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SHORT-TERM TESTS AND MYCOTOXINS

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Abstract - The hepatocarcinogenicity of aflatoxins and sterigmatocystin in a variety of animal species and probably, in man, has led to a search for other carcinogenic fungus derived products. Different biological end-points have been proposed for the various short-term tests designed to detect potential carcinogens: DNA-binding of carcinogens or their metabolites, mutagenicity, induction of DNA repair, enhancement of biphenyl 2-hydroxylase, degranulation of rat liver endoplasmic reticulum, cytogenetic alterations and *in vitro* cell transformation. Based on recent data in the literature, these short-term testing procedures, which appear to be useful for the detection of potential carcinogens and mutagens, and which can be used in the studies of the mechanism of action of chemical carcinogens and mutagens, are limited in that some of the factors which determine the process of cancer development cannot so far be duplicated. Positive results in short-term tests cannot automatically be taken to imply a definite carcinogenic activity in man. Consequently, various short-term tests can be utilised to trace carcinogens or mutagens in man's environment or for prescreening those compounds to be tested for carcinogenic action in animals.

Naturally-occuring fungal metabolites such as aflatoxins and sterigmatocystin are specific examples of toxic and hepatocarcinogenic environmental contaminants which are active in a variety of animal species and probably, in man (Ref. 1). The extreme biological potency of some of these substances has led to a search for other carcinogenic, fungus-derived products. Although long-term tests in animals are at present the only means of proving the carcinogenicity of a chemical, various short-term tests can be utilised to trace carcinogens or mutagens in man's environment or for prescreening those compounds to be tested for their carcinogenic action in animals (Ref. 2). Such pre-selection is necessary in view of the immense number of fungal species and strains and their products that could enter or which already persist in the environment, and in view of the cost and length of animal experiments.

Before discussion of specific applications of short-term tests in the detection of adverse biological effects of mycotoxins, the relevant short-term tests currently being used are reviewed briefly.

Different biological end-points have been proposed for the various short-term tests designed to detect potential carcinogens: DNA-binding of carcinogens or their metabolites, mutagenicity, induction of DNA repair, enhancement of biphenyl 2-hydroxylase, degranulation of rat liver endoplasmic reticulum, cytogenetic alterations and *in vitro* cell transformation. Most of these end-points are based on the interaction of electrophilic, carcinogenic or mutagenic metabolites with nucleic acids and proteins (Ref. 3). A question currently under discussion is whether the recently developed short-term tests in which mammalian metabolism is taken into account can be of value in a long-term programme of cancer prevention which involves the identification of, and minimizing human exposure to, environmental carcinogens and mutagens by systematic screening of the environment. In this respect, two major aspects of currently used short-term tests will be emphasized: (a) their predictive value for assessing possible carcinogenic risk of chemicals and (b) their utility for investigating the mode of biological action of chemicals in mammals and in man.

The somatic-cell theory of carcinogens put forward by Boveri (Ref. 4) and Bauer (Ref. 5), has recently gained attention, since it is becoming evident that the majority of chemical carcinogens, which had been considered non-mutagenic, are mutagens and that many mutagens, the carcinogenicity of which has not yet been investigated by long-term testing, have now been shown to be carcinogens. This convergence of mutagens and carcinogens has resulted from the discoveries that many such chemicals require metabolic activation in order to exert their biological activity and that reaction with DNA is common to the majority of chemical mutagens and carcinogens (Ref. 6). These similarities have led to the suggestion that there is an

empirical relationship between carcinogenesis and mutagenesis.

Many of the mutagenicity systems currently used involve different genetic indicators, ranging from microorganisms to mammals, in combination with an *in vivo* or an *in vitro* activation system. Each system has its individual advantages and disadvantages, because of the limited range of genetic lesions which can be detected in a given assay (Ref.7). Since none of the genetic alterations can be linked to the onset of carcinogenic processes in mammalian cells, the validity of these tests can be ascertained only by screening carcinogenic and non-carcinogenic classes of chemicals.

