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Recent Aspects of Mutagenicity Tests and Their Application to the Screening of Environmental Carcinogens

Motoi ISHIDATE

Jr. Head, Division of Mutagenesis, National Institute of Hygienic Sciences
Tokyo 158, JAPAN

Mutagenicity tests have been used as a short-term screening tool to predict possible carcinogenicity of chemicals found in the environment. These tests can be also used for the evaluation of the potential of genetic hazard from chemicals such as food additives, drugs, pesticides and other chemicals widely used in our daily life. If a chemical induces a mutagenic response in somatic cells, this would suggest potential for carcinogenicity in animals. By the same token, if a mutagenic effect is produced in germ cells, adverse genetic changes might possibly develop in the progeny even in the absence of a carinogenic effect. If the chemical has an effect on the fetus, it would be considered a potential teratogen in humans. I was assighned to talk about mutagenicity and would like to review mutagenicity from various aspects centering around its test methods.

There are a number of methods used in mutagenicity testing. We have to select the most reliable tests among them which can be applied to carcinogenicity screening and to the evaluation of chemical safety. For this purpose, we need adequate background data on well known carcinogens in each test system, in order to obtain information about any false negative or false positive results. This kind of study will provide a data base for use in selection of a minimum set of mutagenicity tests required by the government for the registration of new products.

Mutagenicity tests can be utilized in the field of fundamental research to elucidate the mechanisms involved in carcinogenicity, genetic toxicity, and teratogenicity of the given chemical. Possible metabolites or ultimate forms can be detected with mutagenicity tests if an appropriate metabolic activation method is included in the test system. Such studies are extremely important for the evaluation on chemical safety especially from a regulatory point of view.

Mutagenicity tests are broadly classified into three groups methods for detecting gene muta-

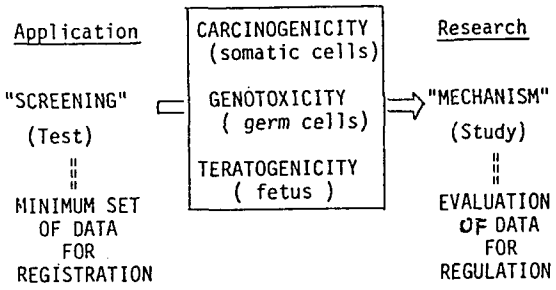

Fig. 1. Mutagenicity tests on chemicals.

tion; mehtods for detecting chromosomal damage in terms of chromosomal aberrations; and methods for detecting primary DNA damage. Each has a different genetic endpoint; systems using prokaryotes and eukaryotes, *in vitro* as well as *in vivo*, are used. This table was based on the proposed guideline prepared under the USA-TSCA(The Toxic Substance Control Act, 1982).

Table 1. *U.S.A.* : TSCA (chemicals) and FIFRA (pesticides)*

1. Gene mutation tests
1) Bacteria or other microorganisms
2) Drosophila
3) Mammalian cells in culture
4) Specific locus mutation in mice
2. Chromosomal aberration tests
1) Eucaryotic microorganisms
2) Drosophila
3) Mammalian cells *in vitro* (incl. SCE)
4) Mammals
a) Micronucleus test
b) SCE *in vivo*
c) Bone marrow or germ cells *in vivo*
d) Dominant lethal
e) Heritable translocation
3. Other tests
1) DNA-repair
2) Aneuploid and non-disjunction
3) Cell transformation
4) Sperm abnormality (Tests for target organs)

* EPA, 1982 ; Gene-Tox Program
Nat. Tech. Inform. Service

Table 2. Selection and utilization of mutagenicity tests

1. Single test approach	
2. Multiple-test approach	
a) Three Tier scheme (Bridges, 1973)	
b) Battery scheme	
8 tests requirement	(EPA, 1978)
4 tests requirement	(UK, 1979)
c) Hierarchical approach	(Bora, 1976)
i) Preliminary phase	
ii) Secondary phase	

Until some time ago, a single test approach had been dominant: chemicals were tested by only one assay system, such as the Ames test. However, this approach is no longer supported by the specialists, since one test alone can not detect all potential effects.

Later, a multiple-test approach was proposed in which several different assays were combined in a series. The three tier scheme introduced by Dr. Bridges is one of these; testing begins in relatively simple organisms and proceeds gradually to more complex systems. The evaluation, however, still includes the concept of the single test approach. Each progressive step depends on the results of the first test. If the first test is erroneous, then the error would be carried forward to the following steps.

