carcinoma of the tongue (Ward et al., 1975) and oesophagus (Butler et al., 1969). Tumours at various sites have been induced in several species of test animals by intratracheal, subcutaneous, or intraperitoneal administration of aflatoxins. The experimental results obtained by intratracheal administration are of particular interest in connexion with reported effects in man associated with airborne aflatoxins (section 3.5.2). Squamous-cell carcinoma of the trachea developed within 37–62 weeks in 3/6 rats given a mixture of aflatoxins (containing B₁, B₂, G₁, and G₂) at 300 µg per animal intratracheally twice weekly, for 30 weeks. Four of these rats also developed hepatomas within 49–62 weeks (Dickens et al., 1966).

Prenatal and early postnatal exposure. In a study of these effects, 6 groups of 10 female, Wistar rats were each fed a diet containing 25% or 50% groundnut meal contaminated with aflatoxin B₁ at 10 mg/kg and aflatoxin B₂ at 0.2 mg/kg. Dams received this diet from day 10 of pregnancy to parturition (exposure of offspring *in utero*); from 1 day post partum to 10 days post partum (exposure via milk); or from day 10 of pregnancy to 10 days post partum (exposure *in utero* and via milk). Among 113 male and 95 female offspring observed for up to 36 months, 1 male exposed *in utero*, 1 female exposed via milk, and 2 females exposed *in utero* and via milk developed malignant liver tumours (Grice et al., 1973). No liver tumours were seen in control offspring (50 male and 50 female rats).

3.4.2.4 Teratogenicity

The teratogenicity of aflatoxins has been reviewed by Ong (1975). The effect of aflatoxin B₁ on embryos in hamsters was studied by Elis & Di Paolo (1967) who reported that a single intraperitoneal injection of aflatoxin B₁ at 4 mg/kg body weight, given on day 8 of pregnancy, resulted in a high proportion of malformed and dead or reabsorbed fetuses. Approximately 50% of the fetuses in aflatoxin-treated mothers and over 85% in control mothers were normal. A dose of 2 mg/kg did not have any effects. In studies by Di Paolo et al. (1967), no teratogenic effects were observed when 12 pregnant C₃H mice were given repeated daily intraperitoneal injections of aflatoxin B₁ at 4 mg/kg body weight. However, a high proportion of dead or reabsorbed fetuses was observed in aflatoxin-treated mice in comparison with controls.

3.4.2.5 Mutagenicity

Aflatoxin B₁ causes chromosomal aberrations (chromosomal fragments with occasional bridges, chromatid bridges, chromatid breakage) and DNA breakage in plant and animal cells (Ong, 1975). It has also been shown to cause gene mutations in bacterial test systems (Ames test), when activated by microsomal preparations from rat and human liver (Wong & Hsieh, 1976). However, no mutagenic effects were observed in mice exposed intraperitoneally to aflatoxin B₁ at 5 mg/kg body weight (Leonard et al., 1975).

3.4.2.6 Biochemical effects and mode of action

Individual aflatoxins (B₁, B₂, G₁, G₂ etc.) with slightly different chemical structures affect experimental animals (sections 3.4.2.1 and 3.4.2.3) and interact with *in vitro* test systems to different degrees (Wogan et al., 1971; Patterson, 1976). However, as aflatoxin B₁ is the commonest and the most potent, it has been studied extensively and the majority of reported biochemical effects are specifically related to this toxin. As discussed in section 3.3.3, the aflatoxin molecule appears to be metabolically activated before exerting its acute and chronic effects.

Interaction between these activated molecular species and the liver cell apparently occurs at several loci. In the nucleus, DNA-dependent RNA polymerase (EC 2.7.7.6)^a is inhibited (Pong & Wogan, 1970), the toxin binds covalently to DNA *in vitro* and *in vivo* (Clifford & Rees, 1967; Lijinsky et al., 1970; Garner, 1973, 1975; Swenson et al., 1974, 1977), DNA repair is stimulated (Seegers & Pitout, 1973; Stich & Laishes, 1975),

^a The numbers within parentheses following the names of enzymes are those assigned by the Enzyme Commission of the Joint IUPAC-IUB Commission on Biochemical Nomenclature.

and the aflatoxin is activated on the outer nuclear membrane to a form that inhibits RNA synthesis (Neal & Godoy, 1976).

The permeability of mitochondria increases (Bababunmi & Bassir, 1972; Doherty & Campbell, 1973) and electron transport is interrupted with a decline in respiration (Doherty & Campbell, 1972, 1973). Lysosomal membranes are also rendered permeable and unbound acid hydrolases leak out (Tung et al., 1970; Pokrovsky et al., 1972; Adekunle & Elegbe, 1974). Activation of lysosomal enzymes and their effects on cellular structures may be a component of the toxic mechanism of aflatoxins (Pokrovsky et al., 1977).

Aflatoxin is metabolized in the endoplasmic reticulum (section 3.3.3) and the unmetabolized toxin competes with steroid sex hormones for polysome-binding sites (Williams & Rabin, 1971). The reticulum degranulates (Theron, 1965; Theron et al., 1965; Butler, 1971, 1972) with the breakdown of polysome profiles (Villa-Trevino & Leaver, 1968; Pong & Wogan, 1969; Godoy et al., 1976) and the formation of helical polysome forms (Sarasin & Moule, 1976). RNA polymerase is inhibited (Pong & Wogan, 1970) and the toxin binds covalently to RNA (Swenson et al., 1977). Many metabolic functions are inhibited, including protein synthesis, enzyme induction (Wogan & Friedman, 1968; John & Miller, 1969; Kato et al., 1970) and the synthesis of blood clotting factors II and VII (Bassir & Bababunmi, 1969). Glucose metabolism via the 6-phosphate pathway (Brown & Abrams, 1965; Feuer et al., 1965; Shankaran et al., 1970), and the synthesis of fatty acids and phospholipids are also depressed (Clifford & Rees, 1969; Kato et al., 1969; Black et al., 1970; Donaldson et al., 1972; Lo & Black, 1972). Furthermore, feed-back control of cholesterol synthesis is lost (Horton et al., 1972), a change considered characteristic of the pre-cancerous state.

It has been shown that aflatoxins have immunosuppressive properties, probably related to their inhibitory effect on protein synthesis. Thus, at levels in poultry feed of 0.25–0.5 mg/kg, aflatoxins have been found to reduce resistance to infection by *Pasteurella multocida*, *Salmonella* spp., Marek's disease virus, coccidia, and *Candida albicans* (Brown & Abrams, 1965; Smith et al., 1969; Pier & Heddleston, 1970; Hamilton & Harris, 1971; Edds et al., 1973).

In the liver cytoplasm, there is a transient stimulation followed by a depression of glycogenolysis and the pentose shunt pathway of glucose metabolism (Shankaran et al., 1970). Aflatoxins also compete with further steroid binding sites in the cytoplasm, notably NADP-linked 17-hydroxy steroid dehydrogenase (EC 1.1.1.148) (Patterson & Roberts, 1971).

Thus, acute hepatocellular necrosis appears to result from the interaction of aflatoxins at a number of intercellular sites whereas the mutagenic

(section 3.4.2.5) and carcinogenic (section 3.4.2.3) properties of aflatoxins probably depend upon metabolic activation to a DNA alkylating agent presumably the 2,3-epoxide (section 3.3.3).

3.4.2.7 *Factors modifying the effects and dose-response relationships of aflatoxins*

Numerous reports have dealt with factors of various types that modify the carcinogenic and other toxic effects of aflatoxins in experimental animals. These include host factors, particularly the sex-linked and endocrine characteristics, and interactions with other environmental factors. The effects of nutrients deserve particular attention in view of nutritional deficiencies occurring in certain parts of the world, where aflatoxins exposure can be considerable. The more recently discovered effect of exposure to (artificial) sunlight might also be of interest in this respect.

The mechanisms by which hormones, nutrition, and other factors influence aflatoxin carcinogenesis are not known but are thought to include effects on DNA synthesis and cell division and differentiation, and/or effects on aflatoxin metabolism, and excretion. Animals with a severely restricted energy food intake do not grow and are less susceptible to the action of many carcinogens than normal animals. The retardation of growth induced by severe protein deficiency and also by hypophysectomy may explain reduced tumour incidence under these experimental conditions. Conversion of aflatoxin B₁ to a bacterial mutagen is different in microsomal liver preparations from rats fed a marginal lipotrope diet compared with those from normal rats. Excretion of mutagens in the urine is also different in lipotrope-deficient rats (Suit et al., 1977).

Changes in aflatoxin B₁ metabolism and reduced levels of hepatic macromolecule-bound aflatoxin B₁ adducts were reported in rats pretreated with phenobarbital (Garner, 1975; Swenson et al., 1977), and also in hypophysectomized animals (Swenson et al., 1977).

Sex-linked differences and endocrine status. Several studies indicate that in comparison with males, female rats are more resistant to both acute toxic and carcinogenic effects of aflatoxins. Thus, with a single administration of aflatoxin B₁ by gavage, the LD₅₀ was estimated to be 7.2 mg/kg body weight (fiducial limits 5.36–8.23) in male rats and 17.9 mg/kg body weight (fiducial limits 14.4–22.5) in female rats (Butler, 1964). The sex-dependent influence of vitamin A deficiency on the acute toxicity of aflatoxins is discussed later in this section.

In another study (Newberne & Wogan, 1968a), Fischer rats of both sexes were kept on diets containing aflatoxin B₁ levels of 0.015, 0.3, or 1.0 mg/kg and killed successively for histological examination. The early hepatic

lesions, considered as precancerous, appeared with almost the same rate of incidence and at approximately the same time in both sexes; however, there was a considerably longer period between the appearance of the precancerous lesions and progression to liver carcinomas in females than in males. At a dietary level of aflatoxin B₁ of 1 mg/kg, males developed carcinomas after 35 weeks of exposure while tumours in females were observed only after 64 weeks. A similar, if much less pronounced, sex-linked difference was observed at the 2 lower aflatoxin levels. Calculations of the approximate total intake of aflatoxin B₁ before the appearance of tumours (based on average intake of food containing a known quantity of aflatoxin) and the time over which these total amounts were consumed are given in Table 10. In a study by Ward et al. (1975), male rats (F344, NIH) kept on a diet containing aflatoxin B₁ at 2 mg/kg, died with malignant haemorrhagic liver tumours significantly earlier than females. Kidney tumours observed in male but not female rats exposed to aflatoxins (Butler et al., 1969) have already been discussed in section 3.4.2.3.

Newberne & Williams (1969) reported that fewer male rats (Charles River CD) fed for more than a year on a diet containing aflatoxin B₁ at 0.2 mg/kg and diethylstilboestrol at 4 mg/kg developed liver tumours (8/40) than those fed the same aflatoxin diet without the estrogen (25/35).

In a study on the influence of hypophysectomy on aflatoxin carcinogenesis, male albino (MRC) rats were fed a diet containing an aflatoxin B₁ level of 4 mg/kg. All of 14 control rats developed liver tumours in 49 weeks whereas none of 14 hypophysectomized rats developed liver tumours in the same period. However, tumours of extrahepatic tissues (4/14 carcinomas of retro-orbital lacrimal glands—see also section 3.4.2.3) were observed in the hypophysectomized aflatoxin-treated rats (Goodall & Butler, 1969).

Nutritional factors (food components). The influence of nutritional factors on the effects and dose-response relationships of aflatoxins has been reviewed by Wogan (1973, 1977), Newberne (1974, 1976), Newberne & Rogers (1976), and Newberne & Gross (1977).

(a) *Dietary protein and lipotropic agents.* Rhesus monkeys were given daily doses of 100 µg aflatoxin per animal by stomach tube. Two animals,

Table 10. Sex-linked difference in the carcinogenicity (liver cell carcinoma) of dietary aflatoxin B₁^a

Dietary level of aflatoxin B ₁	Approximate total intake of aflatoxin B ₁ before the appearance of tumours		Average time (days over which the total amount was consumed)	
	males	females	males	females
1 mg/kg	2.9 mg/rat	5.9 mg/rat	245 days	448 days
0.015 mg/kg	95 µg/rat	115 µg/rat	476 days	560 days

^a Data from Newberne & Wogan (1968a).

fed a severely protein-deficient ration (1% casein) for 8 weeks before and during aflatoxin administration, developed fatty liver and biliary proliferation and fibrosis and died with gastrointestinal haemorrhage within 30 days of aflatoxin treatment. Two animals fed a control ration (16% casein) survived in apparent good health up to the termination of the experiment (35 days of aflatoxin treatment) (Madhavan et al., 1965a).

In a study on weanling male rats given 50 µg aflatoxin per animal per day for 20 days, 2/6 animals, fed a diet containing 4% casein, died on days 18 and 19 of the experiment. Extensive liver damage was found in these rats and in others in the 4%^a casein group within 20 days of aflatoxin treatment. All 12 rats fed a diet containing 20% casein survived similar aflatoxin dosing with only mild changes in the liver (Madhavan & Gopalan, 1965). In another study by Madhavan & Gopalan (1968) 2 groups of 12 male rats were fed the 2 diets (5%^a or 20% casein) for 2 years from weaning and were given, from the beginning of the experiment, 232 daily doses of 5 µg aflatoxin per animal or 225 daily doses of 10 µg aflatoxin per animal. All 12 animals fed the 20% casein diet survived the period of aflatoxin dosing and 50% developed hepatomas; lung metastases were observed in 2 of these rats. Five of the 12 animals fed on the 5% casein diet died during the period of aflatoxin dosing. No hepatomas but one renal cell carcinoma were found in the remaining 7 rats. Both diets used in these experiments were supplemented only with 0.01% choline.

In experiments by Newberne & Wogan (1968b), rats fed a diet containing 9% protein developed a higher incidence of liver tumours (11/15) in a shorter period of time (8 months) than rats fed a diet containing 22% protein (incidence 7/14 after 10 months). Both groups of rats were given a total dose of 375 µg of aflatoxin B₁ per animal by gastric intubation, over 3 weeks, at the beginning of the experiment.

In studies examining lipotropic effects on aflatoxin activity, a diet marginal in methionine and choline, deficient in folate, and high in fat protected male rats against the acute toxicity of a single dose of aflatoxin B₁; however, susceptibility to the toxic effects of repeated doses of aflatoxin B₁ increased, and the carcinogenicity of aflatoxin B₁ was enhanced. The experimental diet (Rogers & Newberne, 1971; Rogers, 1975) contained peanut meal (12%, alcohol extracted), gelatine (6%), casein (3%, vitamin free) and fibrin (1%) as sources of protein, with a supplement of L-cystine (0.5%). This diet is marginally but not severely deficient in threonine, tryptophan, and arginine as well as in methionine. The diet contained

^a According to Madhavan & Gopalan (1968) the composition of the diets used in both studies was the same. However, their 1965 paper gives 4% whereas that of 1968 gives 5% as the level of casein in the diet.

choline chloride (0.2%), and was high in fat (beef fat 30%; corn oil 2%). The control, nutritionally complete diet contained casein (22%, vitamin free) as the protein source, 15% or 16% oil (corn or mixed vegetable oil) and 0.3% choline chloride. Both diets were adequate in other essential nutrients.

As shown in Table 11, the marginal lipotrope diet fed for 2 weeks protected rats against acute aflatoxin B₁ toxicity. The rats that died had haemorrhagic necrosis of the liver with various degrees of bile duct proliferation and, occasionally, haemorrhagic necrosis in the adrenals and kidneys. The surviving rats (killed 2 weeks after aflatoxin administration) had focal necrosis of hepatocytes, bile duct proliferation, and an increase in the size of periportal hepatocytes. Approximately 20% of the rats fed the marginal lipotrope diet and given a single dose of aflatoxin B₁ had focal areas of abnormal hepatocytes which showed an increased uptake of ³H thymidine (section 3.4.2.3) (Rogers & Newberne, 1971).

