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会　長　　池　田　正　之

（東北大学医学部）

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PLENARY LECTURES

PL1 DISPOSITION AND METABOLISM STUDIES OF XENOBIOTICS IN THE ENVIRONMENT: FUNDAMENTALS FOR SAFETY ASSESSMENT

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INTRODUCTION

The hazard to be caused by the environmental chemical will depend on its intrinsic toxicity, and on the amount thereof as well to which living organisms are actually exposed. Thus to accurately assess the hazard, it is essential to elucidate the environmental behavior of the compound in question. Namely, in the complex net work of the environment the translocation of the compound from one ecocompartment to another ought to be clarified, as well as the transformation in these ecosystems determined by a variety of biotic and abiotic factors (Miyamoto, 1987). To this end a number of detailed laboratory studies on the disposition and fate of the compound are routinely carried out nowadays, such as those listed below, and if necessary, a wide range of field monitoring trials are supplemented.

Translocation
  Evaporation, Run off, Leaching
Transformation
 Abiotic
  Hydrolysis, Photolysis
 Biotic
  (Absorption, Retention, Storage, Elimination, Metabolism)
  Mammals, Ruminants, Birds, Plants, Soil microorganisms, Aquatic organisms, etc.

Through these undertakings the following aspects should be borne in mind:

1. Whether the xenobiotic is similarly metabolized in any ecocompartments;

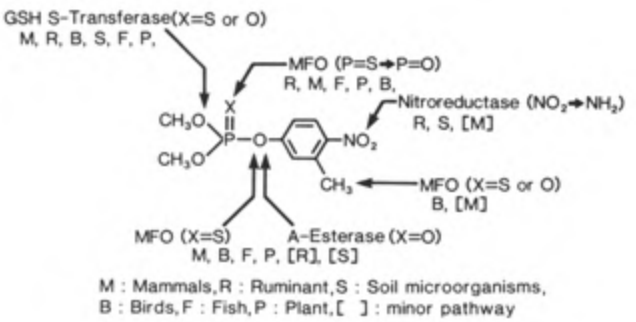
2. Whether it is converted to the biologically active products;
3. Whether any toxicologically significant metabolites are translocated to and/or bioaccumulated in specific organisms.
   Also,
4. Attention should be paid to the possible modification of metabolic pathways at massive dosages, in other words toxicokinetic consideration on linearlity of dose and bioreactions;
5. Possible differential metabolism owing to chirality of the molecule should be taken into account.

The importance of such studies is especially profound in the case of those compounds exemplified by pesticides that are intentionally applied in the field, and as a result they often leave in the environment inevitable residues and contaminants with biological activity. Therefore in the following discussion to be associated mainly with risk assessment to humans, therefore aside from consideration of the effects on other living organisms, the pesticide is referred to as a representative example and the above points are dealt with in some details. However, needless to say, the principles and considerations are applicable to any environmental chemicals.

DISPOSITION AND FATE IN THE ECOSYSTEMS

The organophosphorus insecticide is generally regarded to be of shorter persistency, and actually the metabolic studies reveal that it is quite extensively degraded, and substantially identically in many of the ecocompartments. Fenitrothion, O,O-dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate, affords one such example, which is metabolized through cleavage of P-O-aryl linkage, O-demethylation and oxidative desulfuration (Fig. 1, Hosokawa, 1974; Kikuchi et al., 1984; Mihara et al., 1978; Mihara et al., 1979; Mikami et al., 1985b; Miyamoto, 1964; Miyamoto and Sato, 1965; Miyamoto et al., 1966; Miyamoto et al., 1968; Miyamoto et al., 1976; Miyamoto et al., 1977; Miyamoto et al., 1979; Ohkawa et al., 1974; Takimoto et al., 1976; Takimoto and Miyamoto, 1976; Takimoto et al., 1984; Takimoto et al., 1987a; Takimoto et al., 1987b; Takimoto et al., 1987c). However, exceptionally in ruminants its nitro group is reduced to amino group due to

