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PAST AND FUTURE IN MYCOTOXIN TOXICOLOGY RESEARCH

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Abstract - Before 1960 the toxicology of mycotoxins was mainly of concern to veterinarians, since outbreaks of mycotoxicoses resulted occasionally in considerable losses of livestock. By a wider use of biotests, preferably in mammals a further decline of such intoxications probably will occur. Following the discovery of the carcinogenicity of some aflatoxins the focus turned to human health. Screening tests for carcinogenicity are still in full development. The test used most frequently is the Ames-test on microorganisms. Unfortunately many problems still must be resolved, before an extrapolation of results from these tests to man becomes possible. The examination of the carcinogenic activity of mycotoxins in long-term animal experiments is often difficult due to lack of resources, lack of test material and the toxicity of the compounds which precludes the administration of sufficiently high dose levels. The available data regarding a possible carcinogenic activity of several important mycotoxins such as the trichothecenes or patulin do not fulfil the currently used criteria. Therefore further studies are needed. A new approach is the determination of the binding capacity to DNA of suspected carcinogens, which seems to correlate well with the carcinogenic potency. By this method a high carcinogenicity of aflatoxin M_1 can be deduced, however, the macromolecular bound residues of aflatoxin B_1, which may occur in tissues of domestic animals most probably do not show a carcinogenic activity. Although many questions are still unanswered, it seems that the numerous mycotoxins identified since 1960 are less toxic or carcinogenic and occur less frequently in food than aflatoxins.

Before 1960 knowledge of the toxic effects of mycotoxins originated mainly from intoxications in man and domestic animals and only occasionally were further toxicological and chemical studies subsequently initiated to elucidate the cause of the disease.

Especially in the late medieval age the mycotoxicosis called ergotism led to thousands of victims. This disease, which is due to contamination of rye with claviceps purpurea, is characterized by central nervous symptoms such as tinnitus, convulsions and painful vascular spasms which eventually lead to necrosis of fingers and toes. At that time relief from pain was not sought from scientists but from the religious. Monasteries and places of pilgrimage were found which were dedicated to Saint Anthony. This explains the other frequently used names of the disease "Saint Anthony's fire" or "Holy fire". The causal relationship between ingestion of contaminated rye bread and the disease was discovered as early as 1673 by the French scientist Denis Dodart. However, the disease continued to occur regularly for at least a further century. Today accidental contamination occurs rarely and the symptoms are generally weak. Various alkaloids with a common basic structure are responsible for the symptoms (for a comprehensive review see (1)).

Another important mycotoxicosis examined before 1960 is the toxic yellowed rice disease (2) which was encountered especially after World War II in Japan. Since its toxicity was known such contaminated material was either used for industrial production of alcohol or was blended with intact material, so no human fatalities occurred. However, once when contaminated rice was given to chickens 3 000 out of 14 000 died from acute liver damage. One gram of rice-cake contained as many as 15 000 colonies of Penicillium islandicum, the responsible toxic substances most probably being lukeoskyrin and cyclochlorotine, both of which have been shown to be hepatotoxic and hepatocarcinogenic in experimental animals. A connection between the high rate of liver diseases and liver cancer in certain areas of Asia and the intake of rice contaminated with these mycotoxins cannot be disproved unequivocally.

Another mycotoxin found in yellow rice is citreoviridin. This was claimed to be the cause of cardiac beriberi, a cardiovascular disease which occurred in Japan during the second half of the nineteenth century.

A further important disease was the alimentary toxic aleukia which was mainly observed in people of eastern countries eating grain which had been left in the field over winter and so became contaminated by Fusaria species (3). The most toxic substances produced by these fungi are trichothecenes, and since they are highly cytotoxic the first symptoms arise at the place of contact - stomatitis and gastrointestinal irritation accompanied by bloody diarrhoea.

Several decades ago a similar disease called stachybotryocosis was responsible for heavy losses among horses in Russia (4). A second class of biologically active compounds from Fusaria is represented by the zearalenones. They exhibit an estrogenic activity and are responsible for many cases of infertility, especially in pigs (5). Even now damage to livestock produced by various Fusarium-toxins is frequent and of considerable economical importance.