In place of this gradual step-up approach, a concurrent implementation of various methods, which is called the "battery system", has been proposed. The USA-EPA and UK-EMS (Environmental Mutagen Society) have proposed 8 and 4 test systems, respectively, to evaluate the mutagenic activities of chemicals in general. It may be true that a greater number of tests will produce more information useful for the evaluation of chemical safety. However, industrialists were strongly opposed to doing so many tests as a rule; the EPA guideline has since been slightly modified ans simplified.

The third category utilizes the "hierarchical approach". In this, the preliminary phase includes at least two tests with different genetic endpoint. The secondary phase is carried out after evaluation of the results from the primary phase. Thus, this is a combined concept of the tier system and the battery system. I think this concept is quite reasonable and the idea has

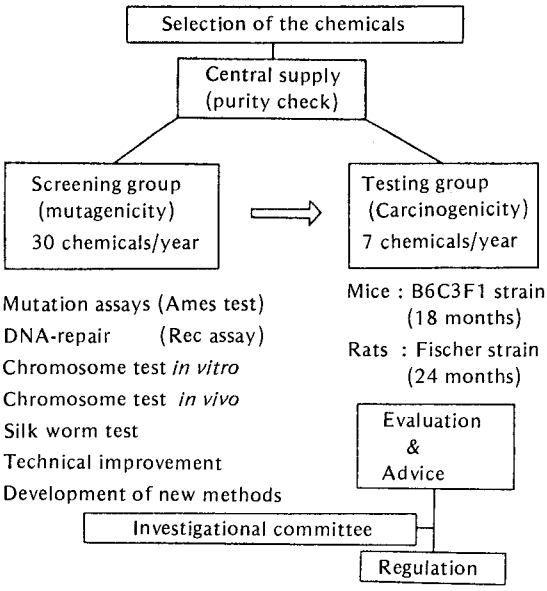

Fig. 2. Cooperative program on short-term assays for carcinogenicity of chemicals.
—Under projects of the ministry of health and welfare —

thus far been accepted in Japan, and taken into consideration in preparing the guidelines not only for medical drugs, but also for pesticides and other chemicals.

In 1973, the Japanese Ministry of Health and Welfare orgainzed a national project team in order to develop effective methods for short-term testing of environmental carcinogens. The team was divided into three groups. The first, the chemical group, aimed at selection of items to be tested from a variety of chemicals. About 30 different compounds were selected every year. The second, the screening group, undertook the performance of mutagenicity tests such as the Ames test, the DNA repair test (Rec-assay), the chromosomal aberration test *in virto* and *in vivo*, and the mutation assay in silkworms. The third, the testing group, conducted long-term carcino-genicity tests on such chemicals which were positive in the mutagenicity tests. The testing group began the evaluation of 7 compounds every year, using mice (1.5 year studies) and rats (2 year studies) according to international carcinogenicity test guidelines.

In 10 years, about 200 compounds have been screened for mutagenicity and of these, 28 compounds have been tested for carcinogenicity. In the case of food additives, all data obtained from this project are carefully considered and evaluated by the Food Sanitation Investigation Council of the Ministry of Health and Welfare of Japan.

The summary of results from the above mentioned collaborative studies is shown in this slide. Compounds listed in the left column are those found positive in both mutagenicity and carcinogenicity tests. AF-2 was positive in all mutagenicity tests and was also positive in the carcinogenicity test with rats (R) and mice (M). Barbital was positive in the chromosomal aberration test but carcinogenic only in mice. BHA was positive in only the rec-assay, but induced tumors in rats. Hydrogen peroxide was weakly positive in the Ames test, but clearly positive in the chromosome test; it was carcinogenic only in mice. Phenacetin was both mutagenic and clastogenic (positive in the chromosome test) after metabolic activation, and was carcinogenic in both rats and mice. Potassium bromate was weakly positive in the Ames test, and strongly positive in the chromosome test; it was carcinogenic in rats. Sulpyrin was mutagenic and also carcinogenic in mice.

Other compounds listed in the right column, such as caffeine, caramel, etc., were positve in mutagnicity tests, but all negative in carcinogenicity tests in rodents.