Fig. 1. Site of metabolic attack of fenitrothion

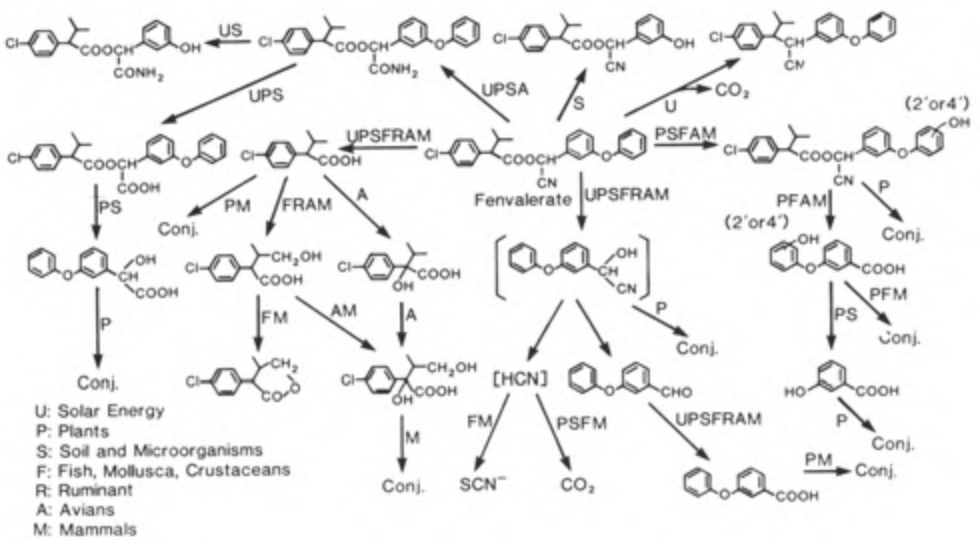


microbial activity in lumen, giving rise to aminofenitrothion and a variety of its derivatives. Since these amino analogs of fenitrothion are formed in other mammals in little amounts, therefore rather foreign to rats and mice and presumably to man, the possible contamination of dairy milk and meat with such amino compounds is tested by giving fenitrothion subacutely to dairy cows. During and immediately after 28 day feeding of 100 ppm in diet of fenitrothion (calculated maximum ingestion based on plant residues), milk as well as representative edible tissues contains aminofenitrothion, fenitrooxon (active metabolite) as well as fenitrothion below the detection limit of 0.01-0.05 ppm (Taylor, 1980).

When the synthetic pyrethroid, one major group of insecticides nowadays, has been introduced for agricultural purposes some ten years ago, the environmental behavior had been scarcely understood, and therefore the extensive investigations have been conducted aimed at obtaining enough information relevant to the risk assessment. Fig. 2 shows the metabolic pathways of fenvalerate elucidated by exhaustive radiotracer studies. The phase I reactions encountered in fenvalerate are ester bond cleavage via microsomal carboxyesterase and other esterases, and oxidation at various sites of alcohol and acid moieties prior to or after hydrolysis (Boyer and Lee, 1981; Boyer and Potter, 1981; Edwards et al., 1987; Gaughan et al., 1977; Kaneko et al., 1981; Kaneko et al., 1984; Leahey, 1985; Mikami et al., 1980; Mikami et al., 1984a; Mikami, 1987; Miyamoto et al., 1981; Miyamoto and Mikami, 1983; Mumtaz and Menzer, 1986; Ohkawa et al.,

1978; Ohkawa et al., 1979; Ohkawa et al., 1980a; Ohkawa et al., 1980b; Potter, 1976). Briefly, there is no appreciable qualitative difference in metabolic attacks. The cyano group released is converted to thiocyanate and carbon dioxide in animals, whereas in plants some portions of the liberated cyano group are incorporated to amino acids, and eventually to carbohydrate and protein.

Fig. 2. Degradation of fenvalerate in the environment



It appears that any of the degradation products are pharmacologically inactive, or inferior to the parent pyrethroid. This implys that all the metabolic attacks described in Fig. 2 lead to detoxication of fenvalerate.

The phase II reactions or conjugations taking place in several phase I reaction products depent on the structure of the primary products and also on the species. For instance the conjugates of 3-phenoxybenzoic acid derived from the alcohol moiety of fenvalerate are quite variable, e.g., conjugated with glycine, glutamic acid, taurine, 1,2- & 1,3-dipalmitines, glucuronic acid, etc. (Cheng and Abell, 1986; Crayford and Hutson, 1980; Curl et al., 1987; Huckle et al., 1981a; Huckle et al., 1981b; Huckle et al., 1982; Hutson and Casida, 1978).

The plant conjugates should be dealt with somewhat carefully, in that they usually stay in harvested substrates which might ultimately be ingested by mammals. In a variety of plants such as cotton, tomato, cabbage, bean and cucumber 3-phenoxybenzoic acid derived from fenvalerate is conjugated with mono-, di- and tri-glucosides including malonylglucose and glucosylxylose with certain species differences in the predominant products. Similarly the acid moiety of fenvalerate yields even the more number of glucoside conjugates, in addition to the aboves, such as sophorose and gentiobiose (Mikami et al., 1984b; Mikami et al., 1985a; Mikami et al., 1985d). These plant conjugates are demonstrated to be well bioavailable in mammals: Oral administration of these plant conjugates to rats reveals stepwise removal of sugar moieties and release of the aglycones, which are then metabolized through the pathways already demonstrated in the metabolism of fenvalerate such as 3-phenoxybenzoic acid and its sulfate. These results implicate no need of additional toxicology studies with the plant conjugates (Mikami et al., 1985e).

Due to its extremely low solubility in water, fenvalerate is tightly adsorbed to the agricultural soil, thus with no leaching activity from the soil, and gradually decomposed by soil microbial activity, with a half-life of several weeks. And as such there is no substantial uptake of fenvalerate by plants from the treated soil (Ohkawa et al., 1978).

However, for certain types of compounds it happens that plants take up their residues from the soil. For example, a paddy field herbicide bromobutide, N-(1-methyl-1-phenylethyl)-2-bromo-3,3-dimethyl butanamide, tends to leach from soil due to its larger water solubility, and yields debromo analog residues in rice plant, which are actually not plant metabolites, but they are formed in soil by microbial activity, since by hydroponic culture no debromo analog is found in plant tissues (Kakuta et al., 1985; Mikami et al., 1982). It is proved that the removal of bromine atom from bromobutide and metabolism of the debromo analog proceeds significantly in aminal body (Figs. 3 & 4, Isobe et al., 1983; Isobe et al., 1984), which implies no need of additional toxicity studies with the debromo analog.