Table 11. Effect of a marginal lipotrope diet on the acute toxicity of aflatoxin B₁ in rats^a

Aflatoxin B ₁ (mg/kg)	Route of administration	Diet	No. of rats	Mortality at 2 weeks (%)
<i>Sprague-Dawley</i> (males)				
7	Intragastric	control	5	60
		marginal lipotrope	5	0
9	Intragastric	control	5	80
		marginal lipotrope	10	0
7	Intraperitoneal	control	5	100
		marginal lipotrope	5	0
<i>Fischer</i> (males)				
7	Intragastric	control	10	100
		marginal lipotrope	10	0

^a From: Rogers & Newberne (1971).

Although resistant to the toxicity of a single dose of aflatoxin B₁, rats fed the marginal lipotrope diet for 2 weeks before and also during repeated aflatoxin exposure (136 males) were highly sensitive to repeated daily doses of aflatoxin B₁ at 25 µg per animal. One half of these rats died, most of them after having received 8 or 9 doses of aflatoxin B₁ (200 or 225 µg total). Various degrees of necrosis were observed in the livers with extensive proliferation of bile duct cells. The mortality in rats fed the control diet (66 males) was only 4% during the administration of the total dose of 350 µg of aflatoxin B₁ (Rogers & Newberne, 1971).

Enhanced aflatoxin B₁ carcinogenicity in rats fed marginal lipotrope diets, observed repeatedly in several experiments reviewed by Newberne & Gross (1977), is demonstrated in Fig. 4 (Rogers, 1975). Cumulative probability of death from a tumour was calculated here by the method described by Saffiotti et al. (1972) i.e., from the number of animals at risk and the number

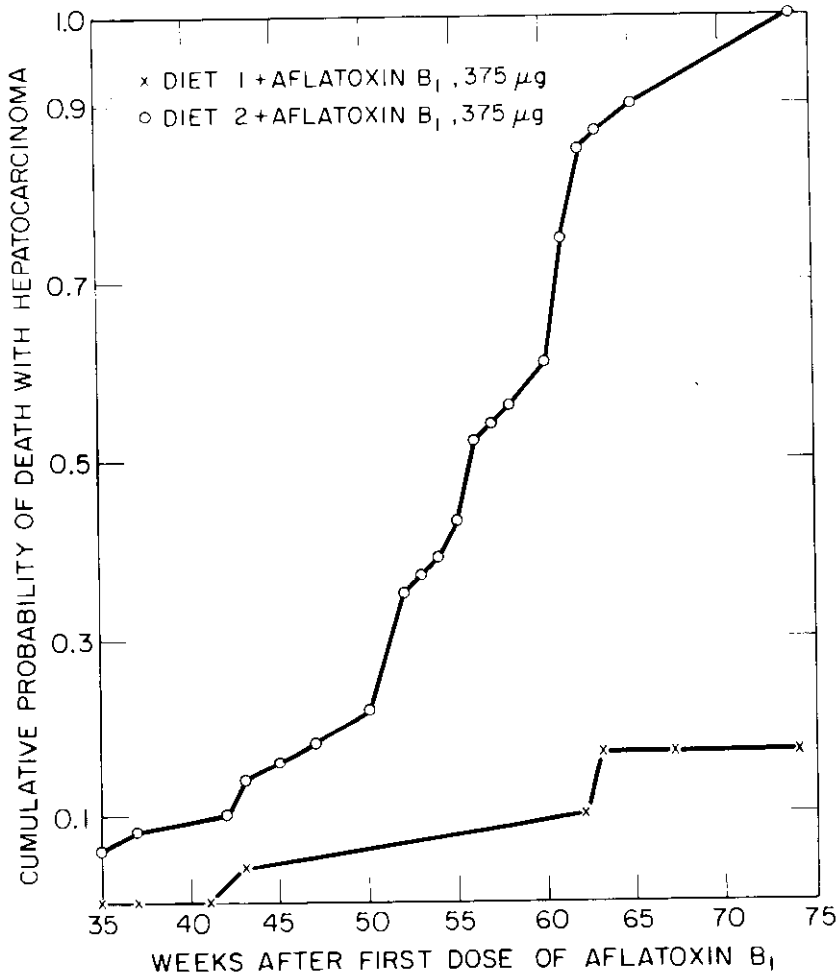


Fig. 4. Cumulative probability of death from hepatocarcinoma in rats fed a control, adequate diet (diet 1), or a marginal lipotrope, high fat diet (diet 2) and treated with aflatoxin B₁ (From: Rogers, 1975).

of deaths from tumours each week. A total dose of aflatoxin B₁ of 375 µg, divided in 25 intragastric doses of 15 µg/animal per day over 7 weeks, was given to male Fischer rats. Hepatocarcinomas developed in 87% of 52 animals fed the marginal lipotrope diet and in only 11% of 27 rats fed the nutritionally complete diet ($P < 0.001$). Twenty-seven percent of tumours in rats fed the marginal diet metastasized to other abdominal organs or the lung; no metastases were detected in rats fed the control diet (Rogers, 1975). The groundnut meal fed in these experiments did not contain detectable aflatoxins.

Further studies were carried out to determine how far the high fat content of the marginal lipotrope diet contributed to the enhancement of carcinogenesis. Male Fischer rats were fed the marginal lipotrope diet, the nutritionally complete control diet, or the control diet with substitution of the fat from the marginal lipotrope diet (30% beef fat, 2% corn oil) for the fat in the control diet (15% mixed vegetable oils). Each rat was given a total of 375 μg of aflatoxin B_1 intragastrically, in divided doses over 7 weeks and kept until moribund or dead, or until 90 weeks after treatment, and then necropsied. Hepatocarcinoma incidence was based on the number of rats that survived until the first death with hepatocarcinoma, i.e., 27-34 rats per group. Incidences were found of 39% in rats fed the marginal lipotrope diet, 15% in control rats, and zero in rats fed the control diet with beef fat (30%) and corn oil (2%) substituted for vegetable oil (15%) (Rogers et al., unpublished data). Thus the high fat content of the deficient diet inhibited, rather than contributed to the enhancement of aflatoxin carcinogenesis.

An enhancing effect on aflatoxin carcinogenicity was observed in the experiments in which male rats (Charles River CD Sprague-Dawley) were fed a low lipotrope diet containing 20% isolated soybean protein and supplemented with 0.1% DL-methionine and 0.1% choline chloride. Aflatoxin B_1 was given intragastrically during the early weeks of the experiment in a total dose of 240 μg /animal (divided in 24 daily doses of 10 μg , 5 days a week). Liver cell carcinomas were observed 5/17 animals fed this diet. No tumours were found in rats given the same dose of aflatoxin B_1 and fed the same basal diet supplemented with 0.6% DL-methionine, 0.6% choline chloride, and vitamin B_{12} (50 μg /kg diet) (Newberne et al., 1968).

It should be noted that a more severe lipotrope deficiency may decrease rather than increase the incidence of liver carcinoma in aflatoxin-treated rats. A decrease in liver carcinoma was observed in aflatoxin-treated, male, Sprague-Dawley rats with severe lipotrope deficiency particularly if penicillin, at a level of 0.1% were added to the diet. The expected penicillin-induced inhibition of cirrhosis development was not observed and the interactions between penicillin and aflatoxins have not been elucidated. Both diets used in these experiments, the control, adequate, and the highly lipotrope-deficient contained alcohol-extracted groundnut meal (25%) and casein (6%) as protein source. No cystine or methionine was added, and choline and vitamin B_{12} were added to the control diet at levels of 0.3% and 50 μg /kg, respectively. When rats were killed 12 months after receiving a total dose of 375 μg aflatoxin B_1 per animal (divided into 15 daily intragastric doses of 25 μg per animal), hepatomas were found in 64% (9/14) control animals and in 41% (7/17) lipotrope-deficient animals. In rats fed diets containing penicillin, hepatomas were found in 70% (14/20) of the

controls and in only 17% (3/18) of the lipotrope-deficient animals (Newberne & Rogers, 1971).

The different effects of marginal and severe lipotrope deficiencies on aflatoxin carcinogenesis are of interest in connexion with apparent discrepancies between different studies on aflatoxin carcinogenesis in rats fed protein-deficient diets containing different levels of lipotropic agents discussed earlier.

Vitamin B₁₂ is a weakly lipotropic factor that has recently been found to increase tumour incidence in aflatoxin-treated rats. In a study by Temcharoen et al. (1978), male Fischer rats were fed diets containing 20% or 5% casein with or without vitamin B₁₂ (50 µg/kg diet) for 33 weeks. The composition of the diets, with the exception of the contents of casein (and dextrin) and vitamin B₁₂, was similar to that used in previous studies by Wogan & Newberne (1967) and Newberne & Wogan (1968a). Choline chloride was added at the level 0.036%. The casein content was similar to that in diets used in the study by Madhavan & Gopalan (1968) mentioned earlier. A mixture of crystalline aflatoxins (containing aflatoxins B₁ and G₁ approximately in the proportion of 1:1 and about 5% of aflatoxins B₂ and G₂) was added to the diets at the level of 1 mg/kg. As shown in Table 12, vitamin B₁₂ supplementation increased liver tumour incidence in rats fed a diet containing aflatoxin and 20% casein. Severe protein deficiency affected the growth of the animals, the body weight of rats fed 5% casein being reduced to about one third of the controls at the termination of the experiment. Liver cirrhosis was observed only in the aflatoxin-treated, protein-deficient rats. As suggested by Temcharoen et al. (1978), a high incidence of hyperplastic nodules and cholangiofibrosis in the protein-depleted, aflatoxin-treated animals may indicate that the carcinogenic process was retarded but not entirely absent.

(b) *Vitamin A.* In a study by Reddy et al. (1973), male and female albino rats fed a vitamin A-deficient diet for 9 weeks after weaning or fed the same diet with a daily oral supplement of 30 µg (100 IU) of vitamin A/rat were given a preparation of crystalline aflatoxins containing aflatoxins B₁ (44%), G₁ (44%), and B₂ and G₂ (2%) in a single intraperitoneal dose of 3.5 mg/kg body weight. High mortality was observed in vitamin A-deficient males (Table 13). The vitamin A-deficient females and all the vitamin A-supplemented animals did not show any adverse reactions 40 h after aflatoxin injection. Histologically, severe liver damage was observed in vitamin A-deficient, aflatoxin-treated male rats, in contrast to minimal liver damage in female rats and male rats given vitamin A.

In a study on rats fed diets containing aflatoxin B₁ in the range of 15–100 µg/kg and vitamin A in deficient, adequate, or excessive levels over a 2-year period, the vitamin A-deficient animals developed a similar incidence of liver

Table 12. The effects of dietary protein and vitamin B₁₂ on aflatoxin-induced liver changes^a

Experimental diet (for details see text)	No. of animals at the beginning/ termination of the experiment	Average body ^b weight (g) at the termination of the experiment	No. of animals with			
			cholangio- fibrosis	cirrhosis	hyperplastic nodules	hepatoma
1. 5% casein	12/6	85	0	0	0	0
2. 5% casein + B ₁₂	12/12	91	0	0	0	0
3. 5% casein + aflatoxins	25/23	125	9	21	17	3
4. 5% casein + B ₁₂ + aflatoxins	25/24	129	8	12	15	1
5. 20% casein	12/9	328	0	0	0	0
6. 20% casein + B ₁₂	12/10	369	0	0	0	0
7. 20% casein + aflatoxins	25/24	409	0	0	0	1
8. 20% casein + B ₁₂ + aflatoxins	25/25	375	0	0	4	6

^a Modified from: Temcharoen et al. (1978).^b Calculated from data of the authors on average liver weight and liver weight/100 g body weight.

Table 13. The effect of vitamin A status on the acute toxicity of aflatoxins in male and female rats^a

Sex	Vitamin A daily supplement (IU/rat per day)	No. of animals	Mortality 40 h after aflatoxin injection	Vitamin A liver content (IU/whole liver) means \pm SEM ^c
Male	0	6	100%	36.5 \pm 6.16 ^b
	100 ^d	6	0%	2128.1 \pm 153.56
Female	0	6	0%	18.6 \pm 3.42
	100 ^d	6	0%	2299.1 \pm 111.49

^a From: Reddy et al. (1973).

^b Vitamin A values for 5 animals only.

^c SEM = standard error of the mean.

^d 30 μ g.

tumours to the other 2 groups but had an increased incidence of colon carcinomas (Newberne & Rogers, 1973). Thus, in a group of 50 male rats (Charles River CD Sprague-Dawley) exposed to a dietary level of aflatoxin B₁ of 100 μ g/kg and 5 μ g vitamin A (retinyl palmitate) per animal per day, colon tumours and liver tumours were observed in 6 and 11 rats, respectively, whereas with daily intakes of 50 or 500 μ g retinyl palmitate per rat, no colon cancers were observed and the incidence of liver tumours was 24/50 or 19/50, respectively. The results of a further study (Newberne & Suphakarn, 1977) are shown in Table 14. Charles River CD Sprague-Dawley male (M) and female (F) rats were fed a diet containing an aflatoxin B₁ level of 1 mg/kg (AFB₁) and 3 different dietary levels of vitamin A (retinyl acetate). Again, there was an increased incidence of colon carcinomas in vitamin A-deficient rats. Excessive vitamin A did not give protection against aflatoxin carcinogenesis in either the liver or the colon.

Table 14. Vitamin A status, aflatoxin B₁, and liver and colon tumours in rats

Dietary retinyl acetate (mg/kg)	AFB ₁	No. rats at risk	Sex	Tumour incidence (%)		
				liver	colon	both
control						
3.0	0	24	M	0.0	0.0	0.0
3.0	0	26	F	0.0	0.0	0.0
3.0	+	24	M	87.5	4.1	4.1
3.0	+	24	F	79.1	8.3	8.3
low						
0.3	0	10	M	0.0	0.0	0.0
0.3	0	12	F	0.0	0.0	0.0
0.3	+	66	M	89.4	28.8	25.7
0.3	+	42	F	76.2	28.6	11.9
high						
30.0	0	23	M	0.0	0.0	0.0
30.0	0	20	F	0.0	0.0	0.0
30.0	+	26	M	92.3	7.7	7.7
30.0	+	31	F	83.9	9.7	6.4

From: Newberne & Suphakarn (1977).

(c) *Selenium*. A single oral dose of aflatoxin B₁ given to rats at 7 mg/kg bodyweight was less toxic in animals fed a high selenium (selenite) diet containing selenium at 1 mg/kg (2-week mortality 7/28) than in animals fed diets adequate or marginal in selenium, containing selenium (selenite) at 0.1 or 0.03 mg/kg feed, respectively (2-week mortalities 20/20 and 28/29). However, a further increase in selenium intake (5 mg/kg feed) reaching toxic levels predisposed the liver to aflatoxin injury and together with aflatoxin exposure resulted in kidney lesions (tubular necrosis at the cortico-medullary junction) (Newberne & Conner, 1974). The incidence of liver tumours in Sprague-Dawley rats given a total of 500 µg of aflatoxin B₁ intragastrically, over a 4-week period, was not influenced by dietary selenium (as selenite) contents ranging from 0.03 to 5.0 mg/kg (Grant et al., 1977).

(d) *Cyclopropenoid fatty acids (CPFA)*. Cyclopropenoid fatty acids (CPFA) which occur for example in cottonseed oil, enhanced tumour induction in trout by both aflatoxin B₁ and aflatoxin M₁ (Sinnhuber et al., 1968, 1974). Young trout were fed a purified diet which contained an aflatoxin B₁ concentration of 4 µg/kg with or without the addition of CPFA at 220 mg/kg diet. Hepatomas were found in 27/30 fish fed CPFA and necropsied after 6 months; at 9 months, 20/20 bore hepatomas. Corresponding incidences of hepatomas in fish that did not receive CPFA were 0/30 and 4/20, respectively (Sinnhuber et al., 1968). In later experiments, fish were fed aflatoxin M₁ at the rate of 4 µg/kg diet, with or without the addition of CPFA at 100 mg/kg. The incidences of hepatomas in CPFA-fed fish were 6/40 at 4 months and 42/63 at 12 months. Corresponding incidences in fish not fed CPFA were 2/40 and 6/40 respectively (Sinnhuber et al., 1974).