When we look at the total picutre, 27% of the

Table 3. Collaborative studies on short-term and long-term tests for environmental carcinogens (Ministry of Health & Welfare)

Carcinogenicity tests in rats (R) and mice (M)			
Positive results		Negative results	
AF-2	(R, M)	Acid red	Sodium nitrite
Barbital	(M)	BHT	Sodium erythorbate
BHA	(R)	Caffeine	Sodium hypochlorite
Hydrogen peroxide	(M)	Caramel	Thiram
Phenacetin	(R, M)	Erythrosine	DDVP (Dichlorvos)
Potassium bromate	(R)	i-Butyl p-hydroxy-benzoate	Aspirin
Sulpyrin	(M)	Potassium sorbate	Acetaminophen
		Potassium metabisufite	Nitrofurantoin
		Sodium benzoate	
		Sodium nitrate	
7 (27%)		19 (73%)	

All compounds shown in the table gave positive results in at least one type of mutagenicity test.
Three other compounds (diphenyl, sodium propionate and n-butyl p-hydroxybenzoate) which were not mutagenic were all negative in the carcinogenicity test.

Table 4. Mutagenicity tests on food additives currently used in Japan
— Bacterial mutation assays (Ames test) —

	Food additive	Dose (mg/plate)		*S. typhimurium* strain used	No. revertants induced/mg
Synthetic	Cinnamic aldehyde	0.1	(−S9)	TA 100	1790
	Hydrogen peroxide	0.2	(−S9)	TA 100	535
	Calcium hypochlorite	1.0	(+S9)	TA 100	491
	Chlorine dioxide	0.4	(−S9)	TA 100	428
	Sodium chlorite	0.3	(+S9)	TA 100	293
	L-Cysteine mono hydrochloride	1.0	(+S9)	TA 100	291
	Sodium nitrite	10.0	(+S9)	TA 100	47
	Sodium hypochlorite	5.0	(+S9)	TA 100	46
	Potassium bromate	3.0	(+S9)	TA 100	44
	Fast Green FCF	10.0	(+S9)	TA 100	35
	Erythorbic acid	50.0	(+S9)	TA 100	4
Natural	Beet Red	50.0	(+S9)	TA 100	3
	Cacao pigment	50.0	(−S9)	TA 1537	3
	Caramel	50.0	(−S9)	TA 100	2

compounds which were mutagenic (or clastogenic) were positive in carcinogenictiy tests in rats and/or in mice. It was concluded from this study that the majority of carcinogens may be mutagenic but that not all mutagenic substances were carcinogenic. The 27% correlation sounds very low and was quite unexpected. However, this value seems to be significant from a viewpoint of screening for carcinogens, since much lower percentages might be expect-

ed if one were to perform carcinogenicity tests on compounds which had been selected at random the environment without testing for their mutagenicity.

There are 347 synthetic food additives currently used in Japan. In addition, more than 300 food additives derived from natural sources are also used. Preliminary mutagenic screening has been carried out at our laboratory since 1979. We have published data on about 200 food additives (Food Chem. Toxicol., 1984). In this Table 4, some food additives which were positive in the Ames test are listed. The mutagenic potential was calculated as the number of revertants induced per mg; cinnamic aldehyde was the highest. Other additives, such as hydrogen peroxide, calcium hypochlorite, chlorine dioxide, sodium chlorite, and ℓ-cysteine monohydrochloride, were moderately active. Natural food additives were relatively weak, even though tested at higher dose levels.

Table 5. Specific mutagenic activities of the compounds isolated from pyrolysates and well known carcinogens

Revertants/μg			
S. typhimurium TA98		*S. typhimurium* TA100	
MeIQ	661,000†	AF-2	42,000†
IQ	433,000†	MeIQ	30,000†
MeIQx	145,000†	Aflatoxin B_1	28,000†
Trp-P-2	104,200†	MeIQx	14,000†
Glu-P-1	49,000+	4NQO	9,900*
Trp-P-1	39,000†	IQ	7,000†
AF-2	6,500*	Glu-P-1	3,200+
Aflatoxin B_1	6,000†	Trp-P-2	1,800†
Glu-P-2	1,900+	Trp-P-1	1,700†
4NQO	970*	Glu-P-2	1,200+
B (a) P	320†	MNNG	870*
AαC	300**	B(a)P	660†
MeAαC	200**	MeAαC	120**
Lys-P-1	86**	Lys-P-1	99**
Phe-P-1	41**	Phe-P-1	23**
DEN	0.02**	AαC	20**
DMN	0.00**	DMN	0.23**
MNNG	0.00*	DEN	0.15**

* Without S9 mix
† 10 μl, + 30 μl, ** 150 μl S9/plate.

TR value : Nub

Table 5 shows the specific mutagenic activities of substances isolated from well-known carcinogens and from pyrolysates of broiled fish and meat. It should be noted that the activities in both TA98 and TA100 are shown in the unit of revertants induced per μg, not per mg of samples. The activities are extremely high when compared with those found in food additives.