The bacterial mutation system with *Salmonella typhimurium* strains uses histidine auxotrophic mutants, developed by Ames *et al.* (Ref.8), which revert to prototrophy by single base-pair substitution or frame-shift mutations. Most of the theoretically possible types of point mutations can be detected with a set of these tester strains in which additional mutations in one of the genes for excision repair enzymes has increased the sensitivity. Penetration of larger molecules through the bacterial cell wall is facilitated by the use of deep-rough mutants with an altered lipopolysaccharide coat. The introduction of plasmids carrying antibiotic-resistance genes increases the rate of mutations induced by certain chemicals. (Ref. 9).
The genetically well-defined *Escherischia coli* WP2 and K12 strains are also utilised for the detection of mutagens (Ref. 10-11) . Fungi such as *Neurospora crassa* permit a refined genetic analysis of the spectrum of mutations induced by carcinogens or mutagenic chemicals (Ref. 12). Yeasts, such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*, permit the scoring not only of forward and reverse mutations but also of gene conversion and mitotic recombination, genetic events which occur in mammalian cells as well (Ref. 13).

The use of *Drosophila melanogaster* has recently received much attention, since the work of Vogel (Ref. 14) has shown that of the variety of genetic lesions which can be measured, recessive lethal mutations correlate best with the carcinogenic activity of chemicals. It has been demonstrated with various classes of different groups of chemicals which require metabolic activation that *Drosophila* possesses enzyme systems which resemble those of mammalian microsomes. The close proximity of the site of action and the genetic target cells renders this system particularly valuable for the detection of short-lived, mutagenic metabolites. One example in mammalian somatic cells is the system developed by Chu (Ref. 15), which utilizes V79 Chinese hamster cells and a nutritional marker, such as azaguanine-resistance, or a drug resistant, such as oubain, to score both forward and reverse mutations.

Most of the above genetic indicators, except *Drosophila melanogaster*, do not possess the metabolic capacity of mammals to convert chemicals that are not active *per se* into mutagenic and/or carcinogenic metabolites. Three current methods combine the genetic indicator with mammalian enzymes: In the host-mediated assay, the genetic indicator organisms are injected into rodents which are then treated with the chemicals under test by a different route of administration (Ref. 16-11), the indicator organisms are then recovered and scored for mutants. The limitations of the host-mediated assay, which are reflected by the necessity of administering the chemical in doses well above the LD_{50}, has focused attention on tissue or microsome-mediated mutagenicity assays. In these, the genetic indicator is incubated *in vitro* in the presence of subcellular tissue fractions from experimental animals or man with the appropriate cofactors for enzymatic conversion of the compound under test. After incubation, the cells are analysed for mutations and survival (Ref. 17). Alternatively, a mammalian organism (including man) is exposed to the test compound, and body fluids, such as urine or blood, are collected. The mutagenicity of urinary metabolites, for example, excreted as conjugates can be detected by treating urine extracts with hydrolyzing enzymes in the presence or absence of an *in vitro* metabolic activation system and bacterial or yeast tester strains (Ref. 18-19).

The induction of DNA-repair processes is another end-point used in short-term tests because of the covalent reaction of carcinogens or metabolites with nucleic acids. Some of the short-term tests involve the use of bacteria deficient in the repair of DNA, such as those lacking DNA polymerase I (Pol^-) (Ref. 20), or bacteria deficient in genetic recombination (Rec-) (Ref. 21). These bacteria are killed more easily by agents which damage DNA than wild-type bacteria. DNA-repair synthesis can readily be determined in mammalian cells by the unscheduled uptake of tritiated thymidine (Ref. 22). This autoradiographic procedure allows a qualitative evaluation of DNA-repair synthesis in fibroblasts, peripheral lymphocytes and biopsy specimens from rodents or humans. The indicator cells can easily be combined with an *in vitro* metabolic activation system. Another test, for compounds which are metabolically activated in extra-hepatic target tissues, involves a combined *in vivo* and *in vitro* system (Ref. 23): the test compound is administered to mice, and the DNA damage produced *in vivo* in various organs is determined *in vitro* by autoradiography after tritiated thymidine uptake.