On the other hand, the enhancing effect of CPFA on aflatoxin hepatocarcinogenesis was not clearly evident in several studies on rats (Friedman & Mohr, 1968; Lee et al., 1969a; Nixon et al., 1974). No significant increase in liver tumours was observed in Wistar male (M) and female (F) rats when sources of CPFA such as food grade cottonseed oil (CSO) or *Sterculia foetida* oil (SFO) were added at levels of 10% and 0.04%, respectively, to diets containing aflatoxin B₁ at concentrations of 20 or 100 µg/kg. Feeding these diets for different periods (generally exceeding 500 days) at the aflatoxin level of 20 µg/kg resulted in hepatomas in 4/36 rats (M: 2/17; F: 2/19) with CSO exposure, in 1/37 rats (F: 1/19) with SFO exposure, and in 0/38 rats without CSO or SFO in the diet. With an aflatoxin B₁ exposure level of 100 µg/kg diet, the incidences of hepatomas in the CSO group, SFO group, and the group without CSO or SFO were 15/36 (M: 12/17; F: 3/19), 17/37 (M: 10/17; F: 7/18), and 15/35 (M: 7/17; F: 8/18), respectively. In Fischer rats, CSO was tested only in combination with the lower concentration of aflatoxin; with an aflatoxin B₁

level of 20 $\mu\text{g}/\text{kg}$ diet, the hepatoma incidence was 6/31 (M: 4/15; F: 2/16) with CSO, and 6/28 (M: 5/13; F: 1/15) without CSO exposure (Nixon et al., 1974).

Other chemicals. Sodium phenobarbital given to rats in the drinking water (1 g/litre) for 9 weeks, together with a diet contaminated with aflatoxin B₁ (at the level of approximately 5 mg/kg) resulted in a lower incidence (11/20) and delayed appearance of liver tumours within the following 2 years compared with the incidence in rats fed aflatoxin alone (17/20) (McLean & Marshall, 1971). The effects of phenobarbital were confirmed in experiments by Swenson et al. (1977) in which 2 groups of 18 male Fischer rats were each given a diet containing aflatoxin B₁ at a level of 0.3 mg/kg and drinking water with or without sodium phenobarbital (1 g/litre) for a period of 15 months. Examination by laparotomy at the end of aflatoxin exposure revealed liver tumours in 11 aflatoxin-treated controls (61%) and in only 2 (15%) rats exposed to aflatoxin with phenobarbital. When the experiment was terminated 5 months later, hepatocellular carcinomas were detected in 18 (100%) aflatoxin-exposed rats and in 12 (67%) rats given aflatoxin and phenobarbital. On the other hand, no effect on aflatoxin hepatocarcinogenesis was observed in rats fed a similar aflatoxin diet with the addition of benz(a)anthracene (70 mg/kg) or ascorbic acid (25 g/kg) (Swenson et al., 1977).

Viral infection. Liver tumours were observed in 2/7 marmosets fed aflatoxin B₁ at a concentration of 2 mg/kg feed and injected with hepatitis-type candidate virus (G. Barker strain) during aflatoxin exposure. The animals survived 3–94 weeks of treatment. Tumours were found in 3/9 marmosets given the aflatoxin diet only (Lin et al., 1974). Exposure to both agents produced more severe effects on the liver (cirrhosis) than exposure to aflatoxin B₁ alone.

Exposure to artificial sunlight. The effects of exposure to artificial sunlight on acute aflatoxin toxicity (Newberne et al., 1974) and carcinogenicity (Joseph-Bravo et al., 1976) were studied recently, using a long-arc xenon source and filter combination, which had a spectral distribution in the ultraviolet and visible ranges closely approximating the 6000 K colour temperature of natural light. The rats, located 1.5 metres from the source, were kept at an illumination level of about 29 600 lx corresponding to an irradiance level of approximately 160 W/m² over a 2-h period. In groups consisting of 40 Sprague-Dawley male rats each, 18 control rats died within 2 weeks of a single intragastric dose of aflatoxin B₁ at 7 mg/kg body weight whereas 23 of the rats exposed to artificial sunlight for 2 h after aflatoxin administration died. When an excessive dose of riboflavin was given intragastrically 30 min before aflatoxin, the corresponding 2-week mortalities in animals unexposed and exposed to artificial sunlight were 20/40 and 30/40,

respectively (Newberne et al., 1974). In the second study, a total dose of 375 µg of aflatoxin B₁ was given to male Sprague-Dawley Charles River CD rats, in the form of 15 doses of 25 µg per rat, administered intragastrically over a 3-week period. Thirty min after each aflatoxin administration, half of the animals were exposed for 2 h to artificial sunlight as described earlier. When the animals were killed 53 weeks after the last aflatoxin dose, benign or malignant liver tumours were found in all 11 non-irradiated animals and in only 5/12 of the irradiated group (Joseph-Bravo et al., 1976).

3.5 Effects in Man—Epidemiological and Clinical Studies

3.5.1 General population studies

3.5.1.1 *Liver carcinogenesis*

The data on aflatoxins and human cancer available before October 1975 were reviewed by IARC (1976) and several other reviews have been published more recently (Linsell & Peers, 1977; Shank, 1977; Van Rensburg, 1977). A positive association between aflatoxin ingestion and liver cancer in man has been found in population studies in which estimates of aflatoxin intake and the incidence of primary liver cancer were made concurrently.

In a study in different parts of Uganda, it was found that increased frequencies of detectable aflatoxin contamination of food samples (range: 10.8%–43%) were associated with increased incidence of primary liver cancer (range: 1.4–15.0 cases per 100 000 total population per year) (Alpert et al., 1971). Four hundred and eighty samples of foods were analysed from 8 areas in Uganda; the total intake was not calculated. Within Swaziland, Keen & Martin (1971a, b) showed regional differences in liver cancer frequencies consistent with regional differences in the frequency of aflatoxin contamination of groundnuts. From the results of a questionnaire study, they also suggested that tribal differences in the preparation of groundnuts for food, and in eating habits, resulting in higher aflatoxin exposure, could explain the apparently higher liver cancer rate in the Shangaans living in Swaziland compared with the Swazis.^a

In the studies conducted by Shank et al. (1972a, b) in Thailand, Peers & Linsell (1973) in Kenya, Van Rensburg et al. (1974) in Mozambique, and

^a A positive association of liver cancer incidence variations with the availability of aflatoxin-contaminated staple foods was also reported recently from the Philippines (Bulatao-Jayme et al., 1976).

Peers et al. (1976) in Swaziland, actual concentrations of aflatoxin in meals about to be eaten (food on the plate) were related to the incidence of primary liver cancer in the areas where the meal samples were collected. These studies are summarized in Table 15. A linear regression between the incidence of liver cancer and the logarithm to the base 10 of the estimated dietary intake of aflatoxin was found within the range of the aflatoxin exposure levels and the liver cancer incidence rates existing in the areas studied.^a Within Kenya and Swaziland, Peers & Linsell (1977) demonstrated a steeper rise in liver cancer incidence with increasing aflatoxin intake in men than in women (Fig. 5). A similar difference seems to exist also in the other areas studied (Shank, 1977).

Table 15. Summary of available data on aflatoxin ingestion levels and primary liver cancer incidence^a

Country	Area	Aflatoxin Estimated average daily intake ^b in adults—ng/kg body weight per day	Liver cancer	
			No. of cases registered	Incidence per 10 ⁵ of total population per year
Kenya	High altitude	3.5	4	1.2
Thailand	Songkhla	5.0	2	2.0
Swaziland	High veld	5.1	11	2.2
Kenya	Middle altitude	5.9	33	2.5
Swaziland	Mid veld	8.9	29	3.8
Kenya	Low altitude	10.0	49	4.0
Swaziland	Lebombo	15.4	4	4.3
Thailand	Ratburi	45.0	6	6.0
Swaziland	Low veld	43.1	42	9.2
Mozambique	Inhambane ^c	222.1	— ^d	13.0

^a From: Peers & Linsell (1977).

^b Excluding any aflatoxin present in native beers.

^c Revised incidence estimate taken from Van Rensburg (1977).

^d Number of cases not available, probably >100.

The possibility that hepatitis B virus infection may confound the relationship between aflatoxin ingestion and liver cancer incidence has been considered (Linsell & Peers, 1977). Hepatitis B infection is common in countries with a high incidence of primary liver cancer and evidence of prior exposure to hepatitis B virus is more common in individuals with liver cancer in these countries than in normal subjects (Vogel et al., 1970; Reys & Sequeira, 1974; Prince et al., 1975; Chainuvati et al., 1975). Nevertheless, the present evidence favours aflatoxin as a possible major disease

^a When Stoloff & Friedman (1976) compared published reports on the incidence of cancer in rural and urban areas of southeastern USA, and in southeast states compared with other areas in the USA, they found lower incidences of liver cancer in the rural areas and in the southeast states, respectively, even though they expected that long-term exposure to aflatoxins would be higher in these areas.

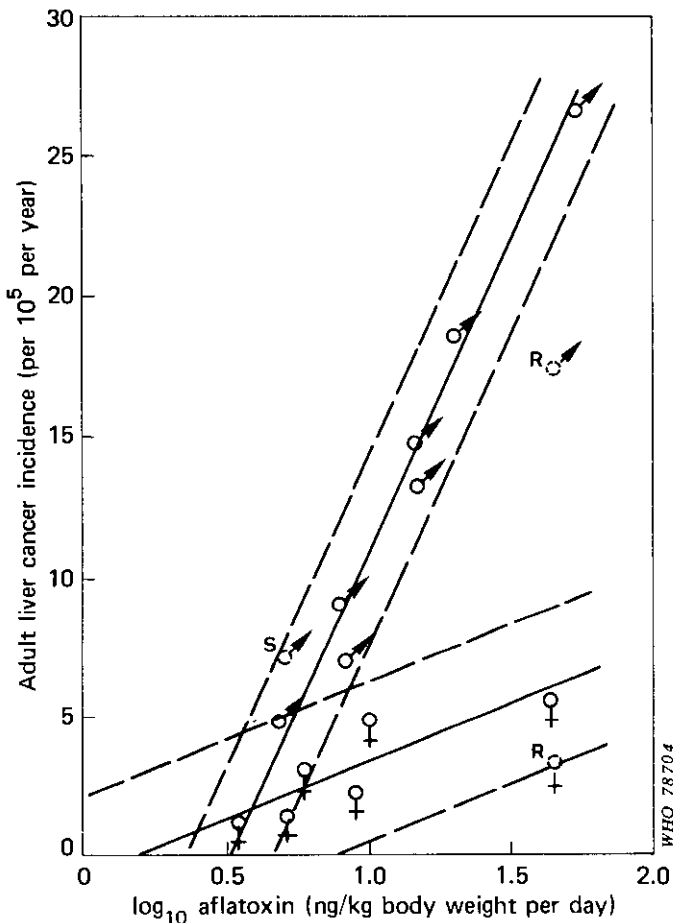


Fig. 5. Correlation of liver cancer with aflatoxin ingestion: Combined data of the Muranga (Kenya) and Swaziland studies (From: Peers & Linsell, 1977).

determinant in primary liver cancer but hepatitis B virus may well be a cofactor in the etiology (Peers & Linsell, 1977).

Two studies discussed by the Task Group reported the presence of aflatoxins in the tissues of cancer patients.

Pang et al. unpublished data^a reported the results of a 2-year study in Indonesia in which the aflatoxins contents were determined in liver tissue biopsy specimens from 71 patients with primary cancer of the liver (histologically verified hepatocellular carcinoma in 62 patients and

^a PANG, R. T. L., HUSAINI, S. N., & KARYADI, D. (1974) Aflatoxin and primary hepatic cancer in Indonesia. Paper presented at the *Vth World Congress of Gastroenterology*, 13–19 October 1974, Mexico.

cholangiohepatocellular cancer in 7 patients). Dietary history indicated consumption of contaminated food, many patients having eaten groundnuts, almost daily, since childhood. Great variation was found in the aflatoxin contents of food samples (type of food and number of analyses not given) with aflatoxin B₁ levels ranging from 17 to 1190 µg/kg, and aflatoxin G₁ levels ranging from 5 to 690 µg/kg. In extracts of liver tissue biopsy samples obtained soon after the first visit to the hospital, spots corresponding to aflatoxins were chromatographically detected for 41 patients (57.7%) but not in extracts of liver tissues from 15 patients without liver cancer serving as controls. The authors also reported the more frequent presence of aflatoxins in the urine of liver cancer patients compared with the controls. Aflatoxin B₁ at an estimated level of 520 µg/kg fresh weight was detected in the liver of a resident of the USA, suffering from carcinoma of the liver and the rectum (Phillips et al., 1976). No attempt was made to associate the aflatoxin with cancer in this case.

3.5.1.2 *Other effects reported to be associated with aflatoxins*

Reye's syndrome. The possibility that some cases of Reye's syndrome (encephalopathy with fatty degeneration of the viscera) (Reye et al., 1963), might be due to aflatoxin ingestion was first suggested by Becroft (1966), who subsequently reported the presence of aflatoxins B₁ and G₁ in the livers of 2 children who had died from Reye's syndrome in New Zealand (Becroft & Webster, 1972). Following this report, Dvoráčková et al. (1974) in Czechoslovakia and Chaves-Caballo et al. (1976) in the USA detected aflatoxins in the livers of patients with Reye's syndrome. More recently, in the USA, Hogan et al. (1978) detected aflatoxin B₁ in the blood serum of 2 patients with Reye's syndrome.

A dietary source of aflatoxin was not identified in any of these case reports. Aflatoxins were not found in the livers of 5 further subjects with Reye's syndrome in the USA (Shank, 1976).

Clustering of Reye's syndrome cases, observed in north-east Thailand, occurred mainly in the villages but not within families and was geographically and seasonally related to high levels of aflatoxin contamination of market food samples (Olson et al., 1971; Bourgeois, 1975). In 2 cases, the presence of heavy aflatoxin contamination in food, eaten 2 or 3 days before death, was demonstrated (Bourgeois et al., 1971; Bourgeois, 1975).

Shank et al. (1971a) reported trace amounts of aflatoxin B₁ in tissues, body fluids, gastrointestinal contents or stools of 22/23 Thai patients who had died from Reye's syndrome and 11/15 who had died from other causes. More than trace amounts of both aflatoxin B₁ and B₂ were found in at least 2 liver specimens (47 and 93 µg aflatoxin B₁/kg, respectively) from 2 of the 23 patients who had died from Reye's syndrome but not in any of the

specimens from patients dying from other causes. These 2 series of patients are not entirely comparable, however, because of differences in the frequencies with which the various tissues and body contents were examined.

Twenty-seven cases of Reye's syndrome collected over a 5-year period (age range: 3 days to 8 years) were investigated by Dvořáčková et al. (1977). Aflatoxin B₁ was found in the liver in all cases and aflatoxin M₁ in 4 cases. No aflatoxin was found in the livers of 25 children, who had died from other causes. Contamination of milk powder with aflatoxin B₁ in the home was suggested to be the source of exposure in 5 of the cases. No aflatoxin M₁ was found in this milk.

With the exception of the previously mentioned studies from Thailand (Shank et al., 1971a; Shank, 1977), cases of Reye's syndrome have not been reported from other countries in which food is commonly contaminated with more than traces of aflatoxins. As Reye et al. (1963) initially suggested, encephalopathy with fatty infiltration of the viscera is probably a syndrome of varied etiology.

Other liver diseases. Instances of human liver disease, other than Reye's syndrome and cancer, that had apparently followed presumed dietary exposure to aflatoxins are summarized in Table 16. This table also gives the levels of aflatoxins found in the suspected foods. In these reports, the data are insufficient to establish a definite association or causal relationship. In the studies of Ling et al. (1967), however, there was a geographical and temporal association between the availability of mouldy food for consumption and the development of disease.

The recent outbreak of acute toxic hepatitis in India (Krishnamachari et al., 1975a,b; Tandon et al., 1977, 1978) is described in detail, because of the number of people affected and because of the evaluation of this outbreak by the Task Group (see section 3.6.2).