We also applied *in vitro* chromosomal aberration tests as one of the complementary tests for the Ames test. We used a Chinese hamster fibroblast cell line, CHL, which was originally derived from lung tissue. This cell line is now commercially available in Japan. Chinese hamster cells are very useful material for chromosome study, because they have relatively samll numbers of large chromosomes and they grow rapidly in a simple medium like MEM with 10% calf serum. Chromatid type breaks or exchanges induced by a carcinogen, MNNG, can be easily found in the metaphase figure on right side. Human diploid cells, e.g. lymphocytes, can also be used for chromosomal aberration tests, but they are relatively in sensitive to chemicals when compared with Chinese hamster cells.

Some food additives which were positive in our *in vitro* chromosome tests are listed in the Table 6. The clastogenic potenital was estimated by the TR value which represents the expected frequency of cells with only exchange type

MNNG untreated cell.

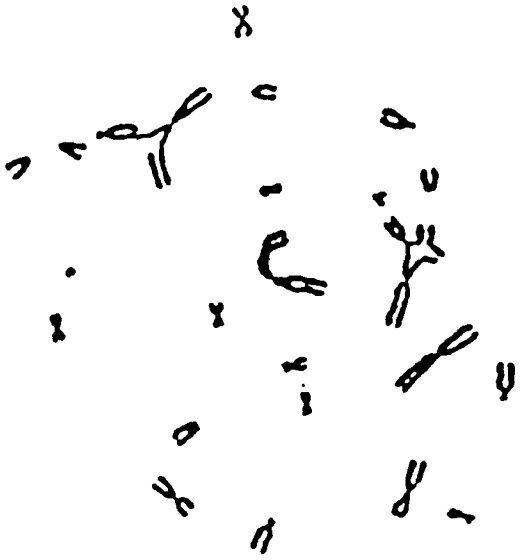

Fig. 3. MNNG treated cell.

Table 6. Chromosomal aberration tests *in vitro* on food additives currently used in Japan
—with a Chinese hamster cell line (CHL)—

Food additive	Dose (mg/ml)	TR-value
Synthetic		
Cinnamic aldehyde	0.015	2133+
Propyl gallate	0.04	800
Sodium 5'-guanylate	1.0	576
Sodium chlorite	0.02	500+
1-Perillaldehyde	0.05	240
Calcium hypochlorite	0.06	166+
Potassium bromate	0.25	116+
Eugenol	0.125	112
Sodium nitrite	1.0	52+
Ammonium chloride	0.4	35
Sodium hypochlorite	0.5	18+
Riboflavin	0.3	13
Ethyl p-hydroxybenzoate	0.25	12
Iron and sodium succinato chl	2.5	7
L-cysteine monohydro-chloride	2.0	6+
Hydrogen peroxide	0.25	1+
Natural		
Curcumin	0.03	400
Caffeine	1.0	35
Perilla pigment	1.25	13
Lacchaic acid	2.0	6
Cellulase	4.0	5
Caramel	8.0	2+

+ : Also positive in Ames test.
TR value : Number of metaphases with exchange type aberrations expected at a unit dose, mg/ml

aberrations at a dose of 1 mg/ml. Cinnamic aldehyde was again positive in this test and the TR value was extremely high. Other additives such as propyl gallate, sodium 5' guarylate, 1-perillaldehyde, and eugenol are clastogenic, even though they were all negative in the Ames test.

Natural food additives such as curcumin, caffeine and perilla pigment are also positive. It can be noted that synthetic additives are more clastogenic than additives of natural origin.

Well-known carcinogens were all positive in this chromosome test, as shown in the Table 7. The carcinogens listed in the left column were positive in both the Ames test and the chromosome test. The TR values of these compounds are very high. Some weak carcinogens, such as hydrogen peroxide, maleic anhydride, ethionamide, isoniazid, benzene, barbital or urethane were positive in only the chromosome test

Table 7. Clastogenic potential of carcinogens (TR values) – The incidence of exchange-type aberrations

Chrom. test (+)	Ames. test (+)	TR value*	Chrom. test (+)	Ames. test (−)
4-NQO		30,000		
Trp-P-1		12,800		
ENNG		9,600		
MNNG		6,200		
Trp-P-2		4,670		
Furylfuramide (AF-2)		4,100		
MNUR, Acrylonitrile		3,400		
β-Propiolactone		2,270		
Styrene oxide, B(a)P		1,570		
Propane sultone		730		
MNU, 3'-Me DAB, DMBA		580		
Rhodamine B		183		
BNU		155		
		144	Hydrogen peroxide	
Potassium bromate		116		
2-Me DAB, DMN		71		
BBNU		48		
		40	Maleic anhydride	
DBN, 2AAF		33	Ethionamide	
		7	Isoniazid, Benzene	
		1	Barbital	
		0.5	Urethane	

$$\text{* TR value} = \frac{\text{No. of cells with exchange type aber.}}{\text{The most effective dose (mg/ml)}}$$

and their TR values are relatively low.