Fig. 3. Residues of bromobutide and its debromo analog in rice grains

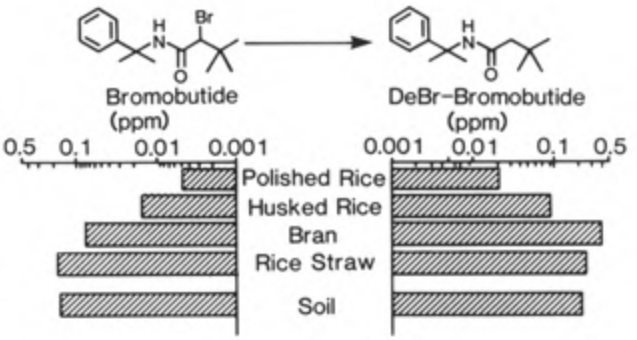
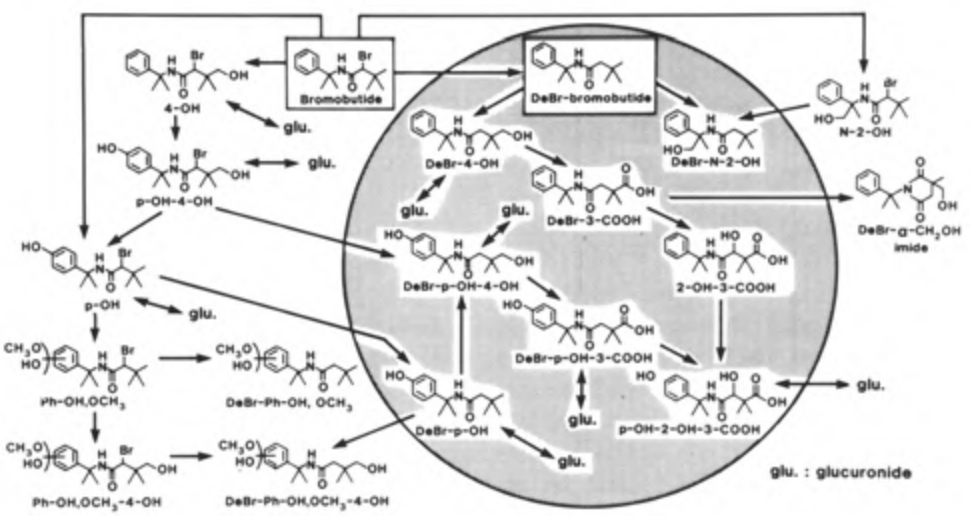


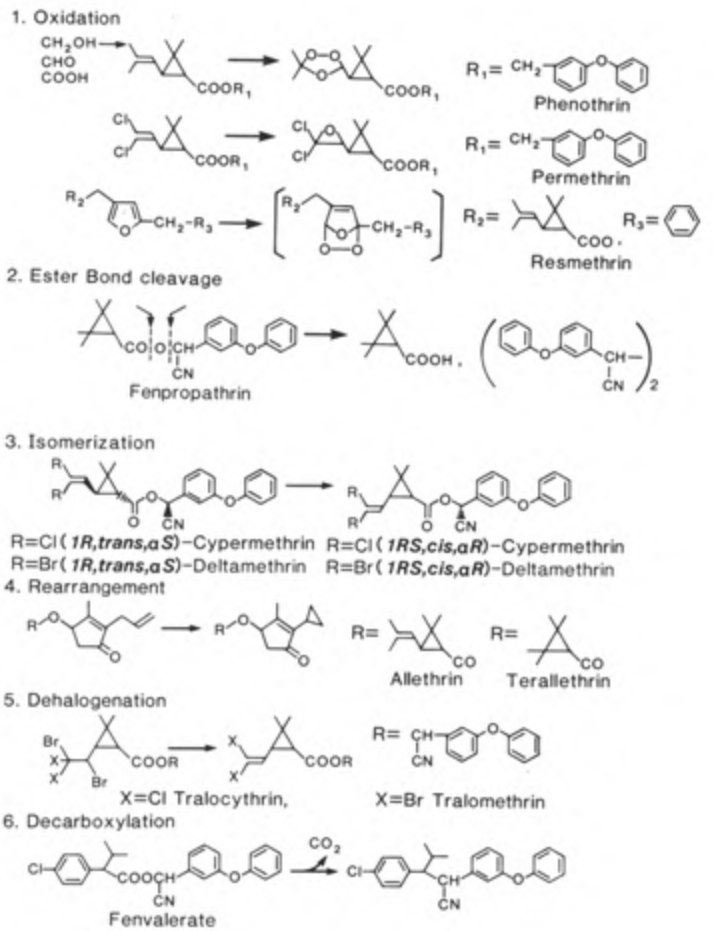
Fig. 4. Biotransformation pathways of bromobutide and deBr-bromobutide (in the circle) in rats and mice



As is well known, photolysis is one of the major governing factors to determine the environmental concentration of a chemical. Usually photochemistry on soil surface and in water is pursued, but for certain compounds with a high vapor pressure and also for those

persistent in the environment vapor phase photolysis will be carried out. Often the photoproducts as well as decomposition pathways are different from those in biospheres due to larger energy utilized in the presence of oxygen, and also due to naturally occurring photosensitizers and quenchers (Fig. 5, Ruzo, 1983; Ruzo and Casida, 1981). Photolysis of fenvalerate affords a typical example. Decarboxy fenvalerate, present in water and on plant surface, formed via intermediary radiacls is unique, since it is not included among the metabolic

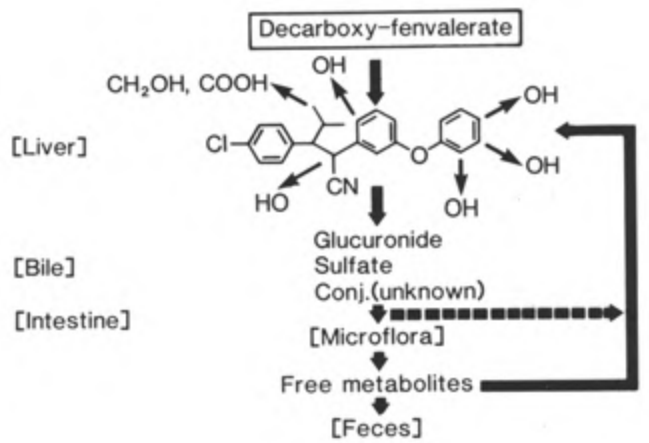
Fig. 5. Photochemical reactions of pyrethroids



patterns in mammals, therefore quite new to them, which means the situation requiring additional metabolic study (Mikami et al., 1985c).