During the last 2 months of 1974 an outbreak of epidemic jaundice with a high mortality rate affected more than 150 villages in adjacent districts of 2 neighbouring states, Gujarat and Rajasthan, in north-west India. Three reports from 2 independent studies of this outbreak were available for evaluation by the Task Group (section 3.6.2.1). The first, preliminary report (Krishnamachari et al., 1975a) mentioned 397 patients in both affected states with 106 deaths. In a later more detailed paper (Krishnamachari et al., 1975b), the same group reported 277 cases in the Panchamahals district of the state of Gujarat with 75 deaths, and 126 hospitalized patients with 38 deaths in the Banswada district of the state Rajasthan. In an even later study, a different group (Tandon et al., 1977) reinvestigating the outbreak in Rajasthan, reported 994 affected individuals with 97 deaths in the Banswada and Dungarpur districts of Rajasthan. As reported by Krishnamachari et al. (1975b), the outbreak started almost simultaneously

Table 16. Reports of liver disease (other than Reye's syndrome and cancer) in individuals exposed to aflatoxins in food

Country	No. of cases of liver disease	Age group	Suspected aflatoxin vehicle	Estimated aflatoxin concentration (mg/kg)	Nature and outcome of liver disease	Reference
Senegal	2	4-6 years	groundnut meal	0.5-1	Hepatitis leading to hepatatic fibrosis in one case.	Payet et al. (1966)
China (Province of Taiwan)	26	all ages	rice	0.2	Acute liver disease, 3 deaths in children.	Ling et al. (1967)
Uganda	1	15 years	cassava	1.7	Acute hepatitis leading to death.	Serck-Hansen (1970)
India	20	1.5-5 years	groundnut meal	0.3	Hepatomegaly. Hepatic failure and death in 3 cases. Subsequent cirrhosis in some cases (juvenile).	Amla et al. (1971)
India	several hundred	infants not affected	maize	0.25-15	Acute toxic hepatitis, more than 100 deaths.	Krishnamachari et al. (1975a,b); Tandon et al. (1977)

in all affected villages, with only a few households affected in each village and several members of the same household becoming ill in some instances. Cases were confined to rural areas and to tribal populations whose staple food, particularly during the period October–February, was locally grown maize. The outbreak commenced with the consumption of recently harvested, badly stored maize, which had been affected by unusual rainfalls in October 1974. Although the maize was visibly spoiled, it was consumed, leaving relatively better cobs for seed purposes and for later use. Suspecting that the outbreak could have been caused by the massive consumption of maize heavily contaminated with fungi, Krishnamachari et al. (1975b) determined the mycoflora and the aflatoxin contents of 10 food samples. *A. flavus* was detected in all 5 samples of maize that were obtained from households affected with the disease, and the aflatoxin B₁ levels in these samples ranged from 0.25 mg/kg to 15.6 mg/kg. In contrast, only traces of aflatoxin were found in maize supplied to a hostel by local shops in one affected village and no aflatoxin was detected in 4 samples of other foodstuffs from the same source. *A. flavus* was not found in these 5 food samples of commercial origin. Assuming a daily local consumption of maize of up to 400 g per adult per day, and with aflatoxin contamination up to 15 mg/kg, Krishnamachari et al. (1975b) concluded that the affected people could have been exposed to considerable quantities of aflatoxins (up to 6 mg/day), for several weeks.

One liver sample obtained at necropsy and 7 blood serum and 7 urine samples collected from affected persons were analysed for aflatoxin content. No information is given on the stage of the disease at which the samples were collected or on the time that had elapsed since the last exposure to food suspected to be contaminated by aflatoxins. Traces of aflatoxin B₁ were reported in only 2 blood serum samples, with negative results for all the other human tissue and fluid samples (Krishnamachari, 1975b).

Tandon et al. (1977) reinvestigated the outbreak in Rajasthan, presenting results of a retrospective epidemiological survey in an area, where the largest number of patients with jaundice had been reported. Statements on dietary history obtained from members of 47 affected families (304 household members, 70 of whom had manifested the disease) and 29 other families (185 members with no case reported) did not indicate any difference in the reported consumption of mouldy maize between affected and non-affected families or between affected or non-affected members of the same household. However, *A. flavus* was detected in 85% of mouldy maize samples collected from 14 affected families compared with 12% in samples obtained from 17 families without manifestation of the disease and 3% in samples obtained from 2 grain dealers. Aflatoxins B₁ and G₁ were detected in 13 out of 14 samples from affected families and in 17 out of 19 samples

from the other families investigated in this area. Aflatoxin B₁ levels ranged from 0.1 to 0.6 mg/kg in all positive maize samples, with the exception of 2 from affected families where levels of 0.9 and 1.1 mg/kg were found. Information is not given in the paper concerning the time at which the samples were collected in relation to the occurrence of the disease, whether the samples were analysed for *Aspergillus* and aflatoxin contamination, and how far the aflatoxin levels found reflected the levels that could have occurred in maize actually consumed before and during the outbreak.

According to Krishnamachari et al. (1975b), all the cases occurred among subjects whose staple food was maize. Even if maize were also the staple food in the community studied by Tandon et al. (1977), statements obtained from members of families studied indicated that 31% of the members of non-affected families and 16% of members of affected families reportedly did not consume maize. From 70 cases of illness, 10 patients (14%) were reported not to have consumed maize at all but the paper does not indicate the staple food that they consumed instead.

In another part of the study by Tandon et al. (1977), clinical data on 200 hospitalized patients were analysed, using hospital records (176 cases) or direct clinical observations (24 patients). The disease had a subacute onset starting with fever (in 86% patients) followed by rapidly developing jaundice (98% cases) and ascites (74%). In the patients where ascites was not massive it was possible to detect hepatosplenomegaly. Vomiting, at the onset or at the time of reporting to the hospital, was present in 46% of cases. Leukocytosis (mainly an increase in polymorphonuclear leukocytes) was detected in the initial stage in 87% of patients. Raised levels of predominantly direct reacting bilirubin and alkaline phosphatase (EC 3.1.3.1) were found in blood serum; transaminase elevation was only mild or moderate and even normal levels were observed in blood samples collected from 11 patients. From the 200 hospitalized patients studied, 10% died in hospital, usually within 6 weeks of the onset of illness.

This description of the principal signs and symptoms is in good agreement with cases reported in the same outbreak by Krishnamachari et al. (1975b). Both Tandon et al. (1977) and Krishnamachari et al. (1975a,b) pointed out that two-thirds of affected people were males. The disease was not reported in infants at all, in the study of Krishnamachari (1975a,b). The youngest patient reported by Tandon et al. (1977) was 2½ years old but very few cases were observed below the age of 5 years. Both studies pointed out the concurrent liver disease with jaundice and ascites observed in village dogs fed food remnants from households (see also section 3.4.1).

Results of histopathological liver examinations are available from 10 patients in this outbreak. In one necropsy sample described by Krishnamachari et al. (1975a,b), microscopic examination revealed

extensive bile duct proliferation with periductal fibrosis and cholestasis. Apparently normal liver cells were observed over wide areas, occasionally replaced in some areas by multinucleated giant cells or hepatocytes with foamy cytoplasm. Tandon et al. (1977, 1978), who examined liver biopsy specimens obtained from 8 patients and one liver specimen from autopsy, pointed out that several histopathological liver changes were characteristic. These included: (a) oedema and collagenization of the central veins (thrombosis was not observed); (b) cholangiolar proliferation; (c) moderate to severe ballooning of the hepatocytes (giant cell transformation of the liver cells); (d) perisinusoidal fibrosis; (e) cholestasis; and (f) cirrhosis with reverse lobulation. On the basis of liver histopathology, Tandon et al. (1977, 1978) excluded any possibility of viral hepatitis and pointed out that the cholangiolar proliferation and syncytial giant cell transformation of hepatocytes (as well as the high prevalence of icterus in affected people) were also not pathognomonic of the veno-occlusive disease of the liver.

In one study by Maleki et al. (1976), an attempt was made to assess the urinary excretion of aflatoxins in patients suffering from cirrhosis of the liver, who came from a rural area of Iran where this disease is considered to be frequent without any clear association with high consumption of alcohol or hepatotoxic spices. The authors reported that the urine of 6/25 patients with a clinical diagnosis of cirrhosis contained aflatoxin M₁ whereas no aflatoxin M₁ was detected in the urine of 30 non-cirrhotic patients. The urine for aflatoxin analysis was collected within 2 days of admission to hospital. The patients came from villages near Isfahan, Iran where exceptionally high levels of aflatoxin M₁ in cow's milk had been reported (section 3.2.2.10).

3.5.2 Occupational exposure

Three available papers deal with occupational exposure to aflatoxins. Eleven out of a group of 55 workers, exposed for 2–9 years to dust containing aflatoxins in a mill crushing groundnuts and other oil seed, developed cancer of various organs within the observation period of up to 11 years. Primary liver cancer (cholangiocarcinoma) was reported in one of these patients. Two other workers were diagnosed to have died of another liver disease. On the basis of airborne dust determinations at various work places and dust analysis for aflatoxins (see section 3.2.4), the authors calculated that airborne aflatoxin levels could have ranged between 0.87 ng/m³ and 72 ng/m³. Assuming respiratory exposure to airborne aflatoxins in the range of 39 ng to 3.2 µg per worker per week, the authors concluded that, depending on the length of employment, the total amount of airborne aflatoxins, to which the patients had been exposed during the whole period of work in the mill, could have ranged in individual cases from 160 to 395

µg. In an age-matched group of 55 workers from a different factory in the same area, 4 cancer patients were found and no cases of liver cancer or death from other liver diseases were recorded (Van Nieuwenhuize et al., 1973).

Dvořáčková (1976) reported that 2 men, who had previously been carrying out the same type of work on a method of sterilizing Brazilian groundnut meal contaminated by *A. flavus*, died with a diagnosis of pulmonary adenomatosis. Analysis of lung samples obtained at autopsy from one of these patients suggested the presence of aflatoxin B₁. Deger (1976) observed that carcinoma of the colon developed in 2 research workers, who, for several years, had been involved in the same type of work in the same laboratory, purifying substantial amounts of aflatoxins for research purposes. No other people were involved in this work in the institute.

3.6 Evaluation of the Health Risks of Exposure to Aflatoxins

3.6.1 Human exposure conditions

The main source of human exposure to aflatoxins is contaminated food. Two pathways of dietary exposure have been identified: (a) direct ingestion of aflatoxins (mainly B₁) in contaminated foods of plant origin such as maize and nuts and their products; and (b) ingestion of aflatoxins carried over from feed into milk and milk products including cheese and powdered milk, where they appear mainly as aflatoxin M₁.

Exposure by pathway (a) is likely to be much greater than by pathway (b) irrespective of some toxicological differences between aflatoxins B₁ and M₁. In tropical countries, where optimal conditions for fungal growth exist, many components of the diet may become contaminated. In the surveillance of the first of these pathways of exposure, sampling is very important, as errors from this source are much greater than those from the analytical methods used. Since surveillance programmes have been established in only very few countries (section 3.6.1.2), information on dietary exposure to aflatoxins is not yet available on a worldwide basis.

Occasionally, workers may be exposed to the dust of agricultural commodities that contain aflatoxins. The only available quantitative data on such exposure (section 3.2.4) indicate that the concentration of aflatoxins in air under these conditions may be of the order of 0.1 µg/m³.

3.6.1.1 Sources and levels of aflatoxins in food

Aflatoxins are fungal products of some moulds that belong to two species: *Aspergillus flavus* and *A. parasiticus*. These moulds are found all

over the world, except in polar regions, but their growth and the formation of aflatoxins require humidity and temperature conditions (section 3.2.1.1) that are prevalent in tropical and subtropical areas, but may occasionally be found in colder regions such as Northern Europe. The formation of aflatoxins takes place mainly during harvesting and storage, but there is evidence that attacks by insects carrying fungal spores can result in the pre-harvest formation of aflatoxins (section 3.2.1.2).

Although aflatoxin-producing moulds can grow on a large variety of foodstuffs, particularly on plant products, it appears that certain foodstuffs are more suitable substrates for aflatoxin formation than others and, thus, may be contaminated more frequently and with higher levels. These include oil seeds (groundnuts, some other nuts, cottonseed) and some cereals (maize) (section 3.2.2).

Existing data on the contamination of foodstuffs by aflatoxins have been obtained by means of various analytical procedures (section 3.1.2). Collaboratively tested methods are now available, and as more countries develop adequate laboratory facilities and make use of these methods, more comparable survey data should become available.

Several species of cereals and nuts can be contaminated with aflatoxins and, from published survey reports, it appears that contamination is highest in groundnuts, Brazil nuts, maize, and maize products (section 3.2.2). An extremely high value of about 3500 $\mu\text{g}/\text{kg}$ was reported in a single sample of groundnuts imported into Europe for feed, and in a survey in Thailand, an average concentration of about 1500 $\mu\text{g}/\text{kg}$ was recorded. However, average values of 5 $\mu\text{g}/\text{kg}$ or less are more common in countries where control measures have been implemented. Peanut butter can also contain aflatoxins, depending on the quality of the groundnuts used in its manufacture. Roasting of groundnuts reduces but does not eliminate aflatoxins contamination (section 3.2.3); in general, cooking of food is not a safeguard against aflatoxin exposure.

There is reliable evidence showing that the level of aflatoxin M_1 in milk is directly related to the daily intake of aflatoxin B_1 in dairy feeds, (section 3.3.4.1) and it is generally recognized that groundnut and cottonseed meals, and maize are the major sources of this contaminant. However, the level of aflatoxin M_1 in milk is approximately 300 times lower than the level of aflatoxin B_1 in the feed consumed. Several surveys of liquid and dried milk powder have been carried out throughout the world (section 3.2.2.10). The highest reported level of aflatoxin M_1 in cow's milk exceeds 10 $\mu\text{g}/\text{litre}$, but, in countries where the quality of dairy products is strictly controlled, levels of 0.1 $\mu\text{g}/\text{litre}$ or less are commoner.

Animal experiments indicate that, in addition to the carry-over into milk, residues of aflatoxins may be present in the tissues of animals that consume

contaminated feed (section 3.2.2.10). However, the Task Group was not aware of any data from surveys for aflatoxin residues in meat and meat products.

3.6.1.2 *Dietary intake and levels in human tissues*

Dietary exposure will depend on the levels of aflatoxins in food and on food consumption patterns. It is evident that the contamination of staple foods is of major concern, and that population segments exposed to monotonous diets based on such staple foods are at particular risk. Two methods are available for assessing dietary exposure to aflatoxins: (a) determination of contamination levels in major food commodities, combined with nutritional surveys; and (b) analysis of food eaten ("food-on-the-plate" analysis).

In principle, the second method provides a better estimate of aflatoxin intake but involves many practical difficulties.

Even though there are many reports from different parts of the world on the presence of aflatoxins in individual food items (section 3.2.2), the use of food consumption data for the assessment of aflatoxin intake seems to be limited, at present, to certain parts of the USA. "Food-on-the-plate" analysis data on aflatoxins are available only for certain restricted areas in the regions of the world where the incidence of liver cancer is high (section 3.5.1.1).

A comprehensive nutritional and commodity survey conducted in the southeastern states of the USA gave an estimated average level of aflatoxin B₁ in groundnuts and groundnut products of 2 µg/kg, and an average level of 5–10 µg/kg for maize products. Based on these data, the estimated average daily intake of aflatoxin B₁ in these areas of the USA was reported (FDA, 1978) to amount to 2.73 ng/kg body weight (maximum 9.03 ng/kg). In certain areas of Thailand and East Africa, the estimated average daily intake based on "food-on-the-plate" data ranged from 3.5 to 222.4 ng/kg body weight (see section 3.5.1.1). However, during the outbreaks of acute liver disease in south-east Asia, much higher estimates of daily intake were obtained (up to 120 µg/kg body weight), and levels of aflatoxin B₁ up to 15 mg/kg were found in the contaminated maize consumed (section 3.5.1.2).

Aflatoxin B₁ has been found in the liver and other tissues of human subjects at levels up to 500 µg/kg or more (section 3.3.2.2). Some of these cases occurred in Europe and North America indicating that, at least in some individuals, significant intake of aflatoxins might occur in these areas.