When the mutagenic activity in the Ames test is compared with the clastogenic potential in the *in vitro* chromosome test for several compounds, we find a nearly liner relationship.

Caramel A-D (different brands), acid red or cochineal are positive in both the Ames test and the chromosome test, but they are all in a group showing weak activity (upper right corner). On the contrary, potent carcinogens such as MNNG, ENU (ethylnitrosourea), 4-NQO, AF-2, captan or AAF (acetylaminofluorene) are all in a group showing strong activity (lower left corner).

The potential in the Ames test varies from 10^{-8} to 10^{0}; the potential in the chromosomal aberration tests varies from 10^{-4} to 10^{2}. The D_{20} in this figure means the concentration (mg/mℓ) at which 20% of metaphases showed aberrations.

The development of appropriate short-term tests is an urgent problem not only in Japan, but also in other countries. The International Program for Chemical Safety (IPCS) organized a new project called "CSSTT" (Collaborative Study on Short-Test for Genotoxicity and Carcinogenicity). The purpose of this project is to recommend complementary assay systems for routine use in conjunction with bacterial mutation assays such as the Ames test. Ten compounds were distributted to about 60 investigators throughout the world. Eight substances are organic carcinogens known to be either inactive or difficult to detect as positive in the Ames test. The other two compounds, caprolactam and benzoin, are both non-carcinogenic.

Results from this program have been compiled into a book and published quite recently. Several Japanese investigators have contributed in this program.

Fig. 6 represents one set of experimental results which was obtained from the Ames test group and the *in vitro* chromosome test group. The black column shows results from the Ames test. Most of the substances produced negative results (graphs fall below the central line). The dotted columns represent results from the chromosome test. Most of the carcinogens were positive (graphs extend above the central line).

This indicates that such *in vitro* chromosome tests may be a very useful complementary test for the Ames test. When condidering the total

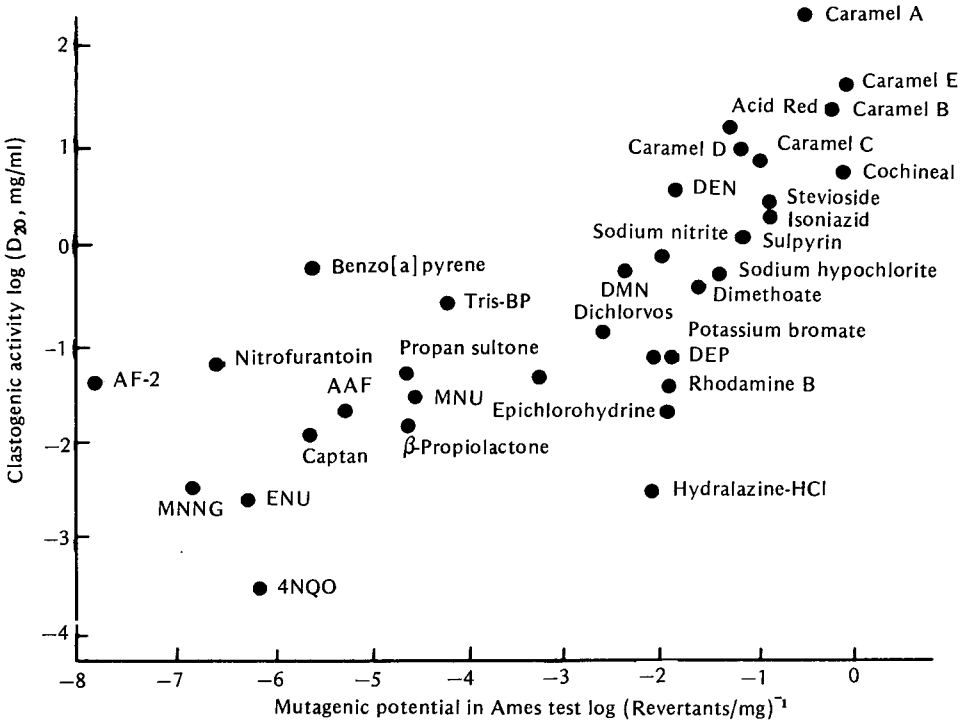

Fig. 4. Quantitative relationship between clastogenic activity in the chromosomal aberration test *in vitro* and mutagenic activity in the Ames test.
D_{20} : The dose at which aberrations were detected in 20% of metaphases (mg/ml)