The rat metabolism shown in Fig. 6 reveals that decarboxy fenvalerate orally administered is extensively hydroxylated, and excreted rapidly and completely into feces through entero-hepatic circulation. The decarboxy fenvalerate causes no untoward toxic effects in mammals based on a variety of preliminary toxicity testings (Mikami et al., 1985f). Therefore, its concomitant occurrence will have no bearings on the toxicological profile of fenvalerate in mammals.

Fig. 6. The excretion profiles for metabolites of decarboxy fenvalerate in rats



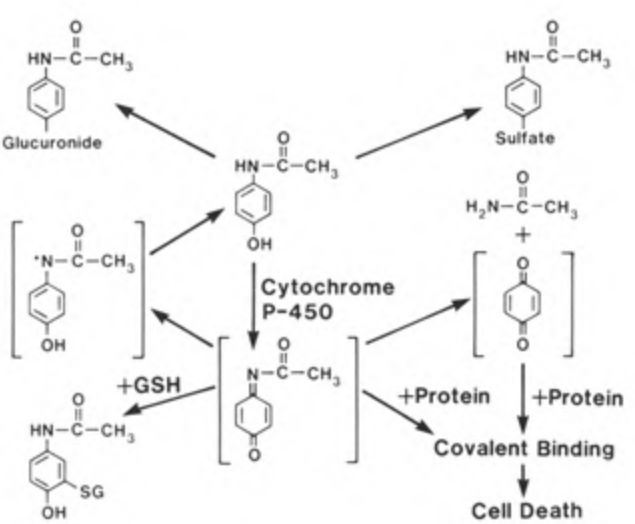
Bioaccumulation potential of a compound through food chain is in parts closely related with its octanol/water partition coefficient. Fenvalerate is very sparingly soluble in water with the same order of DDT, ~10 ppb, having quite a high octanol/water partition coefficient, i.e. log Pow = 6.41. However, it is much less bioaccumulative as compared with DDT, due to its easy biodegradation. In fact, in an aquatic model ecosystem composed of soil sediment, plankton, algae, daphnia, snail and fish, fenvalerate and fenitrothion, which are both biodegradable, show far less bioaccumulation than DDT and its analogs. To be more singificant, once the fish are

transferred to fresh water, both compounds readily disappear from the fish body, whereas DDT and its derivatives are depleted very slowly. Moreover, none of the metabolites of fenitrothion or fenvalerate are bioaccumulative (Miyamote et al., 1979; Miyamoto et al., 1983; Miyamote et al., 1985; Miyamoto, 1987; Ohkawa et al., 1980a). These results are apparently in good accord with the findings in the actual environment.

METABOLISM IN RELATION TO DOSAGE

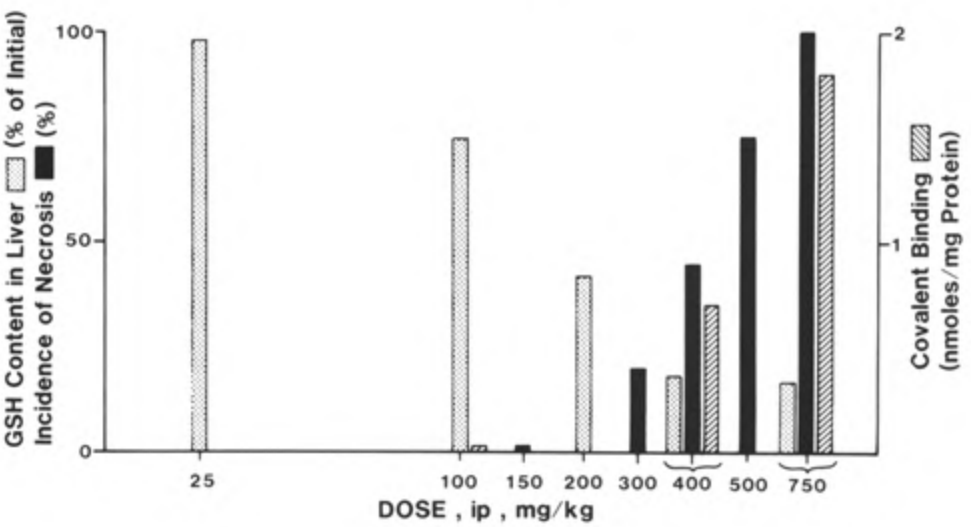
It is generally accepted that absorption, distribution, biotransformation and excretion of a chemical is not necessarily dose-independent (or linear regardless of dosages), and when one metabolic pathway is saturated at higher dosages, then a certain metabolite through another pathway may increase in amounts (Hollingworth et al., 1967). Therefore, if toxicity manifested by higher dosages can be correlated with or ascribed to the presence of a specific metabolite, then toxicokinetic consideration should be necessary to confirm whether the metabolite is formed in enough amounts at lower dosages. Such a

Fig. 7. Postulated pathways of acetaminophen metabolism



consideration is particularly important when toxicity data in animals are to be extrapolated to man, since the doses tested in aminals need not bear any relationship to human exposure levels. One representative example is acetaminophen-induced hepatotoxicity in several mammalian species. As shown in Fig. 7, below certain threshold levels the intermediary N-acethylimidoquinone is conjugated with glutathione. However, at higher dosages glutathione is depleted, with concomitant increase of covalent binding of acetoaminophen (more exactly, reactive quinone metabolites) with hepatic protein, which results in increased incidence of necrosis of hepatic cells (Fig. 8, Mitchell et al., 1973).