3.6.2 **Acute effects of exposure**

Cases of acute human intoxication (section 3.5.1.2) have been reportedly

associated with dietary aflatoxin levels substantially higher (in the mg/kg range) than the levels thought to be associated with liver cancer ($\mu\text{g}/\text{kg}$ total food range). The Task Group was not aware of any long-term follow-up study of human populations in which acute intoxication was reported to have occurred.

3.6.2.1 *Acute liver disease*

The association of an outbreak of liver disease in turkeys with aflatoxins (section 3.4.1) was of basic importance in the recognition of aflatoxins as an environmental hazard.

Similar outbreaks of acute liver disease associated with the ingestion of aflatoxins were also observed in other species. The hepatotoxicity of aflatoxins has been confirmed by animal experiments and dose-response relationships have been obtained for different species (section 3.4.2).

Acute aflatoxicosis in man has rarely been reported but such cases may not always have been recognized. Apart from the death of 3 children in the Province of Taiwan, China and one child in Uganda, where acute liver necrosis was associated with the ingestion of rice and cassava contaminated with aflatoxins at levels of 200 $\mu\text{g}/\text{kg}$ and 1700 $\mu\text{g}/\text{kg}$, respectively, the most convincing case of association of aflatoxins with acute liver disease was an epidemic of toxic hepatitis in north-west India in 1974 (section 3.5.1.2). In this epidemic, several hundred villagers who consumed maize, presumably contaminated with aflatoxins at levels up to 15 mg/kg, exhibited signs and symptoms of poisoning and more than one hundred people died. Estimated daily ingestion of levels up to 6 mg per person were reported, corresponding approximately to dose rates up to 120 $\mu\text{g}/\text{kg}$ body weight per day. Such dose rates exceed those required to produce liver damage in non-human primates and this provides additional support for the assumption that this epidemic was indeed related to aflatoxin ingestion. Although the role of aflatoxin was not unequivocally demonstrated, the Task Group agreed that this incident represented the most acceptable evidence to date of acute human aflatoxicosis, supported by the information on liver histology, the space-time clustering of the cases, and the deaths among village dogs due to a similar form of acute toxic hepatitis. Examination of tissues and body fluids for aflatoxins was limited to 15 samples (1 necropsy liver sample, 7 urine samples, and 7 blood samples); aflatoxin was detected only in 2 blood samples. However, available animal data suggest that the detectable residues of mycotoxins may remain in tissues and body fluids for only a relatively short time after ingestion and that, therefore, the absence of residues does not exclude the possibility of prior exposure.

In the light of two earlier similar occurrences of aflatoxin intoxication

associated specifically with children, it is somewhat surprising that, in the Indian epidemic, infants were completely spared and children under the age of 5 years were less commonly affected than adults.

Similar epidemics could be expected in the future if the unusual harvesting circumstances, considered in this case to be responsible for the high contamination of the staple diet, recurred. The paucity of reports of epidemics of this type would suggest that massive contamination of human staple food is a rare occurrence.

3.6.2.2 *Reye's syndrome*

This syndrome (section 3.5.1.2) is found in many countries of the world and unlike liver cancer does not show a geographical association with areas of high aflatoxin intake. Out of four countries in which the relationship between aflatoxins and Reye's syndrome has been studied (Czechoslovakia, New Zealand, Thailand, and the USA) only Thailand belongs to an area with high aflatoxin levels in food. With the exception of the Thailand cases, the Task Group was not aware of any other similar reports of Reye's syndrome in association with aflatoxins in countries thought to be at increased risk from aflatoxin exposure.

A disease showing many similarities to Reye's syndrome has been experimentally demonstrated in macaques (section 3.4.2.2). This resulted from single doses of aflatoxin B₁ ranging from 4.5 to 40.5 mg/kg body weight. Reports on numerous experimental studies in different animal species, including other nonhuman primates, did not mention brain lesions; it is, however, possible that brains were not examined for abnormalities.

Among the reports on the presence of aflatoxins in the tissues of patients with Reye's syndrome, two studies deserve attention because of the number of cases included, and because control subjects were available (section 3.5.1.2).

In Thailand, appreciable amounts of aflatoxins were detected in autopsy specimens of 6 out of 23 cases of Reye's syndrome and trace amounts (1–4 µg/kg) were found in a further 16 cases. Similar trace amounts of aflatoxins were detected in specimens from 11 out of 15 children who had died from other causes.

In a systematic study over several years in Czechoslovakia, aflatoxin B₁ was unequivocally demonstrated in the liver tissue of 26/27 cases, and M₁ in 4 cases (in one case the liver tissue was not examined for aflatoxins). No aflatoxin was found in the liver tissue of 25 children who died from other causes.

Cases where aflatoxins have been identified in the tissues or body fluids are sporadic, and the Task Group had no indication of the number of

symptomatic cases in which it was not possible to demonstrate the association with aflatoxin exposure. As regards cases in which aflatoxin was detected in the tissues, it cannot be excluded that pathological changes connected with Reye's syndrome could have decreased the clearance of aflatoxins from tissues.

In view of these considerations, aflatoxins cannot be excluded as a contributing factor to Reye's syndrome in some areas, although an exclusive causal relationship cannot be accepted. There is evidence that other factors, particularly influenza B virus, may be associated with this syndrome.

3.6.3. Chronic effects of aflatoxin exposure

3.6.3.1 *Cancer of the liver*

Epidemiological data indicate an association between the level of daily aflatoxin ingestion and the incidence of primary liver cell cancer in certain areas of Kenya, Mozambique, Swaziland, and Thailand (section 3.5.1.1). This relationship is strongly supported by studies in experimental animals. In at least 8 species of experimental animals, aflatoxin has been shown to increase the incidence of liver cancer (section 3.4.2.3).

If the data from the 4 epidemiological studies (section 3.5.1.1) are combined, the best fit to the data points is obtained by a linear regression of the crude liver cancer incidence rates on the logarithms of the dietary aflatoxin intake. The regression has been estimated for dietary intakes ranging from 3.5 to 222.4 ng/kg body weight per day and for crude liver cancer incidence rates from 1.2 to 13 cases/100 000 population per year. At the lower ranges of aflatoxin intake, liver cancer rates are of the magnitude encountered in parts of the world in which liver cancer frequency is considered to be low.

It may be recalled that the liver cancer incidence in rats can be increased by ingestion of diets containing aflatoxin B₁ at a level of 1 µg/kg (Table 7). The estimated levels of exposure to aflatoxins in the USA (see section 3.6.1.2) have been used in the assessment of corresponding life-time liver cancer risks in rats (section 3.4.2.3) (FDA, 1978). For combined rat studies, these risk estimates amounted to 240 and 1100 per 10 000 for aflatoxin exposure levels of 0.1 and 0.3 µg/kg feed, respectively; the estimated life-time risks of primary liver cancer in the human population of the USA from all causes is approximately 161 per 100 000 (FDA, 1978). Comparison of epidemiological and experimental data would seem to indicate that man is not more but probably less susceptible to aflatoxins than the rat.

This conclusion also seems to be supported by studies on the metabolic transformation of aflatoxins (see section 3.3.3).

An association between aflatoxin intake and human liver cancer incidence was established in surveys that included areas with high estimated aflatoxin exposure and high liver cancer incidence. These studies compared the current average aflatoxin intake and the crude liver cancer incidence rate and did not allow for the period of latency between the beginning of exposure and cancer manifestation. The length of this latent period and factors that may modify its length are not well known. However, the studies were conducted in rural areas with stable populations and conditions of exposure thought not to have changed substantially. Data from intervention studies, in which populations are followed up after a reduction in exposure levels has been achieved, are not available.

Published epidemiological studies have been limited in scope and only a single possible etiological factor, i.e., aflatoxin exposure has been examined. Other factors for which there is some evidence of an etiological or modifying role in liver cancer, such as nutritional status, cirrhosis, or viral hepatitis, or the possibility of interactions between these and still other factors have not been considered.

In rats, dietary intake of lipotropes, protein, and vitamin A modified the carcinogenic potential of aflatoxins (section 3.4.2.7). Diet, marginally deficient in lipotropes (choline, methionine, folate), enhanced liver cancer induction by aflatoxin B₁. A diet marginally deficient in protein (9% casein) also increased liver cancer incidence in aflatoxin-treated rats. Severe lipotrope deficiency or severe protein deficiency (4% casein, combined with lipotrope deficiency) decreased cancer incidence in aflatoxin-treated rats. Severe deficiencies which markedly inhibit the growth of experimental animals can reduce the cancer incidence after exposure to different carcinogens. This probably represents a general effect on growth rather than a specific effect on carcinogenesis. Vitamin A deficiency did not influence the incidence of liver cancer in aflatoxin-treated rats but altered the effect of aflatoxin so that the incidence of colon cancers increased. In view of the importance of these results in relation to human health and the shortcomings in some of the experiments reported, further animal studies are necessary to quantify these effects. Future epidemiological studies should consider such interactions.

Other questions raised by the data from animal studies but not demonstrated in the epidemiological studies, and thought by the Task Group to require further study before they can be applied to the human risk assessment, are the possible induction of extrahepatic cancers by aflatoxin (section 3.4.2.3), and the possibility that cancer could be induced by short-time exposure to high concentrations of aflatoxin.

In spite of the existing gaps in knowledge, it should be recognized that in animal experiments there is "strong evidence" of carcinogenicity^a for aflatoxins with established dose-response relationships, and that epidemiological studies in some parts of the world, where liver cancer is more frequent, have indicated a highly significant positive correlation between the crude incidence rate of liver cancer and the estimated current ingestion of aflatoxin in these areas.^b The Task Group, therefore, concluded that aflatoxin ingestion may increase the risk of liver cancer, that the risk depends on the amount of aflatoxin ingested, and that reduction in daily aflatoxin exposure could be expected to reduce the liver cancer risk.

3.6.3.2 *Juvenile cirrhosis in India*

The Task Group concluded that the postulated involvement of aflatoxins in juvenile cirrhosis in India has not been substantiated (section 3.3.4.2 and Table 16 in section 3.5.1.2) and that it is unlikely in view of the epidemiological and morphological evidence available. Preliminary data suggesting the involvement of aflatoxins in the etiology of this disease were not supported by later measurements of aflatoxin exposure and examination of urine and liver specimens.

3.6.4 Guidelines for health protection

The effect of aflatoxins which is of greatest concern is the possible induction of liver cancer in man. Even if it is not possible, at present, to quantify individual risk corresponding to a given exposure to aflatoxins, it is nevertheless prudent to attempt to reduce exposure as much as is practically achievable. Reduction of food contamination by aflatoxins, sufficient to significantly reduce liver cancer risk, would of course significantly reduce the risk of acute toxic effects.

Although there are several aspects of the relationship between aflatoxin exposure and carcinogenic risks in man that require elucidation by further experimental and epidemiological studies, there is, at present, sufficient evidence to justify the implementation or strengthening of national aflatoxin control programmes. It is impractical to insist that staple foodstuffs be

^a "Strong evidence" of carcinogenicity is considered to exist when a chemical has been shown unequivocally to produce malignant neoplasms. Chemicals for which the evidence of carcinogenicity is based solely on the appearance of such neoplastic lesions as lung adenomas or hepatomas in mice belong to the class of chemicals for which there is "weak evidence" of carcinogenicity (IARC, 1977).

^b Of course, this association between liver cancer incidence and aflatoxin does not necessarily mean that these two variables are causally related. However, the existing animal data tend to support a causal relationship, although there may be other factors that contribute to the development of liver cancer in these areas.

aflatoxin-free, but the level of aflatoxin contamination should be reduced gradually by programmes involving the following components: education of farmers to improve crop quality and storage; surveillance of foodstuffs and animal feeds for the presence of aflatoxins; and application of appropriate food-processing technology to separate contaminated, from noncontaminated food elements. These and other measures have recently been discussed elsewhere (FAO, 1977).

Several countries have established tolerance levels for aflatoxins in specific food items (for review see Stoloff, 1977; Krogh, 1978). It should be clearly understood that these tolerance limits are only management tools intended to facilitate the implementation of aflatoxin control programmes, and that adherence to these tolerance limits does not provide an absolute protection against the increased liver cancer risk associated with aflatoxin exposure.

4. OTHER MYCOTOXINS

4.1 Ochratoxins

4.1.1 Properties and analytical methods

4.1.1.1 Chemical properties

The ochratoxins are a group of structurally-related compounds (Fig. 6),

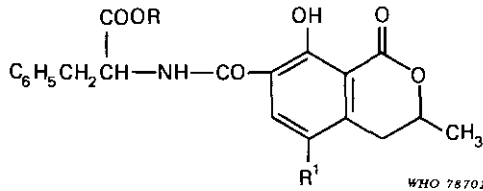


Fig. 6. The chemical structure of ochratoxins: ochratoxin A: R' = Cl, R = H; ochratoxin B: R' = H, R = H; ochratoxin C: R' = Cl, R = C₂H₅; methyl ester of ochratoxin A: R' = ² Cl, R = CH₃; methyl or ethyl ester of ochratoxin B: R' = H, R = CH₃ or C₂H₅.

classified according to biosynthetic origin as pentaketides within the group polyketides (Turner, 1971). The first compound discovered, ochratoxin A, was isolated from a strain of *Aspergillus ochraceus* (van der Merwe et al., 1965). It is a colourless, crystalline compound, exhibiting blue fluorescence under UV-light. The sodium salt of ochratoxin A is soluble in water; as an acid, it is moderately soluble in polar organic solvents (e.g., chloroform and methanol). Some of the chemical and physical properties of three ochratoxins are summarized in Table 17. Only ochratoxin A, and very

Table 17. Chemical and physical properties of some ochratoxins

Ochratoxin	Molecular formula	Relative molecular mass	Melting point °C	Absorption maxima (nm) absorption coefficient (ε)	Reference
A	C ₂₀ H ₁₆ ClNO ₆	403	169 (xylene) 89-95 (benzene)	213(36 800); 322(6400)	Stevn & Holzappel (1967)
B	C ₂₀ H ₁₆ NO ₆	369	221	218(37 200); 318(6900)	van der Merwe et al. (1965)
α	C ₁₁ H ₈ ClO ₅	256	229	212(30 000); 338(5600)	van der Merwe et al. (1965)

rarely ochratoxin B, have been encountered as natural contaminants of foodstuffs, the remaining ochratoxins listed in Fig. 6 have been isolated only from fungal cultures, under laboratory conditions. On acid hydrolysis, ochratoxin A yields phenylalanine and an optically active lactone acid, ochratoxin α, a metabolite which has been found in the urine of test animals ingesting ochratoxin A-contaminated feed. This subject has been reviewed by Chu (1974) and Harwig (1974), and the spectroanalytical parameters have been reviewed by Neely & West (1972).

4.1.1.2 *Methods for the analysis of foodstuffs*

Methods of analysing foodstuffs for ochratoxins have been reviewed by Nesheim (1976). The distribution of ochratoxins in commodities has not been studied in detail and no specific sampling plans have been developed. However, the general principles of sampling, outlined in section 3.1.2.1 for aflatoxins, are also applicable to ochratoxin.

Several chemical methods have been developed, with limits of detection as low as 2 µg/kg (Nesheim, 1976). Ochratoxin A in acidified commodities is readily soluble in many organic solvents, and this characteristic has been used as the principle of extraction in several methods. The most widely used method, for cereals in particular, includes extraction with chloroform-aqueous phosphoric acid followed by cleanup on an aqueous bicarbonate-diatomaceous earth column, and quantitative determination using thin-layer chromatography (Nesheim et al., 1973). This procedure has been recommended by the International Union of Pure and Applied Chemistry (IUPAC) as an international method (IUPAC, 1976), and has a limit of detection of a few µg/kg, when improved by ammoniation.

Minicolumn methods for screening purposes have been developed (Hald & Krogh, 1975; Holaday, 1976), as well as a spectrophotometric procedure based on cleavage of ochratoxin A to form ochratoxin α and phenylalanine (Hult & Gatenbeck, 1976).