There is no other group of chemicals which caused such a high number of deaths in the past than the mycotoxins. Today the situation has changed, now the forerunner is cigarette smoking. But most probably even now victims of mycotoxins, especially among domestic animals, are much more frequent than deaths due to other chemicals such as pesticides, industrial or environmental chemicals.

Following the discovery of the prominent toxic and carcinogenic properties of the aflatoxins in the early 1960s experimental studies were initiated even on mycotoxins, where no clear previous evidence existed on their adverse effects on man and animals under real conditions of life. In contrast to the early phase of retrospective toxicology, this new approach can be characterized as preventive toxicology. To fulfil this highly needed but ambitious task certain requirements must be met:

1º Availability of test systems which are able to detect possible adverse effects in man and domestic animals.

2º Knowledge of the exposure (occurrence and concentrations in various food and feed items).

3º Transmission of scientific conclusions into practical measures.

The following discussion will focus on toxicological test systems, since this is the main area of the toxicologists involvment. However, some contributions from toxicologists are also needed in area 2 and especially 3.

A suitable test system for screening many samples should be broad in scope, rapid and not too expensive. False positive results are more acceptable than false negative ones. By using classical techniques of experimental toxicology positive results have to be confirmed and elaborated in details. The basis for a selection of a screening test is given by the expected adverse effects. This differs in veterinary medicine and in human public health as generally the quality of animal diets is lower than that for man. Consequently the concentrations of mycotoxins in animal feed will be higher, so that there is a definite probability of overt organ damage and severe acute or subacute illness. On the other hand, in man subclinical effects are more likely. Among such effects the most important one regarding human health is the possible carcinogenicity of mycotoxins, a problem which can be neglected in animal health. Since the intake of low levels of a carcinogens for years can finally lead to cancer, even without producing apparent organ damage, such effects potentially could arise from mycotoxins.

Therefore screening tests for organ toxicity as well as for carcinogenicity are needed.

The most widely used organisms for screening tests on organ toxicity in mycotoxicology are (6):

- lower organisms such as Zebra fish larvae (7) or brine shrimps (Artemia salina) (8)
- cultured mammalian cells (9)
- rabbit skin tests (10)
- chicken embryos (11, 12)
- laboratory rodents (mice and rats)

Although the tests on lower organisms or on rabbit skin perform very well with certain classes of mycotoxins, e.g. with Fusaria-toxins (13, 10), they do not respond to several other important compounds such as luteoskyrin and griseofulvin and only moderately to citrinin, patulin, penicillic acid and zearalenone (8). Since physiology and biochemistry of the lower organisms differ greatly from those of mammals, a lack in conformity is not surprising. A disadvantage of the Artemia salina-test is its susceptibility to fatty acids (14). When crude extracts are examined this has to be taken into account. Skin tests on rabbits or other rodents, mammalian cell systems in culture or chicken embryos are able to detect general cytotoxicity. However, effects due to metabolism, absorption, distribution and elim-

ination are not predictable by these systems.

The toxicity of anthraquinone pigments, which we recently isolated from A. glaucus species typically exemplifies this point - they exhibit a high cytotoxicity, are toxic to chicken embryos, and produce lethal local irritations after intraperitoneal injection to mice. No toxicity, however, was found after oral administration, because the compounds are not absorbed from the gastrointestinal tract (15).

A similar cytotoxic effect is shown by the trichothecenes. However, since they are absorbed from the intestine, they also lead to important organ toxicity, the most affected being the rapidly dividing cells in the body, namely the blood forming cells in the bone marrow and the lymphoid organs (16).

All these observations are in favour of performing even preliminary toxicological tests in rodents. Most mycotoxins exhibit a high acute toxicity especially when they are administered by the intraperitoneal route. The LD_{50} is generally lower than 100 mg/kg bw (17). Therefore, performance of tests on acute lethality with extracts of cultures or moulded material is a suitable method for screening purposes. Since there is no need for numerically precise results, the tests can be done with no more than three animals per dose group. In addition there is no reason for not using the same surviving animals several days later for another test. It must be kept in mind, however, that there are some mycotoxins with low acute toxicity - for instance the zearalenones or mycophenolic acid. Such compounds may be missed by the proposed screening procedure. It is likely that a wider use of mammalian biotests will help to reduce outbreaks of mycotoxicoses in animals.