Fig. 8. Acetaminophen-induced hepatotoxicity in mice

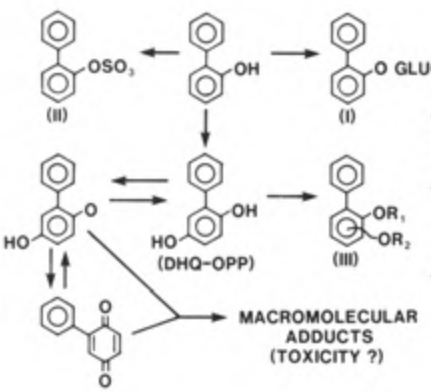


Similar depletion of glutathione responsible for detoxication of oxidized products of bromobenzene (Ugazio, 1978) and chloroform (Pohl, 1979) is conceived for the mechanism of hepatotoxicity of these compounds.

Another noteworthy finding suggesting formation of novel metabolites at massive dosages is carcinogenic o-phenylpnenol (OPP) or its sodium salt (OPP-Na). After gavage with 50 mg/kg or less, most of the administered OPP

or OPP-Na is recovered in F344 rat urine as glucuronide or sulfate conjugates of parent material (Fig. 9). After administration with 500 mg/kg a new metabolite, III, is formed, which upon acid hydrolysis yeilds DHQ-OPP apparently produced by mixed function oxidase (Hiraga and Fujii, 1981; Hiraga and Fujii, 1984; Nakao et al., 1983). It is postulated that potentially reactive metabolites including DHQ-OPP will be associated with bladder carcinogenicity and renal toxicity of OPP and OPP-Na,

Fig. 9. Hypothetical scheme for the metabolism of OPP and OPP-Na in F344 male rats



Dose of OPP-Na (mg/kg)	Metabolite III (% of dosed ^{14}C)
5	<2
50	<2
500	24.6±6.4

since e.g. intravesical application of DHQ-OPP in rat urinary bladder results in DNA damage as well as hyperplasia of the bladder epithelium (Morimoto et al., 1987; Reitz et al., 1983).

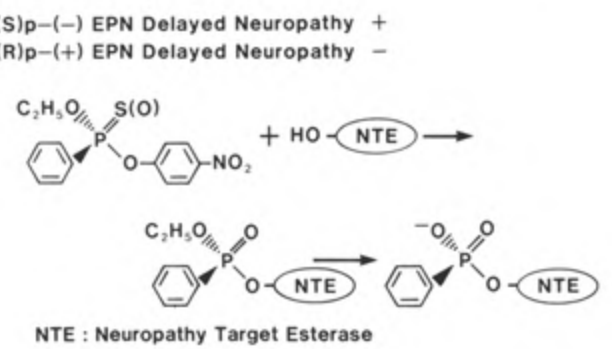
STEREOSELECTIVE METABOLISM

Is is well recognized at present that stereochemical factors play a significant role in dispositon as well as in biological action of xenobiotics. Owing to the progress of chiral synthesis together with refined stereochemical analysis a number of findings are being accumulated indicating differential metabolism of enantiomers of chiral substrates and creation of new asymmetric centers during metabolic processes. The

various drug-metabolizing enzymes such as mixed function oxidases, epoxide hydrolases and glutathion transferases have been demonstrated to exhibit substrate- and product-selective effects. (Cf. Abstract of lectures and posters presented at International Symposium on Methods of Stereochemical Analysis and Their Application into the Biosciences. University of Tübingen, FRG, April 5 to 8, 1988, and Abstract of papers presented at the Second ISSX Meeting, Kobe, Japan, May 16 to 20, 1988). However, there are relatively few studies demonstrating chiral metabolism to be related with toxicity.

Some organophosphorus esters develop delayed neuropathy in mammals, and experimentally the toxicity is developed in adult hens. The delayed neuropathy is associated with inhibition of neuropathy target enzyme, NTE, in brain and spinal cord, followed by aging (removal of one alkyl group from the active site of inhibited enzyme, see Fig. 10). Of the two chiral isomers of EPN, ethyl p-nitrophenyl phenylphosphonothioate, only S(-)-isomer is delayed neurotoxic. It is now inferred that the inhibited NTE by S(-)-isomer is preferentially modified through the aging process. In contrast, although the R(+)-isomer inhibits NTE similarly, the amount of the aged protein never reaches the postulated level for manifestation of delayed neuropathy (Johnson and Read, 1987; Ohkawa et al., 1977).