A number of bioassays involving zebra fish larvae, brine shrimps, and bacteria have been developed, but none of the assays has been used routinely, so far (Harwig, 1974).

4.1.2 Sources and occurrence

4.1.2.1 *Fungal formation*

Ochratoxin A was first obtained from *A. ochraceus*, but subsequent investigations have revealed that a variety of moulds included in the fungal genera *Aspergillus* and *Penicillium* are able to produce ochratoxins (Table 18). The main producers appear to be *A. ochraceus* and *P. viridicatum*. This subject has been reviewed by Krogh (1976a).

Moisture content and temperature. In studies of ochratoxin A production by *A. ochraceus*, optimal production occurred between 20 and 30° C (Schindler & Nesheim, 1970; Bacon et al., 1973). Maximum production was observed at 30° C and a water activity (a_w) of 0.953 (39% of water content, % dry weight). At lower temperatures, such as 15° C, the moisture requirement was higher ($a_w = 0.997$, or 52% moisture) (Table 19).

The genus *Penicillium* includes psychrophilic species, and investigations of the influence of low incubation temperatures have revealed that strains of

Table 18. Ochratoxin-producing fungi^a

<i>Penicillium</i> Link	
Monoverticillata:	
<i>P. frequentans</i> series:	<i>P. purpurescens</i> Sopp
Asymmetrica-Lanata:	
<i>P. commune</i> series:	<i>P. commune</i> Thom
Asymmetrica-Fasciculata:	
<i>P. viridicatum</i> series:	<i>P. viridicatum</i> Westling
	<i>P. palitans</i> Westling
<i>P. cyclopium</i> series:	<i>P. cyclopium</i> Westling
Biverticillata-Symmetrica:	
<i>P. purpurogenum</i> series:	<i>P. variabile</i> Sopp
<i>Aspergillus</i> Micheli	
<i>Aspergillus ochraceus</i> group:	
	<i>A. sulphureus</i> (Fres.) Thom and Church
	<i>A. sclerotiorum</i> Huber
	<i>A. alliaceus</i> Thom and Church
	<i>A. melleus</i> Yukawa
	<i>A. ochraceus</i> Wilhelm
	<i>A. ostianus</i> Vvehmer
	<i>A. petrakii</i> Vörös

^a From: Krogh (1978).

P. viridicatum are able to produce ochratoxin A at 5–10° C (Harwig & Chen, 1974) (Table 19). This indicates that the heavy ochratoxin contamination observed in countries with cold climates such as Canada and the Scandinavian countries is mainly produced by the *Penicillia*.

Table 19. Production of ochratoxin A by *A. ochraceus* and *P. viridicatum* at various a_w and temperatures

(a) <i>A. ochraceus</i> (after 2 weeks of incubation) ^a			
a_w	15° C	22° C	30° C
	mg ochratoxin A/kg		
0.852	0	0	60
0.901	0	46	111
0.953	36	201	302
0.997	81	156	218
(b) <i>P. viridicatum</i> (after 3 weeks of incubation) ^b			
a_w	5° C	12° C	25° C
	mg ochratoxin A/kg		
0.85–0.86		4000	11 000
0.90–0.93		160 000	280 000
0.95–0.97	17 000		
	(after 5 weeks)		

^a Adapted from: Bacon et al. (1973).

^b Adapted from: Harwig & Chen (1974).

4.1.2.2 Occurrence in foodstuffs

Plant products. The occurrence of ochratoxins in foodstuffs has been reviewed by Chu (1974a), Krogh (1976a, 1977a), and Stoloff (1976).

Table 20. Natural occurrence of ochratoxin A in plant products

Commodity	Country	No. of samples analysed	Percentage contaminated	Range of contamination ($\mu\text{g}/\text{kg}$)	Reference
wheat, oats, barley, rye (feed)	Canada	32	56.3	30-27 000 ^c	Scott et al. (1972)
barley, oats	Denmark	33	57.6	28-27 500 ^{b,c}	Krogh et al. (1973b)
malt barley	Denmark	50	6.0	9-189	Krogh (1978)
maize	France	463	2.6	15-200	Galtier (1975)
barley, wheat, oats, rye, maize (feed)	Poland	150	5.3	50-200	Juszkiewicz & Piskorska-Pliszczynska (1976)
mixed feed	Poland	203	4.9	10-50	Juszkiewicz & Piskorska-Pliszczynska (1977)
barley, oats (feed)	Sweden	84	8.3	16-409	Krogh et al. (1974)
maize, wheat, barley	Yugoslavia	47	12.8	5-90	Krogh et al. (1977)
barley	USA	127	14.2	10-40	Nesheim (1971)
coffee beans	USA	267	7.1	20-360	Levi et al. (1974)
maize	USA	283	0.4	110-150	Shotwell et al. (1969c)
maize	USA	293	1.0	83-166 ^a	Shotwell et al. (1971)
wheat (red winter)	USA	291	1.0	5-115	Shotwell et al. (1976a)
wheat (red spring)	USA	286	2.8	5-115	Shotwell et al. (1976a)

^a Two of these samples also contained ochratoxin B.

^b Ochratoxin B, as well as ochratoxin A, detected in 2 additional samples of barley.

^c Most of the samples containing high levels had undergone "heating".

Naturally occurring ochratoxin A was first reported at a concentration of 110–150 µg/kg in one sample of maize included in a survey of 283 samples from commercial markets in the USA (Shotwell et al., 1969c). Data from subsequent surveys of plant products in various areas of the world are summarized in Table 20.

Residues in food of animal origin. In 1971, a farm was traced where pigs had been fed ochratoxin A-contaminated feed. When the bacon pigs, some of which suffered from nephropathy, were delivered to the slaughterhouse, samples of kidney, liver, and adipose tissue were collected for analysis. Residues of ochratoxin A were detected in 18/19 investigated kidneys, at levels up to 67 µg/kg (Hald & Krogh, 1972). Residues were also detected in 7/8 livers, and in all 8 samples of adipose tissue analysed.

Surveillance studies based on data from meat inspection in Denmark have revealed prevalence rates of porcine nephropathy ranging from 10–80

Table 21. Correlation between feed level and tissue levels (residues) of ochratoxin A in pigs^a

Tissue	Regression equation	<i>r</i>
kidney	$y = 2.15 + 0.0123x$	0.86
liver	$y = 0.35 + 0.0095x$	0.82
adipose	$y = 2.51 + 0.0099x$	0.78

^a Modified from: Krogh et al. (1974).

x = ochratoxin A in feed (µg/kg)

y = ochratoxin A residue (µg/kg tissue)

r = correlation coefficient

The regression is calculated on feed levels of ochratoxin A in the range of 200–4000 µg/kg.

cases per 100 000 slaughtered pigs (Krogh, 1976b). A survey of kidneys from pigs with the disease collected at various slaughterhouses, showed that 35% of the affected kidneys contained residues of ochratoxin A, ranging from 2–68 µg/kg (Krogh, 1977b). A similar survey of porcine nephropathy in Sweden revealed that 25% of the affected kidneys contained ochratoxin A at levels ranging from 2 to 104 µg/kg (Rutqvist et al., 1977). The carry-over of ochratoxin A from feed to animal tissues has been elucidated in studies in which groups of pigs were exposed for 3–4 months to dietary levels of ochratoxin A of 200, 1000, and 4000 µg/kg (Krogh et al., 1974). At termination (slaughter), the highest levels of ochratoxin A residues were found in the kidneys (mean level 50 µg/kg at the 4000 µg/kg feed level) with slightly lower levels in the liver, and even lower levels in muscle and adipose tissue. Other tissues were not analysed. There was a high correlation between the feed level of ochratoxin A and the residue levels in the 4 tissues investigated (Table 21). In another study on pigs (Krogh et al., 1976a), a high correlation ($r = 0.74–0.94$) was found between ochratoxin A levels in the kidney and in other organs and tissues including the liver, muscle, and adipose tissue (Table 22 and section 4.1.3.3).

Table 22. Correlation between ochratoxin A mass concentration residues in the kidney and certain other tissues^a

Tissue	Regression equation	<i>r</i>
liver	$y = -0.650 + 0.706x$	0.937
muscle	$y = -0.603 + 0.438x$	0.888
adipose	$y = -0.775 + 0.309x$	0.739

^a From: Krogh et al. (1976a).

x = ochratoxin A mass concentration (µg/kg) in the kidney

y = ochratoxin A mass concentration (µg/kg) in the other tissues

r = correlation coefficient

Ochratoxin A levels of up to 29 µg/kg were found in the muscle of hens and chickens collected in one slaughterhouse (Elling et al., 1975). The birds had been condemned because of nephropathy. In another study, groups of hens were exposed for 1–2 years to dietary levels of ochratoxin A of 0.3 and 1 mg/kg (Krogh et al., 1976c). The kidneys contained the highest residues, with a mean value of 19 µg/kg tissue in the group fed ochratoxin A at 1 mg/kg; the liver and muscle contained lower levels of ochratoxin A residues. Ochratoxins were not detected in the eggs.

4.1.3 Metabolism

4.1.3.1 Absorption

In a study on rats exposed by gavage to a single dose of ochratoxin A at 10 mg/kg body weight, Galtier (1974b) found the highest tissue level of unchanged ochratoxin A in the stomach wall during the first 4 h following administration. The small and large intestine and caecum contained small amounts of unchanged ochratoxin A, and it was concluded that ochratoxin A was absorbed mainly in the stomach. In the caecum and the large intestine, small amounts (1–3% of the total dose), were detected as the isocoumarin moiety (ochratoxin α) most likely as the result of the hydrolysing action of the intestinal microflora (Galtier & Alvinerie, 1976; Hult et al., 1976).

In *in vitro* studies, Pitout (1969) showed that ochratoxin α could also be formed from the hydrolysis of ochratoxin A by carboxypeptidase A (EC 3.4.12.2) and α -chymotrypsin. No quantitative information is available on the rate of absorption of ochratoxin A and ochratoxin α from the gastrointestinal tract.

4.1.3.2 Tissue distribution and metabolic conversion

In slaughterhouse cases of mycotoxic porcine nephropathy studied by Hald & Krogh (1972), residues of unchanged ochratoxin A were found in

all tissues investigated (kidney, liver and muscle), the highest levels (up to 67 $\mu\text{g}/\text{kg}$) occurring in the kidney. In experimental studies on pigs ingesting feed containing ochratoxin A, residues of this toxin were found in all 4 tissues in the decreasing order of kidney, liver, muscle, adipose tissue (Krogh et al., 1974). When rats were exposed perorally to an ochratoxin A dose of 10 mg/kg body weight, Galtier (1974b) recovered 0.3% of the administered dose in the whole kidneys, 0.9% in the whole liver, and 0.6% in the total muscle tissue, 96 h after exposure. Chang & Chu (1977), using a single intraperitoneal injection of 1 mg ochratoxin A per rat (labelled with ^{14}C in phenylalanine), found that the kidney contained twice as much unchanged ochratoxin A as the liver after 0.5 h, amounting to 4–5% of the total dose.

It has been shown by *in vitro* studies that ochratoxin A binds to serum albumin (Chu, 1971, 1974b); this binding has also been observed in *in vivo* studies of rats (Galtier, 1974a; Chang & Chu, 1977). Ochratoxin α has been detected in the urine and faeces of rats intraperitoneally injected with ochratoxin A (Nel & Purchase, 1968; Chang & Chu, 1977), indicating the cleavage of ochratoxin A into ochratoxin α and phenylalanine under these conditions. Studies with ^{14}C -labelled ochratoxin A indicated that some other, not yet identified, metabolites are formed in the body. Less than half of the radioactivity excreted in the urine within 24 h of a single intraperitoneal injection of ^{14}C -phenylalanine-labelled ochratoxin A was identified as ochratoxin A (Chang & Chu, 1977).

4.1.3.3 Excretion

Using ^{14}C -labelled ochratoxin A in studies on rats, it has been demonstrated that this toxin is excreted primarily in the urine (Chang & Chu, 1977) although faecal excretion also occurs to some extent (Galtier, 1974b; Chang & Chu, 1977). Ochratoxin A has been detected in the urine of bacon pigs suffering from nephropathy (Krogh, personal communication).

In a study of the disappearance rates for various tissues, female bacon pigs were fed ochratoxin A at a level of 1 mg/kg feed for 1 month and then kept on a toxin-free diet for another month during which animals were sacrificed at regular intervals (Krogh et al., 1976a). Ochratoxin A disappeared exponentially (Table 23) from the 4 tissues investigated (kidney, liver, muscle, and adipose tissue), with residual life values (RL_{50})^a in the range of 3.3–4.5 days; the toxin could still be detected in kidneys one month after termination of exposure. When the level in the kidney is known, the

^a RL_{50} = half-time of residues calculated from the exponential equations shown in Table 23.

Table 23. The rate of disappearance of ochratoxin A residues from pig tissues after termination of one month exposure to ochratoxin A at 1 mg/kg feed^a

Tissue	Ochratoxin A ($\mu\text{g}/\text{kg}$ tissue) at time t (days) after termination of exposure
kidney	$28.22 \exp(-0.1522t)$
liver	$19.49 \exp(-0.1598t)$
muscle	$12.94 \exp(-0.2096t)$
adipose	$4.62 \exp(-0.0565t)$

^a From: Krogh et al. (1976a).

ochratoxin A residues in the 3 other tissues can be calculated (Table 22). No data are available on ochratoxin levels in human tissues, urine, or faeces.

4.1.4 Effects in animals

4.1.4.1 Field observations

Pigs. The effects of ochratoxins in animals have been reviewed by Krogh (1976a, 1978). Cases of mycotoxic porcine nephropathy, have been regularly encountered in studies in Denmark since the disease was first discovered 50 years ago (Larsen, 1928). The disease is endemic in all areas of the country, although unevenly distributed. Prevalence rates in 1971 varied from 0.6 to 65.9 cases per 10 000 pigs and epidemics were encountered in 1963 and 1971, associated with a high moisture content in the grain caused by unusual climatic conditions (Krogh, 1976b). Extensive etiological studies have revealed that ochratoxin A is a major disease determinant of porcine nephropathy, although other factors such as citrinin are also involved as causal determinants (for review see Krogh, 1976a). Analyses of kidneys from cases of porcine nephropathy collected at slaughterhouses have revealed that 35% of these kidneys contained ochratoxin A in concentrations ranging from 2–68 $\mu\text{g}/\text{kg}$ (Krogh, 1977b). As this compound has not been detected in healthy kidneys, it is indicated that it may play a causal role in the disease.

The morphological changes in the kidneys in cases of mycotoxic porcine nephropathy are characterized by degeneration of the proximal tubules, followed by atrophy of the tubular epithelium, interstitial fibrosis in the renal cortex, and hyalinization of some glomeruli (Elling & Moller, 1973). Although mycotoxic porcine nephropathy has only been reported from one other European country besides Scandinavia (Buckley, 1971), there are indications that this disease also occurs in other countries in Europe and North America.

Poultry. In a preliminary study in Denmark of chickens and hens condemned by meat inspectors because of renal lesions, 29% of 14 birds were suffering from nephropathy associated with ingestion of ochratoxin A (Elling et al., 1975). The morphological renal lesions were characterized by

degeneration of proximal and distal tubules of both reptilian and mammalian nephrons, and interstitial fibrosis.

4.1.4.2 Experimental studies

Acute and chronic effects. The acute and chronic effects of ochratoxins in experimental animals have been reviewed by Chu (1974a), Harwig (1974), and Krogh (1976a). Different species vary in their susceptibility to acute poisoning by ochratoxin A, with LD₅₀ values ranging from 3.4 to 30.3 mg/kg (Table 24). When administered orally to rats, the female is more sensitive to ochratoxin A than the male. The kidney is the target organ, but changes in the liver have also been noted during studies of acute effects.