Two less thoroughly explored short-term tests for the detection of carcinogenic chemicals can at present only be considered as empirical because of the end-points on which they are based. One involves the degranulation of rough endoplasmic reticulum of rat liver *in vivo* and *in vitro* by certain carcinogens. Williams and Rabin (Ref 24) and Purchase and Lefèvre

(Ref. 25) have shown that several carcinogens display a degranulating activity on the membrane-polysome complex of rat liver *in vitro*; since NADPH and oxygen are required and since these are cofactors for mixed-function oxidase, this alteration appears to be due to an action of the metabolically-activated carcinogens, rather than of the parent compounds, interfering with the binding properties of polysomes. Another action of reactive carcinogenic metabolites generated by microsomal mixed-function oxidase is the enhancement of biphenyl 2-hydroxylase activity, as shown by MacPherson *et al* . (Ref. 26): incubation of several carcinogens with hepatic microsomes in the presence of NADPH produced an enhancement of biphenyl 2-hydroxylation but not of biphenyl 4-hydroxylation.

Bioassays in which malignant transformation of cultured cells is used as the end-point can be considered to be the short-term tests which most closely estimate the onset *in vivo* of neoplastic processes by chemicals; the malignancy of the cells is confirmed by inoculation of the treated cells into an appropriate host. At present, there is still the unresolved problem of standardization of techniques: different criteria are used to diagnose neoplastic transformation of cultured diploid cells *in vitro* by morphological changes, e.g., colony-forming capacity in soft agar for epithelial-like cells and criss-cross growths and neoplastic growth pattern in fibroblastic cell lines (Ref. 27).

Since none of the short-term tests available use the appearance of malignant tumours in mammals as end-points, none of them can define a carcinogen. Therefore, their efficiency in predicting the carcinogenic properties of chemicals must be determined by testing large numbers of different classes of carcinogens and non-carcinogens. Data from the *Salmonella*/microsome mutagenicity tests that combine *S. typhimurium* strains with a rat-liver microsome activation system have been compiled and analysed by McCann *et al.* (Ref. 28) and by McCann and Ames (Ref. 29); these are compiled from tests with 300 carcinogens and non-carcinogens differing widely in chemical structure. The results reveal that there is a good correlation between the activities of carcinogens and mutagens; about 90% of the carcinogens tested were shown to be mutagenic; and 94 of the 108 non-carcinogens, including 46 common biochemicals, were without mutagenic activity, corresponding to 87%. Only 10% of carcinogens were not detected as mutagens, and 13% of mutagens which are claimed to be non-carcinogens were carcinogenic.

A more reliable analysis of the relationship between the mutagenic and carcinogenic effects of chemicals is based on results obtained in more than one mutagenicity assay system. Montesano and Bartsch (Ref. 30) have extracted data on genetic activity of 46 *N*-nitroso compounds reported between 1967 and 1975 in six different assay systems, comprising assays with sub-mammalian cells, tissue-mediated assays with sub-mammalian and mammalian cells, host-mediated assays, dominant lethal tests, tests for chromosomal aberrations and tests in *Drosophila*. In order to overcome the specificity of one particular mutagenicity assay, a chemical was designated as being mutagenic if it was positive in at least one of the six test systems; if it was designated as non-mutagenic, it was negative in all six systems. Of 47 *N*-nitroso compounds, 81% were detected as mutagens; three non-mutagenic *N*-nitroso compounds were also non-carcinogens; and 10% of the carcinogenic compounds were not found to be mutagenic. Thus, the results obtained with this class of structurally-related *N*-nitroso compounds in the six assay systems are very similar to the values obtained in the survey of 300 chemicals of different structures tested in one mutagenicity assay system. These data fully justify the use of mutagenicity tests for the detection of potential carcinogens, man-made or of environmental origin.

In order to validate the use of unscheduled DNA repair synthesis as an end-point, San and Stich (Ref. 22) tested 87 substances, including 29 reactive carcinogens,30 precarcinogens and 20 non-carcinogens. They obtained a good correlation between the induction of DNA repair synthesis and the carcinogenic capacity of the chemicals. Similarly, tests in *Drosophila* on about 70 chemicals revealed a good overall correspondence between the induction of recessive lethal mutations and the carcinogenic activity of the chemicals tested (Ref. 14).