Fig. 10. Chirality of EPN isomers and delayed neuropathy



A more distinct example is chiral metabolism of fnvalerate isomers in mammals. When racemic fenvalerate containing four chiral isomers is administered subacutely

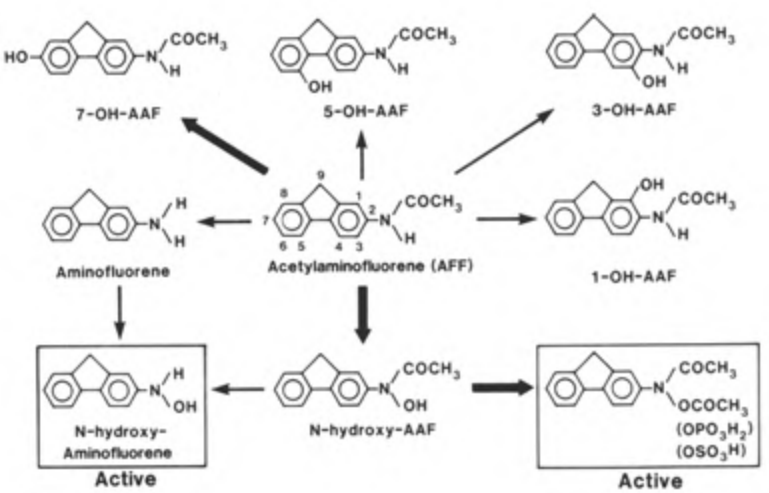
to mice and rats, microgranulomatous changes are observed in several tissues including spleen, lymph node and liver, which are defined as a tissue response to injury caused by a poorly soluble substances. The causative agent stimulates the mononuclear phagocytic system: The mononuclear cells changes their forms, proliferate and migrate locally through tissues. The resultant accumulation of cells are called (micro)granulomata. The granulomatous cells have a tendency to fuse, forming large multi-nucleated cells called giant cells. The administration of the respective chiral isomers of fenvalerate reveals that Bα [2R,αS] isomer is solely responsible for the histological changes. Although general metabolism of the four chiral isomers exemplified in Fig. 2 proceeds basically in similar fashions, Bα gives rise to higher radioactive residues in mammalian tissues. The residual metabolite is identified to be cholesterol conjugate of [2R]-2-(4-chlorophenyl)isovaleric acid (abbreviated as CPIA-cholesterol ester). It has been shown that this cholesterol ester is actually the causative agent of granulomatous changes — through granuloma formation by CPIA-cholesterol administration —, and that membrane bound carboxyesterases, therefore not the three known cholesterol ester synthesizing enzymes, preferentially catalyze transesterification of acyl moiety of Bα with cholesterol, as shown in Fig. 11 (Kaneko et al., 1986; Miyamoto et al., 1986a; Miyamoto et al., 1986b; Takamatus et al., 1987).

Fig. 11. Formation of cholesterol ester of 2R-2-(4-chlorophenyl)isovaleric acid from Bα isomer of fenvalerate

COMPARATIVE METABOLISM IN MAMMALS

It is now obvious that all species do not necessarily respond to xenobiotics in the same way, and often toxicity of a specific compound remarkably varies depending on species. Among a variety of factors underlying such species difference is included the difference in metabolism. For instance the rat seems to be more susceptible to hepatocarcinogenic effect of acetylaminofluorene (AAF) than are the mouse and the hamster, whereas the guinea pig has been resistant. The kinetics of AAF metabolism in primary monolayer culture of hepatocytes reveals that the ratio of reactive metabolites of AAF covalently bound to cellular macromolecules (active metabolites in Fig. 12) to the ring hydroxylated metabolites and their glucuronide conjugates (detoxified products) is far higher in rat, followed by hamster, guinea pig and mouse, which is in apparent accord with the susceptibility to AAF (Holm et al., 1986).

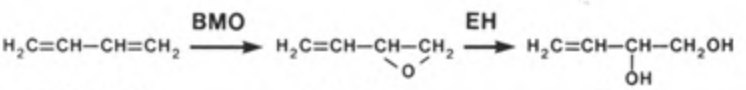
Fig. 12. Metabolism of acetylaminofluorene



When 1,3-butadiene is incubated with postmitochondrial fractions of liver and lung from mouse, rat, monkey or man and an NADPH-generating system, the formation rate of butadiene monoxide is different in the four species, as shown in Table 1. In both liver and lung mouse produces

larger amounts of butadiene monoxide (an activation product) than rat, which apparently corresponds to susceptibility of tumor formation, whereas monkey and human lung tissues do not produce any measurable level of butadiene monoxide. Furthermore, epoxide hydrolase activity (detoxification) determined by hydrolysis of 3-(p-nitrophenoxy)-1,2-propene oxide is quite high in monkey and man as compared with mouse and rat. The results might suggest that the rodent inhalation toxicity data with 1,3-butadiene could not be straightforwardly extrapolated to man (Schmidt and Loeser, 1985).

Table 1. Activity of butadiene oxidase and epoxide hydrolase in postmitochondrial preparations (a,b)



BUTADIENE EPOXIDE DIOL

	Liver		Lung	
	BMO	EH	BMO	EH
mice, ♂	1.00	1.00	1.00	1.00
mice, ♀	1.74	0.79	0.79	—
Rat, ♂	0.66	0.75	0.16	0.50
Rat, ♀	0.54	0.51	0.11	0.75
Monkey, ♂	0.26	11.98	n.d.[c]	2.00
Monkey, ♀	0.27	14.42	n.d.	2.75
Human	0.63	15.81	n.d.	2.50

(a) BMO : Butadiene Monoxidase EH : Epoxide Hydrolase
(b) mice, ♂ =1.00 , (c) n.d. : not Detectable