Table 24 Acute toxicity of ochratoxin A

Animal	LD ₅₀ mg/kg body weight	Route of administration	Reference
mouse (female)	22	intraperitoneal	Sansing et al. (1976)
rat, male	30.3	peroral	Galtier et al. (1974)
rat, female	21.4	peroral	Galtier et al. (1974)
rat, male	12.6	intraperitoneal	Galtier et al. (1974)
rat, female	14.3	intraperitoneal	Galtier et al. (1974)
guineapig, male	9.1	peroral	Thacker (1976)
guineapig, female	8.1	peroral	Thacker (1976)
white leghorn	3.4	peroral	Prior et al. (1976)
turkey	5.9	peroral	Prior et al. (1976)
Japanese quail	16.5	peroral	Prior et al. (1976)
rainbow trout	4.7	intraperitoneal	Doster et al. (1972)
beagle dog, male	< 9 (total dose)	peroral ^a	Szczzech et al. (1973a)
pig, female	< 6 (total dose)	peroral ^b	Szczzech et al. (1973b)

^a All 3 dogs, dosed daily with 3 mg/kg, died within 3 days.

^b Both pigs receiving 2 mg/kg daily were moribund and killed within 3 days, and both pigs receiving 1 mg/kg daily were moribund and killed within 6 days.

The lesions observed in field cases of mycotoxic porcine nephropathy (section 4.1.4.1) have been reproduced by feeding diets containing levels of ochratoxin A identical to those encountered in naturally contaminated products (section 4.1.2.2). Thus 39 pigs fed rations containing ochratoxin A at levels ranging from 200–4000 µg/kg developed nephropathy after 4 months at all levels of exposure (Krogh et al., 1974). Changes in renal function were characterized by impairment of tubular function, indicated particularly by a decrease in Tm_{PAH}/C_{In} ^a and reduced ability to produce concentrated urine. These functional changes corresponded well with the changes in renal structure observed at all exposure levels including atrophy of the proximal tubules, and interstitial cortical fibrosis. Sclerotized glomeruli were also observed in the group receiving the highest dose of ochratoxin A of 4000 µg/kg feed. No other organ or tissue exhibited any changes.

^a Tm_{PAH} = transport maximum for para-aminohippuric acid, C_{In} = clearance of inulin.

Kidney damage, identical to the naturally occurring porcine nephropathy, was produced in another study by feeding pigs (9 animals) with crystalline ochratoxin A in amounts corresponding to a feed level of 1 mg/kg for 3 months. Similar damage was not observed in 9 controls. Significant renal tubular impairment was detected after only 5 weeks of ochratoxin exposure (Krogh et al., 1976b).

In pigs and dogs given high peroral doses, corresponding to feed levels of more than 5–10 mg/kg (levels rarely found in nature) extrarenal effects, in addition to renal lesions, were observed, involving the liver, intestine, spleen, lymphoid tissue, and leukocytes (Szczech et al., 1973a,b,c). Three groups of rats, each consisting of 15 animals were exposed to feed levels of ochratoxin A ranging from 0.2 to 5 mg/kg for 3 months. Renal damage in the form of tubular degeneration was observed at all dose levels (Munro et al., 1974).

Avian nephropathy similar to spontaneously occurring cases (section 4.1.4.1) developed in chickens and hens exposed to dietary levels of 0.3 and 1 mg/kg for 1 year (Krogh et al., 1976c). The renal changes included degeneration of the tubular epithelium, mainly confined to the proximal and distal tubules of both reptilian and mammalian nephrons; impairment of glomerular and tubular function was also observed. Acute necrosis and “visceral gout” was observed in chickens exposed to high levels of ochratoxin A (LD_{50} values) (Peckham et al., 1971). The same authors reported that ochratoxin B, the other naturally occurring ochratoxin was not highly toxic to chickens (LD_{50} : 54 mg/kg); no toxic effects have been reported in other animals.

The toxic effects of ochratoxin A on the renal epithelial cells of the monkey were demonstrated in *in vitro* studies, in the form of abnormal mitotic cells (Steyn et al., 1975).

Teratogenic effects. Intraperitoneal injection of pregnant mice with ochratoxin A at 5 mg/kg body weight on one of gestation days 7–12 resulted in increased prenatal mortality, decreased fetal weight, and various fetal malformations including exencephaly and anomalies of the eyes, face, digits, and tail (Hayes et al., 1974). When rats were treated perorally with ochratoxin A at 0.75 and 1.0 mg/kg body weight on gestation days 6–15, fetuses taken on day 20 showed decreased weight and various anomalies (e.g., open eyes, wavy ribs, and agenesis of vertebrae) (Brown et al., 1976). In hamsters injected intraperitoneally with ochratoxin A at doses of 5–20 mg/kg body weight on one of gestation days 7–9, increased prenatal mortality and malformations were observed, including hydrocephalus, micrognathia, and heart defects (Hood et al., 1976).

Mutagenicity. No data were available on the mutagenicity of ochratoxins.

Carcinogenesis. There have not been any recent data that would change

the conclusion that an evaluation of the carcinogenic risk of ochratoxins cannot be made because of the inadequacy of available studies in terms of the numbers of animals used and survival rates (IARC, 1976).

Biochemical effects. Ochratoxin A affects the carbohydrate metabolism in rats. Thus, a single oral dose of ochratoxin A at 15 mg/kg body weight caused a decrease in the glycogen level in the liver and an increase in the heart glycogen level 4 h later (Suzuki & Satoh, 1973). In a more extensive study on rats, the decrease in liver glycogen level, 4 h after a single oral dose of ochratoxin A at 15 mg/kg body weight, was associated with an increase in serum glucose levels and a decrease in liver glucose-6-phosphate (Suzuki et al., 1975). At the same time, the liver glycogen synthetase (EC 2.7.1.37) activity decreased and the liver phosphorylase (EC 2.4.1.1) activity increased. Three daily oral doses of ochratoxin A at 5 mg/kg body weight caused a decrease in liver glycogen concentration, measured on the fourth day. The decrease was attributed to inhibition of the active transport of glucose into the liver, suppression of glycogen synthesis from glucose, and acceleration of glycogen decomposition.

During *in vitro* studies of rat liver mitochondria, it was observed that ochratoxin A inhibited the respiration of whole mitochondria by acting as a competitive inhibitor of transport carrier proteins located in the inner mitochondrial membrane (Meisner & Chan, 1974). Further experiments with mitochondrial preparations revealed that the mitochondrial uptake of ochratoxin A was an energy-using process that resulted in depletion of intramitochondrial adenosine triphosphate (ATP), and that ochratoxin A inhibited intramitochondrial phosphate transport, resulting in deterioration of the mitochondria (Meisner, 1976). This might explain the degeneration of liver mitochondria observed by Purchase & Theron (1968) in rats exposed perorally to a single dose of ochratoxin A at 10 mg/kg body weight. These authors observed accumulation of glycogen in the cytoplasm of the rat liver cells microscopically. This was in contrast to the previously discussed observations of Suzuki et al. (1975) who found a decrease in glycogen levels.

In a study on mice, Sansing et al. (1976) found that ochratoxin A, administered intraperitoneally at 6 mg/kg body weight, inhibited orotic acid incorporation into both liver and kidney RNA, 6 h after toxin injection. Ochratoxin A acted synergistically, in this respect, with another nephrotoxic mycotoxin, citrinin.

4.1.5 Effects in man

4.1.5.1 *Ochratoxin A and Balkan nephropathy*

Balkan endemic nephropathy is a kidney disease only observed so far in rural populations in Bulgaria, Romania, and Yugoslavia. In the past 2

decades, etiological investigations covering bacteria, viruses, toxic metals, genetic factors, etc. have been conducted but with unconvincing results (reviewed Puchlev, 1973, 1974). Balkan endemic nephropathy is a chronic disease that is commonest between 30 and 50 years of age and progresses slowly up to death. The kidneys are remarkably reduced in size. Histologically, the renal disease is characterized by tubular degeneration, interstitial fibrosis, and hyalization of glomeruli in the more superficial part of the cortex (Heptinstall, 1966). Impairment of tubular function, indicated by a decrease in Tm_{PAH} , is a prominent and early sign (Dotchev, 1973).

The disease occurs endemically and affects females more often than males (Hrabar et al., 1976; Chernozemsky et al., 1977). In Bulgaria and Yugoslavia, a high incidence of urinary tract tumours has been found to be closely correlated with the incidence and mortality rates of Balkan endemic nephropathy (Čeović et al., 1976; Chernozemsky et al., 1977).

Fungal growth in foodstuffs and subsequent mycotoxin formation is influenced by the water content of the foodstuffs, and can be changed by climatic conditions such as heavy rainfalls during harvest. Thus, the observation (Austwick, 1975) of a positive correlation ($r = 0.80$) between excess rainfall and the number of people who died of nephropathy during the succeeding 2 years in the Balkan peninsula might be interpreted as suggesting a fungal involvement in the etiology of endemic nephropathy.

Attention has been called to the striking similarities in the changes of renal structure and function found in Balkan endemic nephropathy and in ochratoxin A-induced porcine nephropathy, suggesting common causal relationships (Krogh, 1974). Furthermore, epidemiological similarities have been noted, in particular, the endemic occurrence (Krogh, 1976b). Preliminary results of a survey of foodstuffs indicate that exposure to food-borne ochratoxin A seems to be higher (12.8% contamination) in an area of Yugoslavia with a high prevalence of human endemic nephropathy than in nonendemic (control) areas (1.6% contamination) (Krogh et al., 1977).

4.1.6 Conclusions and evaluation of the health risks to man of ochratoxins

4.1.6.1 Experimental animal studies

The toxic effects of ochratoxin A have been studied extensively in a variety of experimental animals. All the animals studied so far have been susceptible to orally administered ochratoxin A, but to various degrees, as indicated by the range of LD_{50} values (Table 24). At high levels of ochratoxin A, changes were found in the kidneys and also in other organs and tissues. However, only renal lesions were observed at exposure levels identical to those occurring environmentally. The renal lesions included degeneration of the tubules, interstitial fibrosis, and, at later stages,

hyalinization of glomeruli, with impairment of tubular function as a prime manifestation. Feed levels as low as 200 µg/kg produced renal changes in the course of 3 months in rats and pigs. Field cases of ochratoxin A-induced nephropathy are regularly encountered in pigs and poultry. Ochratoxin A is teratogenic in the mouse, rat, and hamster.

Ochratoxin B, rarely found as a natural contaminant, is much less toxic; the other ochratoxins have never been encountered in natural products.

4.1.6.2 *Studies in man*

The ochratoxin A-induced nephropathy in farm animals is similar to Balkan endemic nephropathy in several aspects. In a preliminary study in an area where Balkan endemic nephropathy is prevalent, the ochratoxin A contamination of food appeared to be more frequent than in control areas. However, the hypothesis that ochratoxin A may be a causal determinant in this disease, needs further support.

4.1.6.3 *Evaluation of health risks*

The nephrotoxic potential of ochratoxin A is well documented from all experimental studies, with a feed level of 200 µg/kg causing nephropathy in pigs and rats. Lower levels have not been tested. Field cases of ochratoxin A-induced nephropathy in farm animals have long been recognized. The toxin has been found in a variety of foodstuffs, with levels in commodities used as feed ranging up to 27 mg/kg, and with levels in foodstuffs used for human consumption in the range of trace to about 100 µg/kg. In one area where endemic nephropathy was prevalent in the human population, home produced foodstuffs were more frequently contaminated with ochratoxin A than those from control areas. However, the total intake of ochratoxin A by man has not been assessed so far, and there is, at present, no proof that ochratoxin A is causally involved in human diseases.

4.2 Zearalenone

4.2.1 Properties, analytical methods, and sources

The properties, analytical methods, sources, and occurrence of zearalenone have been reviewed by Mirocha & Christensen (1974), Pathre & Mirocha (1976), and Mirocha et al. (1977). Zearalenone is a phenolic resorcylic acid lactone (Fig. 5), classified, according to biosynthetic origin, as a nonaketide within the group polyketides (Turner, 1971). Zearalenone (C₁₈H₂₂O₃) is a white crystalline compound with a relative molecular mass of 318, melting point 164–165° C, and absorption maxima (and absorption coefficient) at 236 nm (29 700), 274 nm (13 909) and 316 nm (6020).

Zearalenone exhibits blue-green fluorescence when excited by long wavelength (360 nm) UV-light, and a more intense green fluorescence when excited with short wavelength (260 nm) UV-light.

A number of derivatives of zearalenone have been isolated from fungal cultures (Fig. 7), but none of these derivatives has been encountered, so far, as a natural contaminant of foodstuffs.

A multiple detection method for aflatoxin, ochratoxin, and zearalenone has been developed (Eppley, 1968) and tested collaboratively (Shotwell et al., 1976b). The procedure consists of water-chloroform extraction combined with sequential elution of the mycotoxins from a silica-gel column and the detection limit is in the range of 50–100 µg/kg. A versatile method of analysis for zearalenone has been described by Mirocha et al. (1974) using thin-layer chromatography (TLC), gas-liquid chromatography (GLC), gas-liquid chromatography-mass spectrometry, or a combination of all these methods; the limit of detection is about 50 µg/kg. The derivatives

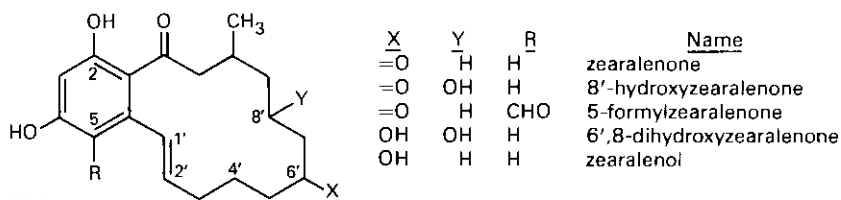


Fig. 7. Zearalenone and selected derivatives.

dimethoxyzearalenone and methyl oxime-di-TMS-zearalenone are used to confirm the identity of zearalenone. Two methods using high pressure liquid chromatography (HPLC) are now available (Scott et al., 1978; Ware & Thorpe, 1978). With HPLC, a concentration of zearalenone in cornflakes of 5 µg/kg could be determined (Scott et al., 1978). Zearalenone is produced by strains of *Fusarium graminearum*, *F. tricinctum*, *F. oxysporum*, *F. sporotrichioides*, and *F. moniliforme*, and a period of low temperature (12°–14° C) during fungal formation seems essential for high yield.

4.2.2 Occurrence

The occurrence of zearalenone in foodstuffs has been reviewed by Stoloff (1976). Zearalenone has been encountered as a natural contaminant, particularly in maize, but occasionally in other cereals and in feedstuffs. In a survey of maize in the USA during the period 1968–69, zearalenone was found in 6 out of 576 (1%) samples at levels ranging from 450 to 800 µg/kg (Shotwell et al., 1971). In 1972, when conditions in the USA were conducive to *Fusarium* ear rot, zearalenone was found in 17% of 223 samples of maize

at levels ranging from 0.1 to 5.0 mg/kg (Eppley et al., 1974). It has also been detected in maize in France and in barley and mixed feed in England, Finland, and Yugoslavia (Stoloff, 1976).

Nine out of 11 commercial corn meal samples in the USA contained zearalenone at levels ranging from 12 to 69 µg/kg (Ware & Thorpe, 1977). The compound has been found in one sample of cornflakes (13 µg/kg) (Scott et al., 1978), and in maize beer, in Zambia, in the range of 0.01–4.6 mg/litre (Lovelace & Nyathi, 1977).

Zearalenone was detected in 11% of 55 samples of Swazi sour drinks, sour porridges, and beers (range: 8–53 mg/kg) and in 12% of 140 beer samples from Lesotho (range: 0.3–2 mg/kg), but such high figures have not been reported elsewhere. No data on consumption were given (Martin & Keen, 1978).