Probably the most important mycotoxins detected in the course of screening programs were the ochratoxins and citrinin, compounds which are responsible for nephrotoxic lesions in pigs. Ochratoxin is possibly also the cause of Balkan endemic nephropathy in humans.

A much more difficult task is the evaluation of possible carcinogenic effects of mycotoxins. For classical long-term bioassays several hundreds of experimental animals are needed. The studies last for up to three years and the interpretation of the results is far from being simple. Nevertheless, results from carefully conducted studies constitute an acceptable basis for the evaluation of risk to man. However, the long-term tests are completely inappropriate for screening purposes.

Based on the widely accepted somatic mutation theory for the induction of at least a considerable part of cancers, tests where the mutagenic activity of a chemical is examined have been proposed for carcinogenicity screening. It is believed that as a first step the genotypic chemical carcinogens bind to DNA (18). Such DNA-damages can then lead to somatic mutations which among others can manifest themselves as malignant transformations. The most famous among these mutagenicity tests is the so called Ames-test on S. typhimurium. Although the qualitative correlation between mutagenicity in microorganisms and carcinogenicity in mammals is surprisingly good (19), the existence of a quantitative relationship is highly questionable (20). As a general rule therefore substances showing positive results in short-term tests are subjected to long-term animal studies.

Performance of appropriate long-term studies with mycotoxins is hampered for various reasons:

a) lack of resources

b) lack of physical facilities and technical expertise

c) lack of test material

d) impossibility for use of high dose levels due to toxicity

It is not surprising therefore that for many mycotoxins, which showed positive results in short-term tests, acceptable long-term bioassays are still lacking.

Various short-term tests have been conducted with mycotoxins. Among the most important mycotoxins the following ones were positive in at least one of these tests:

aflatoxins	citrinin
sterigmatocystins	penicillic acid
(-) luteoskyrin	patulin
(+) rugulosin	mycophenolic acid
cyclochlorotine	several anthraquinones
zearalenones	

For aflatoxins, sterigmatocystins, (-) luteoskyrin, (+) rugulosin and cyclochlorotine long-term tests are available (21). Although they do not fulfil in every respect the currently used criteria they are acceptable, since the potency of these compounds overwhelms the

experimental deficiencies such as small group size or low dose levels. All these five compounds induced mainly liver tumours in rodents.

Zearalenone and zearalenol-b were found to be positive in the so-called Rec-assay on B. subtilis (22). Although this test is positive for many carcinogens it cannot yet be regarded as validated by results from a sufficiently high number of carcinogens and non-carcinogens (20). Therefore, its predictive value is still uncertain. Zearelenone was negative in the Ames-test on S. typhimurium and also in a test on S. cerevisiae (23, 24). If in future results from long-term tests become available, the fact that estrogens generally induce an increase of tumours must be considered (25). It is believed that such an activity is linked to the hormonal action of the compounds. At low concentrations, where such an action no longer exists, probably no tumour induction will take place. So there seems to be no danger for consumers ingesting minute amounts of zearalenones.

Citrinin was positive in the Rec-assay (22), but negative in S. typhimurium and S. cerevisiae (23, 24). However, according to results from Ito kidney tumours have been found (22). This finding is difficult to evaluate in regard to human health. There are several examples of compounds, not otherwise regarded as carcinogens, such as NTA (26) or lead acetate (27), which produce kidney tumours when they are given in dose levels producing severe kidney damage. Citrinin too is a powerful nephrotoxin. Another argument against a specific carcinogenicity of citrinin is given by its high polarity due to the carboxylic group. Most if not all chemical carcinogens are lipophilic. This property enables them to cross the cell membrane and to penetrate into the cell nucleus for interaction with DNA. It is very improbable that citrinin will ever reach the cell nucleus.

Ochratoxin A was tested in the Rec-assay (22), in a mammalian cell line for inducing point mutations (8-azaguanine resistence in FM_3A-cells (9)) and in S. typhimurium and S. cerevisiae (23); it was found to be negative. The available long-term tests on rodents were negative but the studies were not adequate regarding the number of animals, survival rates, low dose levels (21). The chemical structure does not contain elements which raise a suspicion of possible carcinogenic activity.