These two simple examples appear to favor the importance of matabolism as one major governing factor to determine the response of an animal species to a chemical. As is well known, ideally all toxicological information should be obtained in an animal species that responds to the chemical equally to, or similarly to man. Herein lies the necessity for information on comparative metabolism in experimental animals and man. Fig. 13, Tables 2 and 3 illustrate certain aspects of metabolic activities of animal species including man which are arbitrarily chosen from cumulative evidences of biotransformation of

Fig. 13. Relative monooxygenase activity (logarithmic scale) in mammals (male rat = 1.0)

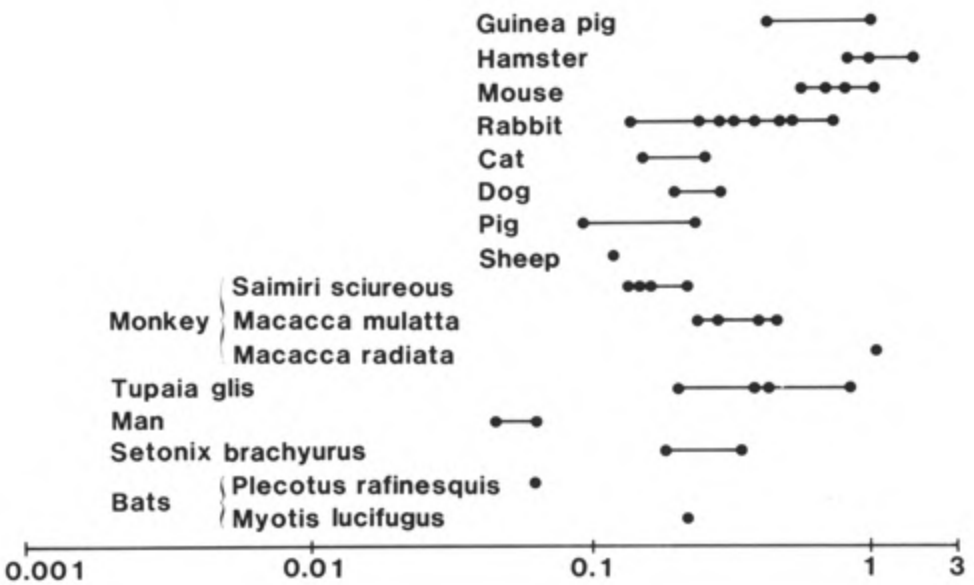


Table 2. Glutathion S-styrene oxide transferase activity in cytosolic fractions of mammalian tissues[a)]

Species	Glutathione Transferase Activity (nmol Product/mg Protein per Min)		
	Liver	Kidney	Lung
Mouse	149	34	15
Hamster	107	14	3.8
Rat	25~224	67~82	6~20
Guinea-pig	237~356	65~93	19~25
Rabbit	27~36	8~18	6.5~8.0
Dog	19	13	n.d.
Pig	11	5.1	1.3
Baboon	7.4	4.2	2.7
Human	25	5.9	6.9

a) Different Activities, from Different References.

Table 3. Species variation in conjugation of phenol[a)]

Species	Glucuronide of		Sulphate of	
	Phenol	Quinol	Phenol	Quinol
Pig	100	0	0	0
Indian Fruit Bat	90	0	10	0
Rhesus Monkey	35	0	65	0
Cat	0	0	87	13
Man	23	7	71	0
Squirrel Monkey	70	19	10	0
Ring-Tailed Monkey	65	21	14	0
Guinea Pig	78	5	17	0
Hamster	50	25	25	0
Rat	25	7	68	0
Ferret	41	0	32	28
Rabbit	46	0	45	9
Mouse	33	14	43	5

a) % of ^{14}C Excreted in 24 Hrs

xenobiotics (Miyamoto et al., 1988). Based on these and other evidences it can be concluded that "man has virtually all of the capabilities that have been demonstrated in experimental animals for the metabolism of xenobiotics, i.e. man, in general, does not suffer from any particular defect in metabolism. However, whilst the metabolic options for a given compound are now largely predictable, the choice which even the rat makes between these options is not. Similarly, it is not possible to predict the choice which man will make from data in animals, as shown in Table 4. Thus, foreign compound metabolism is compound-specific as well as specics-specific. Therefore, if any knowleage of the metabolic fate of chemicals in man shold be needed, it is advisable to study man" (Miyamoto et al., 1988). Obviously data on human exposure obtained e.g. during production and use of the chemical should be collected as part of the continuing evaluation of the safety of compounds in commercial use. However, there is the need, at a relatively early stage, to obtain information on the absorption, distribution, metabolism and elimination of the chemical in human subjects, in order to choose the species that are most likely to have a high predictive value for human response. The real issue with respect to human studies is, as once discussed by Paget (1970), not whether or not human subjects should be used in toxicity experiments but rather

Table 4. Primates varsus non-primates as metabolic models for man

Pattern as Model for Man	Percent of Occasions		
	Rat	Other Nonprimate[a]	Rhesus Monkey
Good	29	32	73
Fair	12	27	19
Poor	20	9	4
Invalid	42	32	4

a) Dog, Rabbit or Guinea Pig

Rating for Species Similarity

Good	Pathways Similar
Fair	Metabolic Pathways Similar, but Significant Differences in Amounts of Metabolites
Poor	Marked Species Differences in Amounts of Metabolites by Similar Pathways
Invalid	Metabolic Pathways Quite Different

whether such chemicals, deemed from animal toxicity studies to be relatively safe, should be released first to controlled, carefully monitored groups of human subjects, instead of being released indiscriminately to large populations with no monitoring and with little or no opportunity to observe adverse effects (Paget, 1970). Needless to say, in actually carrying out the human studies, it is imperative that the ethical consideration should be made, e.g., by following such international codes as The Declaration of Helsinki I & II.