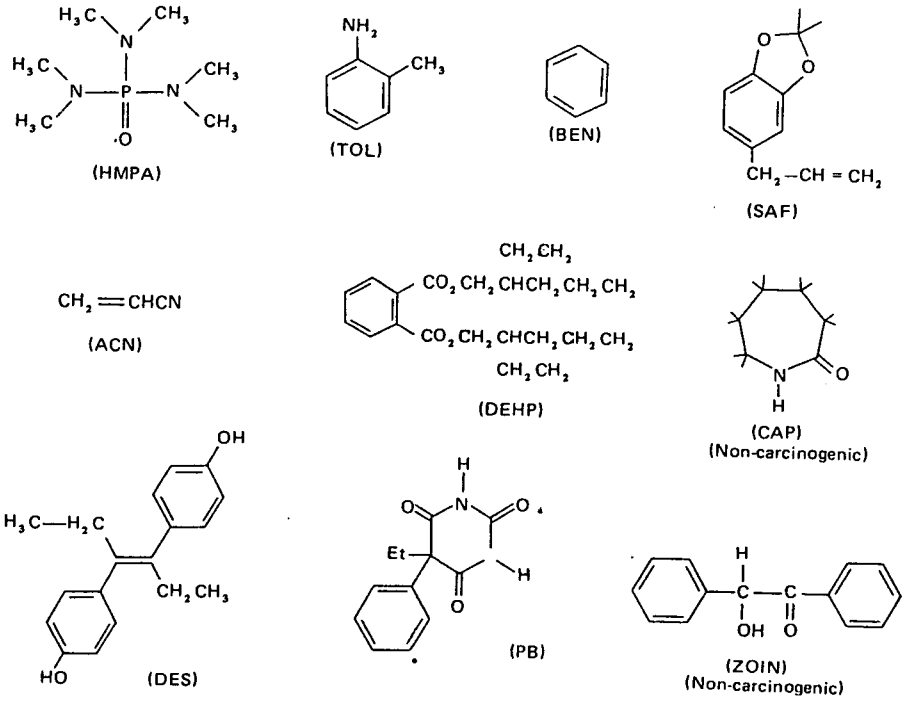

Fig. 5. Selected compounds to be tested in *in vitro* systems (CSSTT).

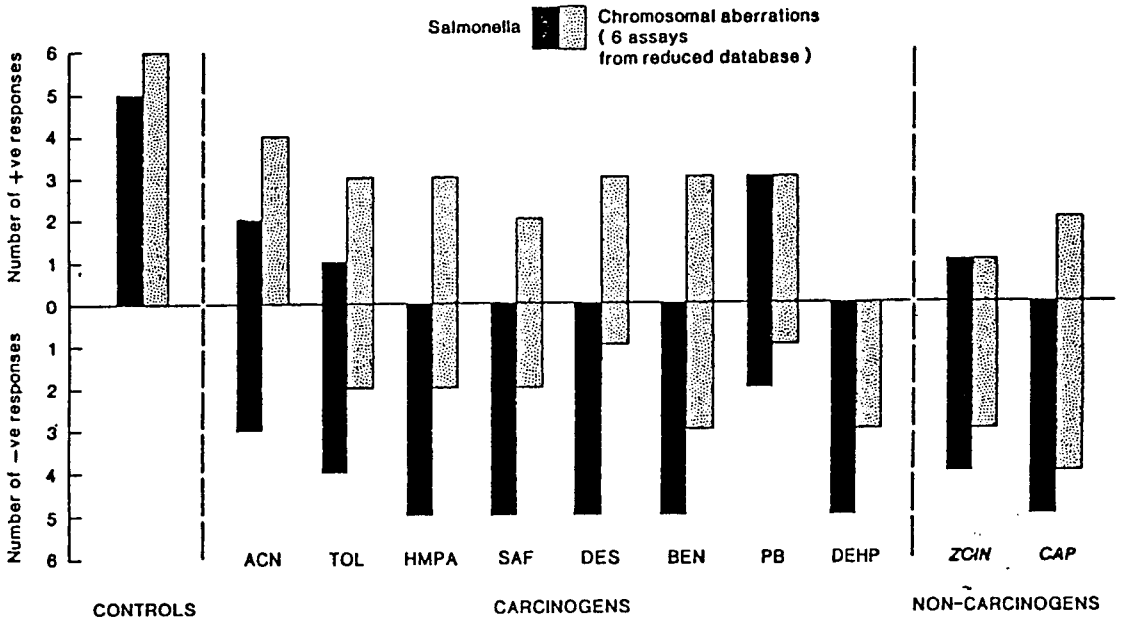

Fig. 6. Comparison of the performance of the Salmonella assay and the 6 chromosomal aberration assays listed in the reduced database (Table 3). Questionable responses have been ignored.