Trichothecenes did not induce an increased number of recessive lethals in drosophila (28). Several trichothecenes were negative in the Rec-test (22), with and without activation, in the Ames-test on S. typhimurium (24, 23) and on S. cerevisiae (23); in addition Fusarenon X was also negative in the test on mammalian cells (9). In another study, however, Fusarenon X was found to be positive in the Ames-test with strain 100 without enzymatic activation at the rather high concentration of 0.5 - 1 mg/plate (29). This result hints to a direct alkylation of DNA by the parent compound. Indeed such a possibility exists due to the epoxide group of the trichothecenes. Many carcinogenic compounds such as aflatoxin, benzo(a)pyrene or vinylchlorid are oxidized in vivo to an epoxide which then in turn reacts with DNA (18). The reactivity of the epoxide group of the trichothecenes was used for the development of a new identification method (30): on thinlayer chromatogrammes 4-(p-nitrobenzyl)pyridine was found to react rapidly with trichothecenes. This compound was introduced several years ago as a prescreening test for the detection of directly acting carcinogens (31). A further indication for the alkylation of biomolecules is the irreversible depigmentation of dark mouse hair (32). Many carcinogens were found to produce depigmentation. The effect probably is due to reaction with thiolcompounds. The symptoms observed after repeated administration of T-2 toxin also raise some suspicion about a possible carcinogenic action of this group, the effects being depression of bone marrow and lymphoid tissues (16). These signs resemble the picture seen after the administration of DNA damaging agents such as ionizing radiation or cytostatics. The existing long-term tests (33) are not adequate, again for the same reasons as stated already for ochratoxin - small number of animals, low survival rate, low dose levels. So the question of the safety of low levels of trichothecenes still remains to be solved.

The case of patulin deserves some discussion. By its lactone group it could very well exhibit some alkylating activity, which is regarded as an essential prerequisite of a chemical carcinogen. The compound was negative in the Ames-test and on S. cerevisiae (23, 24); dominant lethal tests in mice and rats were negative too (34, 35). However, positive results were found in the Rec-assay (22) and in the mammalian cell test (9); it also induces chromosomal breaks (36). In rodents patulin produced locally sarcomas after subcutaneous injection (37). However, nowadays this effect is no longer believed to be a relevant one (21), since rodents generally develop sarcomas even after unspecific irritation of the subcutaneous tissues. Feeding experiments in rodents were negative (38). Unfortunately in the most recent carefully conducted and reported study (39) the dose level administered twice weekly by stomach tube was not more than 2.5 mg/kg. This shows that patulin is certainly not a strong carcinogen, but that a weak activity cannot be excluded. Not only shortage of pure patulin, but also its high toxicity, precluded the use of much higher dose levels. These results have to be viewed in the light of potential exposure of humans. Commercial apple juices sometimes contain up to several hundreds of ppb (40). Thus, an intake of 1 - 10 µg/kg bw twice a week, that is 250 -

2500 times lower than the inactive dose in rat, is quite possible. One would like to have a higher safety factor for potential carcinogens.

Similar toxicological results as with patulin were found with penicillic acid (9, 21, 22, 23, 24). However, since presumably the exposure to this substance is much lower, further examination of this compound has not a very high priority.

Another case, where due to lack of material, adequate long-term tests in rodents have not been performed yet, is that of aflatoxin M_1. Recently in our laboratory we tried to compare the oncogenic activity of aflatoxin B_1 with that of M_1 by using a biochemical approach. The genotypic carcinogens are believed to induce cancer by binding to DNA (18), therefore a correlation should exist between the extent of DNA-binding and the carcinogenic potency. Determination of binding should give some indication of the carcinogenic potency. The binding capacity is expressed as Covalent Binding Index (CBI), which is defined as

$$\frac{\mu\text{mole chemical bound / mole DNA-phosphate}}{\text{mmole chemical / kg body weight}}$$

By examination of the binding capacity of various carcinogens the postulated correlation was verified in our laboratory (for review see Lutz (41)):

TABLE 1. Effectiveness of covalent binding of several xenobiotics to rat liver DNA

Compound	Route	CBI
Strong hepatocarcinogens		
Aflatoxin B_1	p.o.	10 000
Carcinogens, but not hepatocarcinogens		
Benzo(a)pyrene	i.p.	10
Benzene	inhal.	1.7
Doubtful or non-carcinogens		
Ethinyloestradiol	p.o.	1.5
Oestrone	p.o.	1.1
Toluene	inhal.	0.04
Saccharin	p.o.	0.005