The predictive value of certain test systems varies with the class of carcinogen or non-carcinogen assayed. No systematic validation of different short-term assays has so far been carried out for mycotoxins, thus, some of the published data have been collated here (Table 1). For the aflatoxins, there appears to be a good correlation between carcinogenicity and results obtained in rec-assays, mutagenicity assays with *S. typhimurium* and induction of DNA repair synthesis. For other mycotoxins, such as patulin and luteoskyrin, the few existing data, obtained from rec-assays or DNA-repair synthesis, are contradictory.

These results reveal two points: (a) in order to obtain reliable results, and to overcome the specificity of one particular assay system, a battery of short-term tests is needed; and, (b) mycotoxins sometimes exert a high cytotoxic activity, thus preventing the testing of higher concentrations with which mutations or other DNA damage could be detected in certain assay systems. This latter drawback is well illustrated by the way in which aflatoxin 2,3-oxide, the presumed ultimate metabolite of aflatoxin B_1 was detected: Garner *et al* (Ref. 31) showed the formation of a toxic principle of aflatoxin B_1, and it was only years later that mutation induction could be demonstrated.

The second use of short-term tests is in investigating the mode of action of chemical carcinogens. One example is the contribution of short-term assays to studies of the metabolism of

aflatoxin B_1. Lijinsky *et al.* (Ref. 32) showed that the administration of aflatoxin B_1 to rats results in the formation of adducts covalently-bound with hepatic nucleic acids and proteins.

TABLE 1. Mutagenicity of some mycotoxins in different short-term tests

Mycotoxins and Carcinogenicity (a)	Rec. assay *(B. subtilis)* (b)	Mutagenicity		DNA-repair (mammalian cells) (g)
		S. typhimurium	*Drosophila* (f)	
Aflatoxin B_1 +	+	+ *c*	+	+
G_1 +	+	+ *c*	n.d.	+
G_2 -	-	- *d*	n.d.	-
M_1 +	n.d.	+ *c*	n.d.	n.d.
B_2 +	-	+ *c*	n.d.	n.d.
Sterigmatocystin +	+	+ *c*	n.d.	+
Patulin +	+	- *e*	n.d.	n.d.
Luteoskyrin (?) +	+	n.d.	n.d.	-
Rugulosin +	+	n.d.	n.d.	-
Penicillic acid +	+	- *e*	n.d.	n.d.
Griseofulvin +	-	n.d.	n.d.	n.d.

n.d.: not determined

a IARC, (1)

b Ueno and Kobuta, (34)

c McCann *et al.* (28)

d Wong and Hsieh, (35)

e Bartsch and Malaveille, unpublished data

f Lamb and Lilly, (36)

g San and Stich, (22)

Using a microsome-mediated mutagenicity assay with *S. typhimurium* strains, Garner *et al.* (Ref. 34) and McCann *et al.* (Ref. 9) showed that aflatoxin B_1 is converted into a toxic and mutagenic metabolite by the action of mixed-function oxidase. The characterization of the structure of the adducts formed with nucleic acids after mild acid hydrolysis gave strong support to the theory that aflatoxin B_1 2,3-oxide, an extremely reactive intermediate, is formed. The recent work of Swenson *et al.* (Ref. 33) gives further evidence that aflatoxin B_1 2,3-oxide is also involved in carcinogenic processes *in vivo*.

These short-term testing procedures, which appear to be useful for the detection of potential carcinogens and mutagens, and which can be used in studies of the mechanism of action of chemical carcinogens are limited in that some of the factors which determine the process of cancer development cannot so far be duplicated. Positive results in short-term tests cannot automatically be taken to imply a definite carcinogenic activity in man; neither positive nor negative results from such tests can substitute for long-term carcinogenicity tests in animals. Only the probable carcinogenicity of a chemical can be predicted, and this without indication of the target organs in animals or man; nor can the potency of a chemical in a short-term test at present be correlated with its potency as a carcinogen.

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