Table 8. *U.K.* : Recommendation as a "Basic package" (1981) ; Supported by UKEMS, EC-CPMP

* 1) Bacterial mutation
* 2) Chromosomal damage *in vitro*
3) Mutation in mammalian cells *in vitro*
or
Drosophila (Recesive lethals)
4) Chromosomal damage *in vivo* (incl. micronucleus)
or
Dominant lethal in rodents

* Sufficient for chemicals of limited exposure

data base for the gene-mutation assays and chromosomal tests, the latter appear to be more sensitive. There were 22 positive responses out of 43 determinations made on the 8 carcinogens as opposed to only 40 of 103 for the mutation assays. It is therefore recommended that at least tests with different genetic endpoints (gene mutation and chromosomal aberration) should be combined in the routine system of screening for environmental carcinogens.

Table 8 is a guideline for mutagenicity tests on chemicals, which was prepared by the subcommitte of the UK-EMS in 1982. They recommend the combined use of four of tests as shown in the table. For chemicals to which there is only limited industrial exposure, the first two tests are required. As *in vivo* systems, they propose the Drosophila gene mutation test, te bone marrow cytogenetic test (including the micronucleus test), or the dominant lethal test in rodents.

I think that the principle of this guideline is quite reasonable and very practical, so that it may be acceptable even in other European countries.

In the United States, the National Research Council Committee presented a proposal to the EPA as shown in Table 9. They recommend employing a tier approach. Tier I would include three *in vitro* tests: Ames test, a somatic gene

mutation assay, and a chromosomal aberration test. If negative results were obtained from all of three tests, the test substance should be classified as non-mutagenic. If positive results were obtained in more than two tests, then the substance should be considered to be mutagenic. If only one was positive among these, the substance should be subjected to the Drosophila test.

In Japan, a new guideline for mutagenicity tests on new drugs has been established by the Ministry of Health and Welfare, in view of the current world-wide trend.

As shown in the Table 10, this guideline proposes two *in vitro* tests, such as bacterial mutation assay and chromosomal aberration test, as the primary evaluation. If positive results are obtained from either one of these tests, the micronucleus test in rodents should be conducted as an *in vivo* confirmatiory test. Data obtained from other mutagenicity tests, such as DNA repair tests with bacteria of mammalian cells, are also used as additional information when necessary. The bone marrow cytogenetic assay may be acceptable as an alternative to the micronucleus test. All data are evaluated by the Drug Sanitation Committee, not only qualitatively but also quantitatively, together with other toxicological data from animals.

If negative results are obtained in all *in vitro* tests, it can be said that the substance is probably not mutagenic. In this case, perphaps no further tests are required. If a positive result is obtained in either one of the *in vitro* tests but a negaive result in the *in vivo* test (in fact, there are number of such cases), the substance should be classified as a weak mutagen. In this case, further information on the metabolic pathway of the test substance may be required. The third possibility is a positive result in the *in vivo* test. In this case, the substance is judged to be mutagenic, although additional *in vivo* test data are required. If all the subsequent tests are positive, substance is no doubt mutagenic.

Human cancer development is said to depend more on diet than on industrial chemicals. As

Table 9. *U.S.A.* : Nat. Res. Council's committee (1983) (a proposal to EPA by CCEM)

1. Tier I	Bacterial mutation (Ames test)
	Somatic cell mutation (HGPRT, TK)
	Chromosome aberration *in vitro*
2. Tier II	Drosophila test
Evaluation	
	Negative in all T-1 : Non-mutagenic
	Positive in 2-3 T-1 : Mutagenic
	Positive in 1 T-1 : Step up to *Tier II*
Further *in vivo* tests	
	1) Dominant lethal test in mice
	2) Specific locus test in mice

Table 10. A primary evaluation

Gene mutation assays with bacteria	Chromosome tests *in vitro* with mammalian cells
Salmonella/microsome *E. coli*/microsome	Chinese hamster fibroblast Human lymphocyte
Somatic cell mutation Cell transformation etc.	Rec-assay with bacteria UDS test *in vitro* SCE test *in vitro* etc.