For the determination of the binding of aflatoxin M_1 we administered orally to rats radioactively labelled aflatoxin M_1, which was obtained biosynthetically. The CBI of aflatoxin M_1 6 hours later was 1600 (Table 2) (42). Since most probably AFB_1 and M_1 bind to the same sites on DNA a similar pattern of somatic mutations is expected, however, with M_1 the frequency is lower by a factor of 6. With the experimentally suppported assumption of a linear dose-response relationship one can deduce from these results a carcinogenicity of M_1, which is 5 - 10 times lower than that of B_1. But compared with other hepatocarcinogens aflatoxin M_1 still belongs to the strong hepatocarcinogens, therefore the human exposure should be kept as low as possible.

We also determined the DNA-binding of aflatoxin B_1 in different species in order to have some indication of species differences (Table 2) (42).

TABLE 2. Covalent binding indices (CBI) of orally administered aflatoxins in liver-DNA of different species

Compound	Species	CBI
Aflatoxin B_1	rat	10 000
	pig	13 000
	mouse	250
Aflatoxin M_1	rat	1 600

In the mouse binding was much lower than in rats. This is in accordance with the well-known low susceptibility of mice to the carcinogenic action of aflatoxin B_1. Pigs bind aflatoxin B_1 even more than rats. It is difficult to predict where man will be situated, but since mouse liver differs in many other respects from that of other mammalian species, it is more probable that man reacts like rat and pig.

Another application of the DNA-binding test was the determination of a possible carcinogenic activity of macromolecular bound aflatoxin-residues in liver. Such residues are of considerable importance since about 10 % of ingested aflatoxin is bound to liver macromolecules and their biological half live is several days (43).

The main steps of the experiment are outlined in figure 1:

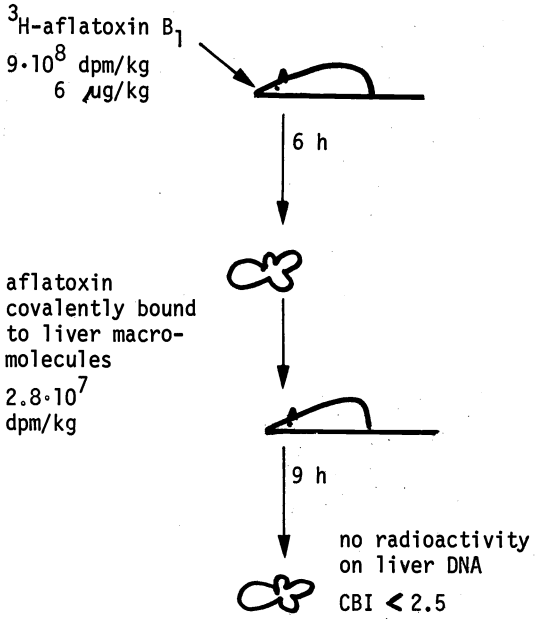

Fig. 1. Binding to liver DNA of aflatoxin residues.

^{3}H-aflatoxin B_1 was orally administered to a rat. After 6 hours the animal was killed and the compounds of low molecular weights were separated from the liver macromolecules by extraction with organic solvents and by dialysis. The macromolecular fraction containing the bound aflatoxin residues was subsequently administered to a second rat. No radioactivity was detectable on the liver DNA of the second rat (44). This means, that the binding capacity of these macromolecular metabolites is at least 4 000 times less than that of aflatoxin B_1. Obviously even after digestion of the macromolecules no aflatoxin-derivatives were formed which could still react with DNA.

By this relatively simple experiment good evidence for the harmlessness of these liver residues was obtained.

We hope that further use of this and similar biochemical approaches could help to answer the many open questions on the carcinogenicity of the mycotoxins discussed above. In addition a wider use of the well established validated short-term tests is advisable especially in the field of mycotoxicology where long-term tests cannot easily be performed. It should be recognized, however, that the probability for the detection of mycotoxins with similar high carcinogenic potency as aflatoxins is quite low, since such compounds would have been found even with simple and not much sophisticated methods. On the other hand due to a considerable exposure of certain population groups to mycotoxins even a low carcinogenic activity could constitute a concern for public health.

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