4.2.3 Effects in animals

4.2.3.1 Field observations

The effects of zearalenone in animals have been reviewed by Mirocha & Christensen (1974). Field cases of the estrogenic syndrome in pigs, associated with the use of mouldy feed, were first observed half a century ago. The disease was characterized by enlarged oedematous vulvae and mammary glands (McNutt et al., 1928). Subsequently, this syndrome has been encountered in a number of countries in North America and Europe and in Australia (Table 25), and in most cases zearalenone has been identified in associated feeds, indicating together with the result of experimental studies (4.2.2.2) a causal role of this compound. Zearalenone feed levels reported to be associated with the syndrome in swine (Mirocha et al., 1977) are listed in Table 26. Cases of reduced fertility in cattle indicated by an increase in the artificial insemination index have been reported to be associated with a feed (hay) content of zearalenone of 14 mg/kg (Mirocha et al., 1968b). Fertility

Table 25. Occurrence of the estrogenic syndrome in pigs in various countries^a

Year	Country	Feedstuff
1928	USA	maize
1937	Australia	maize
1952	Ireland	barley
1962	France	maize
1962	Italy	maize
1963	Yugoslavia	maize and barley
1967	Romania	maize
1968	Hungary	maize
1968	Denmark	barley
1971	Canada	maize

^a From: Mirocha & Christenson (1974).

Table 26. Natural occurrence of zearalenone in feeds associated with hyperestrogenism in swine^a

Feed sample	Level of contamination (mg/kg)
maize kernels (Minnesota) ^b	0.1–0.15
dry sow ration (Vancouver)	0.15
farrowing ration (Vancouver) ^c	0.066
dry sow ration (Vancouver)	0.15
corn kernels (Vancouver)	0.20
dry sow ration (Vancouver) ^c	0.25
lactation ration (Vancouver)	1.00
gestation ration (Vancouver)	0.5
milo (Minnesota)	2.5–5.6
sesame meal (Univ. of Minn.) ^d	1.5
corn kernels (Ohio)	0.12
mixed feed corn (Ohio) ^b	0.12
corn kernels (Minnesota)	6.4
commercial pelleted mixed feed (Minnesota)	6.8

^a From Mirocha et al. (1977).

^b Rectal prolapse in gilts.

^c Diethylstilbestrol was also present in these samples.

^d Associated with hyperestrogenism in turkey poults.

disturbances and prolonged heat in a herd of cattle suggested to be associated with zearalenone-contaminated feed were reported by Roine et al. (1971).

4.2.3.2 *Experimental studies*

Under experimental conditions, pigs (6-week-old-gilts) exposed perorally to zearalenone at 5 mg per animal per day for 5 days developed enlarged vulvae and mammae, and prolapse of the vagina within a few days; effects were reversible on termination of exposure. In another experiment, oral administration of 8 mg of pure crystalline zearalenone to 6-week-old, pre-pubertal gilts (8 doses of 1 mg/day per animal) induced pronounced tumefaction of the vulva (Mirocha & Christensen, 1974). Histological changes in the genital tract of pigs exposed to zearalenone included metaplasia of the epithelium in the cervix and vagina, and oedema in the wall of the uterus (Kurtz et al., 1969).

Observations on pigs (Miller et al., 1973) suggested that ingestion of grain contaminated with zearalenone during late gestation might be related to still-birth and splayleg. Splayleg was observed in the offspring of one sow and one gilt given daily intramuscular injections of zearalenone at 5 mg per animal throughout the last month of pregnancy. However, in the experiment of Patterson et al. (1977), all 7 gilts fed zearalenone at a level of 2 mg/kg feed throughout pregnancy remained clinically normal and embryonic survival rates were not affected by the toxin. Splayleg was diagnosed in only 1/63 piglets and the condition appeared to have resolved

within 24 h. Results in the 2 groups of 3 pigs fed the lower levels were inconclusive (unpublished data, Chang & Kurtz, quoted by Mirocha et al., 1977).

No effects on egg production were observed when laying hens were fed rations containing zearalenone levels of 250 and 500 mg/kg (Speers et al., 1971).

In a 2-generation study on rats exposed to dietary levels of zearalenone corresponding to daily intakes of 0.1, 1.0, and 10 mg/kg body weight, no teratogenic effects were observed at any level of intake but impaired fertility and resorptions and stillbirths occurred in animals receiving 10 mg/kg body weight daily with 56% of dams showing complete litter resorption (Bailey et al., 1976).

Ruddick et al. (1976) found fetal skeleton anomalies in rats exposed orally to zearalenone at doses ranging from 1–10 mg/kg body weight during the gestation period, with defect incidences of 12.8% (11/86) at the 1 mg/kg level, 26.1% (18/69) at the 5 mg/kg level, and 36.8% (28/76) at the 10 mg/kg level. No effect was observed with exposure to zearalenone at a dose of 0.075–0.30 mg/kg body weight.

The results of a study by Mirocha et al. (1968a) in which the dose-effect relationships for zearalenone and estrone were compared with regard to increase in uterus weight, are given in Table 27. The results of a study

Table 27. Effect of peroral dosing of zearalenone and estrone in mice^a

Maternal administered	Total dose (µg)	No. of mice	Mean uterine ratio ± S.E.
control	0	10	0.76 ± 0.06
estrone	0.5	10	1.21 ± 0.12
	1	9	1.19 ± 0.10
	2	9	2.12 ± 0.23
	4	10	2.93 ± 0.21
	12.5	10	1.11 ± 0.04
zearalenone	25	10	1.48 ± 0.18
	50	10	1.80 ± 0.12
	100	9	2.35 ± 0.17

^a From: Mirocha et al. (1968a).

comparing the effects of estrogens and zearalenone in 24 adult female castrated rhesus monkeys with a previous history of regular menstrual cycles are recorded in Table 28.

4.2.4 Conclusions and evaluation of the health risks to man of zearalenone

4.2.4.1 Animal studies

Field cases of the estrogenic syndrome in pigs have been encountered in many countries in association with zearalenone in feeds at levels ranging from 0.1 to 6.8 mg/kg (Table 26), in samples not known to be contaminated

Table 28. The estimated minimum dose of estrogens that will depress serum gonadotropin (FSH or LH) in castrated rhesus monkeys^a

Estrogen administered	Estimated minimum dose (µg/kg)			
	Subcutaneous ^b		Oral ^c	
	LH ^d	FSH ^d	LH	FSH
estradiol-17β	4	1	5	—
diethylstilboestrol	0.5	2	2.5	—
zearalenone	14	56	400	—

^a From: Hobson et al. (1977).

^b Two injections given in oil.

^c Given on 4 consecutive days.

^d FSH = follicle-stimulating hormone; LH = luteinizing hormone.

with other estrogens. The condition has been reproduced experimentally in pigs, with a daily dose of 1 mg/animal for 8 days. Infertility, sporadically encountered in cattle, has been suggested to be causally associated with zearalenone in feed at a reported level of 14 mg/kg of hay.

In one of two studies on teratogenic effects in rats, skeletal defects were detected in fetuses at a daily oral dose of 1 mg/kg body weight.

4.2.4.2 Evaluation of health risks

There are no reports on the adverse effects of zearalenone in man. With the exception of 2 reports from Africa, levels of zearalenone ranging from 12 to 69 µg/kg have been found in a limited number of maize products destined for human consumption. Even assuming that 1 kg of these products is consumed daily, it can be estimated that a 70 kg man would not receive more than 1 µg zearalenone per kg body weight in food. This level of exposure is 400 times lower than the lowest peroral dose causing effects in tests on monkeys (Table 28) and more than 600 times lower than the lowest dose (µg/kg) used in assessing the estrogenic potency of perorally administered zearalenone in mice (Table 27).

However, in the 2 reports from certain parts of Africa, high levels of zearalenone were found in beer and sour porridge prepared from maize and sorghum. Long-term exposure to such contaminated drinks could represent a health hazard.

4.3 Trichothecenes

4.3.1 Properties and sources

The properties and sources of trichothecenes have been reviewed by Bamburg & Strong (1971), Smalley & Strong (1974), and Bamburg (1976).

The trichothecenes possess the tetracyclic 12,13-epoxytrichothec-9-ene skeleton. More than 30 trichothecene derivatives have been isolated from fungal cultures, but, so far, only 4 have been identified as natural contaminants of foodstuffs (Table 29). The compounds have been detected by various methods including thin-layer chromatography, gas chromatography, and bioassays, in particular the rabbit skin test.

One or more of the trichothecenes have been isolated from strains of the following *Fusarium* species: *F. episphae*, *F. lateritium*, *F. nivale*, *F. oxysporum*, *F. rigidisculum*, *F. solani*, *F. roseum*, and *F. tricinctum* (syn. *F. sporotrichioides*). In addition, species of *Cephalosporium*, *Myrothecium*, *Trichoderma*, and *Stachybotrys* have been found to produce trichothecenes.

Table 29. Natural occurrence of trichothecenes

Compound	Concentration (µg/kg)	Commodity	Reference
T-2 toxin	2	maize	Hsu et al. (1972)
T-2 toxin	2.5	barley	Puls & Greenway (1976)
T-2 toxin	0.076	mixed feed	Mirocha et al. (1976)
nivalenol	n.q. ^a	barley	Morooka et al. (1972)
deoxynivalenol	7.3	barley	Morooka et al. (1972)
deoxynivalenol	n.m. ^b	maize	Vesonder et al. (1973)
deoxynivalenol	0.1, 1.0, 1.8	maize (3 samples)	Mirocha et al. (1976)
deoxynivalenol	1.0, 1.0, 0.06	mixed feed (3 samples)	Mirocha et al. (1976)
diacetoxyscirpenol	0.38, 0.5	mixed feed (2 samples)	Mirocha et al. (1976)

^a n.q. = not quoted

^b n.m. = not measured

4.3.2 Occurrence

So far, the trichothecenes have only been found very sporadically in natural products. Naturally occurring trichothecenes include the following, based on chemical identification: T-2 toxin, nivalenol, deoxynivalenol, (vomitoxin), and diacetoxyscirpenol. In available studies, these 4 compounds have only been found in a total of 14 samples (Table 29), sometimes (as in the case of deoxynivalenol) concomitantly with zearalenone. No information is available concerning the number of samples analysed in these studies and therefore on the frequency of positive results.

4.3.3 Effects in animals

4.3.3.1 Field observations

A field case involving the death of 20% of a dairy herd was suggested to

: associated with ingestion of mouldy corn in the feed containing a concentration of T-2 toxin of approximately 2 mg/kg dry weight (Hsu et al., 1972). The lesions in the cattle included extensive haemorrhages on the serosal surface of all internal viscera.

An outbreak of a disease, observed in poultry (ducks, geese), horses, and pigs, was suggested to be associated with mouldy barley containing T-2 toxin at approximately 25 mg/kg (Greenway & Puls, 1976). The lesions in the geese included necrosis of the mucosa of the oesophagus, proventriculus, and gizzards.

Deoxynivalenol (vomitoxin) was isolated from a batch of maize that had caused vomiting in pigs (Vesonder et al., 1973).

4.3.3.2 *Experimental studies*

Acute effects of trichothecenes determined as LD₅₀ values, are listed in Table 30. Sato et al. (1975) studied the effects in cats of T-2 toxin,

Table 30. Acute toxicity of naturally occurring trichothecenes^a

Compound	LD ₅₀ (mg/kg body weight)	Route of administration	Animal
T-2 toxin	3.04	intraperitoneal	mouse
T-2 toxin	3.8	peroral	rat
T-2 toxin	6.1	peroral	trout
T-2 toxin	5.25	peroral	one-day old chick ^b
nivalenol	4.0	intraperitoneal	mouse
diacetoxyscirpenol	10.0	intravenous	mouse
diacetoxyscirpenol	0.75	intraperitoneal	rat
diacetoxyscirpenol	7.3	peroral	rat

^a From: Bamburg & Strong (1971).

^b From: Chi et al. (1977).

administered in several ways. Two cats weighing 1.5 and 4.0 kg were given purified T-2 toxin in single subcutaneous doses of 0.5 and 1.0 mg/kg, respectively. Nausea and vomiting appeared after 1 h and the animals died 20 h after the injection. The autopsy revealed extensive necrosis of the mucosa in the small and large intestine, marked karyorrhexis in the germ centre of the lymph follicles of the spleen and lymph nodes, and diffuse vacuolar degeneration of the renal tubules. Damage to bone marrow cells was observed in the cervical, thoracic, and lumbar vertebrae. In two cats subcutaneously injected with T-2 toxin (repeated doses of 0.1 and 0.05 mg/kg body weight, given over a 4-week period) a decrease in the white blood cell (WBC) count was observed and the WBC value remained low until death occurred during the fourth week of exposure. The clinical signs in the cats were nausea and vomiting a few hours after each injection and ataxia of the hind legs. At the postmortem examination, hypoplasia was observed in the thymus, spleen, lymph nodes, and bone marrow. Other

changes included marked meningeal haemorrhage, extensive bleeding in lung, and vacuolar degeneration of the renal tubular epithelium. A marked decrease in the WBC values was also observed in 3 cats given T-2 toxin subcutaneously at a daily dose of 0.05 mg/kg for 12 days (i.e., total dose 0.60 mg/kg); 2 of these cats died, 4 and 35 days, respectively, after the last injection and one cat survived with the WBC count returning to the normal level 17 days after the last injection. Leukopenia and death were also observed in 3 cats (body weight 2–3 kg) receiving a crude preparation containing 4% T-2 toxin and 1% neosolaniol. This crude preparation was first administered subcutaneously once a week for 5 weeks in repeated doses of 1 mg/kg body weight (corresponding to a repeated dose of T-2 toxin of 0.04 mg/kg). This subcutaneous administration was then followed by daily oral dosing (15 mg of the crude preparation corresponding to 0.6 mg of T-2 toxin per animal per day) for 17 days.

More recently Yagen et al. (1978) have mentioned an experiment, in which oral administration of gelatin capsules containing purified T-2 toxin to 10 cats resulted in vomiting, leukopenia, haemorrhagic diathesis, neurological disturbances, and death. The dose and frequency of administration are not given in the paper.

The effects observed in these experiments, in particular leukopenia, resemble those produced when cats are fed cultures of *F. sporotrichioides*, which is thought to be causally associated with alimentary toxic aleukia (ATA) in man (section 4.3.4).

Feeding laying hens T-2 toxin at a dietary level of 20 mg/kg for 3 weeks, resulted in oral necrotic lesions, decreased leukocyte count, and reduced egg production (Wyatt et al., 1975). In mice, intraperitoneal injection with T-2 toxin at doses of 1.0 and 1.5 mg/kg body weight on one of the days 7–11 of gestation resulted in a number of maternal deaths as well as in an increase in prenatal mortality. Malformations of the fetuses were observed including tail and limb malformations, exencephaly, and retarded jaw development (Stanford et al., 1975).

Daily oral doses of crude or purified T-2 toxin (0.2 mg/kg body weight) continued for 79 days failed to produce ill effects in calves, although the total amount of toxin ingested by one calf was almost 1.8 g (Matthews et al., 1977).

4.3.4 Alimentary toxic aleukia

Studies on alimentary toxic aleukia (ATA), a disease encountered in man in the period 1931–43, have been reviewed by Sarkisov (1954) and more recently by Bilai (1977) and Leonov (1977). The dominant pathological changes were necrotic lesions of the oral cavity, the oesophagus, and stomach, and in particular a pronounced leukopenia. The disease was lethal

in a high proportion of cases. An association was established with ingestion of grain invaded by some moulds, in particular *Fusarium poae* and *F. sporotrichioides*. Effects similar to ATA have been reproduced in cats by feeding cultures of these species (Bilal, 1977). T-2 toxin and other trichothecenes have been identified in a submerged culture of *F. sporotrichioides* by Mirocha & Pathre (1973).

4.3.5 Conclusions and evaluation of the health risks to man of trichothecenes

In recent years, a group of mycotoxins, the trichothecenes, has been isolated under experimental conditions from many fungi, including *Fusarium* species. Isolated field cases of intoxication in farm animals have been suggested to be related to some of the trichothecenes.

About 40 years ago, a disease in man known as alimentary toxic aleukia (ATA), occurred that was suggested to be related to the presence of toxic *Fusarium* species in mouldy over-wintered grain. With improved harvesting, food production, and storage conditions, the disease disappeared and no new outbreaks have occurred. However, present knowledge is not sufficient to establish a causal relationship between any of the isolated trichothecenes and this outbreak. There are no reports on the exposure of man to trichothecenes.

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