A secondary evaluation

Micronucleus test
Dominant lethal test
Drosophila test
Sperm abnormality test
etc.

Evaluation of safety

Carcinogenic hazard Genetically heritable hazard

shown in this Fig. 7, mutagenic involvement in food should be considered from different angles. All kinds of food additives currently used should be checked for their mutagenicity, since consumers are exposed to them all through life.

Table 11. Evaluation of the results obtained from the package of short-term tests

Case	*In vitro*		*In vivo*	Evaluation
	Ames test	Chro-mosome	Micro nucleus	
I	−	−	−	Non-mutagen? [1]
II	+	−	−	Weak mutagen? [2]
	−	+	−	
III	+	−	+	Mutagen? [3]
	−	+	+	
IV	+	+	†	Strong mutagen? [4]

1) No more tests
2) Further studies on metabolic psss-way *in vivo*
3) Additional mutagenecity tests *in vivo*
4) Possibly carcinogenic and/or genetically hazardous

Food contaminants such as mycotoxin, pesticide, packing material, etx., should be examined not only by chemical analysis but also for their mutagenic potential. Different kinds of mutagenic pyrolysates are formed during food processing. Some of them are known to be carcinogenic in rodents. Natural components in plants, e.g. flavonoids, alkaloids, and spice components, are mutagenic. Furthermore, some mutagenic nitrosocompounds are formed directly in the stomach or indirectly in other parts of the digestive tract. Some factors in foods may be involved in promotion or inhibition of mutagenic effects of other components.

In conclusion, mutagenicity tests with diferent genetic endpoints can be recommended as sueful and reliable tests for the screening of environmental carcinogens, and for the risk evaluation of chemicals in general. The mut-

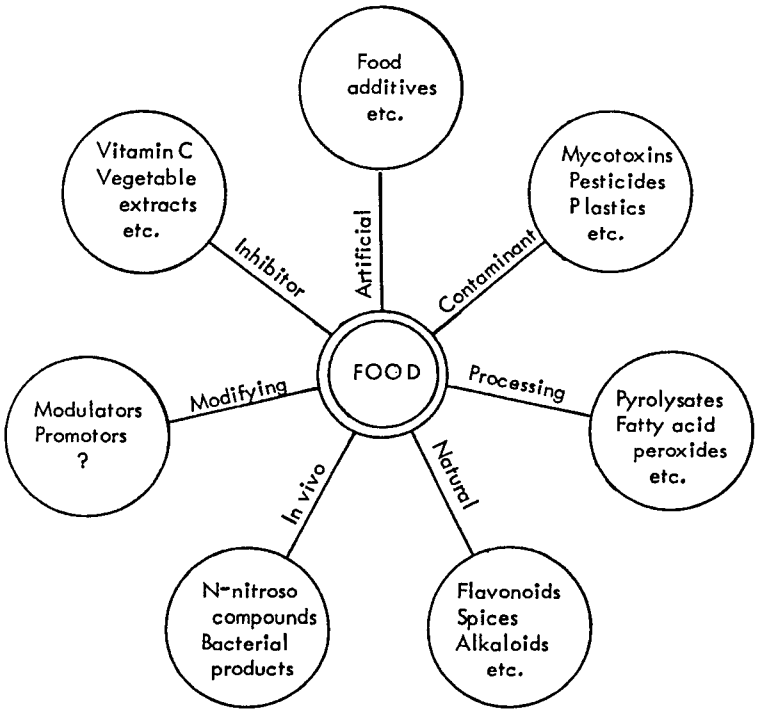

Fig. 7.

agenic potential, however, vary so widely among different chemicals. Therefore, we need quantitative rather than qualitative evaluation of the data obtained from each mutagenicity test. If some positive results were obtained from *in vitro* tests, we need to add *in vivo* tests for confirmation.

Mutagenic substances may not be necessarily carcinogenic, but may be genetically harzadous in mammals. First, we should eliminate substances which showed very potent mutagenic activity from our environment. Second, we need more informations of heritable genetic toxicity of the chemicals which were mutagenic but not carcinogenic in animals. Finally, it should be noted that the development of highly refined short-term tests for genotoxicity will give a great advantage for the fundamental research in the mechanisms of induced mutation and of carcinogenesis of chemicals. In additon, an application of these techniques to the monitoring of human populations for sign of exposure to genotoxic agents, not only industrial chemicals but also air and water pollutants, is extremely important in the future.