CONCLUSION

It is hoped that the above examples clearly demonstrate the importance of knowledge on environmental behavior of any chemicals as the sound basis for risk assessment. Quite obviously without such knowledge on xenobiotics no pile of mammalian and ecotoxicological data may be effectively used. What is more important is that each information is no more discrete, and specific to a given compound. A certain generalization may become possible now, by which environmental fate of a chemical can be presumed fairly exactly. Evidently, however, there are

many areas remaining for future research for more precise knowledge of environmental behavior of chemical, or more broadly future research to elucidate any chemical-biological interactions. Amoung them will be included the followings. Namely,

1. Improved techniques for metabolism studies.
   For example,
   1) more precise, specific analytical procedures to detect minute and short-lived reactive metabolites,
   2) computer-aided analysis for metabolic sites in the molecule,
   3) in vitro systems replacing in vivo studies,
   4) laboratory studies more representative of total ecosystems in the environment, especially simplified and reliable test systems taking into account the relationship between physico-chemical parameters and actual environmental behavior.
2. Further development of biochemical toxicology, i.e., structure-toxicity relationships defined at molecular level; and development of appropriate biomarkers for specific toxicity.
3. Improved scope and detail of human studies including the above 1 and 2, with a view to facilitate extrapolation of animal data to man.

These comprehensive investigations on xenobiotics will ultimately lead to the creation of more bioacceptable chemicals.

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PL2

RISK ASSESSMENT OF ENVIRONMENTAL CARCINOGENS

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This presentation is intended to show the current status and future problems of risk assessment for environmental carcinogens from the aspect of toxicologic pathology.

1. Old concept of carcinogens

Modern laboratory sciences on the chemical carcinogenesis is said to have started with two series of studies concerning the carcinogenicity of coal-tar; induction of skin cancer in rabbits after long-term application of coal-tar by Yamagiwa and Ichikawa (1915) and the isolation/identification of carcinogenic polycyclic aromatic hydrocarbons from coal-tar by Kennaway and his coleagues (1924). Thereafter many carcinogens such as o-aminoazotoluol, dimethylaminoazobenzene or ß-naphthylamine were discovered mostly from industrial chemicals, and until 1951 approximately 350 chemicals were registered as carcinogens. The discovery of carcinogens facilitated experimental research on carcinogenic mechanism leading to two theories, namely two stage carcinogenesis and summation carcinogenesis proposed on the assumption that cancerization is a type of somatic cell mutation. Meanwhile epidemiological investigation disclosed an association of excess incidence of human cancers in certain occupational communities with exposure to related chemicals such as bladder cancers in the dye-stuff industry or skin cancers in the textile industry. Until the middle of this century, these experimental and epidemiological evidences underlay the general concept concerning the carcinogen and the carcinogenic risk of chemicals, 1) the carcinogen is a particular compound capable of producing cancers through altering the genetic materials of target cells and 2) the carcinogen is a hazardous compound to be eliminated from human environment or to be avoided from human exposure. Based on this simple concept the carcinogenic risk assessment of chemicals could be made with the data to classify the chemicals into two categories, namely carcinogens or non-carcinogens.

2. Diversity of carcinogens

In 1960s two kinds of scientific evidences were published raising a posibility that exposure to environ-

mental carcinogens was associated with excess risk of cancers not only within particular small communities but also in general human population. The first one is the epidemiological evidence suggesting that majority of human cancers results from exposure to environmental carcinogens, and the second one is the experimental evidence from short-term mutagenicity tests indicating a wide-spread distribution of mutagens in human environment. Motivated by these scientific evidence, aiming at detection of causative principles for human cancers, a large number of chemicals have been tested for potential carcinogenicity in animals using a standard test method and evaluation criteria of test results proposed by WHO.

The WHO's proposal indicates that a test compound is judged as being positive for carcinogenicity when the tests turn out to meet one of the following 3 criteria; 1) a significant increase in the incidence of the same types of neoplasms as found in the control animals, 2) the occurrence of types of neoplasms not observed in control animals and 3) a decreased latend period for the production of neoplasms in comparison with that in control animals. These criteria are still useful for the evaluation of carcinogenicity testings. However, it must be realized that the carcinogenicity testings have been designed primarily to detect or presume a weak carcinogenicity or non-carcinogenicity of test compounds, so that, theoretically, they can only provide data to show whether or not the test compounds have a potential of inducing tumors in animals.

With accumlation of test data, it has become increasingly apparent that carcinogens designated on the basis of animal tests are quite diverse in nature in terms of both mechanism of action and potencies. For example, there appears to be no definite correlation between mutagenicity test results and animal carcinogenicity test results; some chemicals with siginificant mutagenic activities are incapable of producing tumors in animals while some other chemicals without mutagenic activity can induce tumors in animals. Regarding the potency of carcinogenicity, there can be noted 10 million-fold difference in TD50 value between the strongest carcinogen, aflatoxin B1 and the weakest carcinogen, sodium saccharin. Considering the diversity of carcinogens, apparently, it is neither a scientific way nor a practical manner to treat all carcinogens based on long-term animal tests as

being similarly hazardous to human.

3. Procedures of risk assessment.

Assessment of human cancer risk associated with any particular specified chemical exposure requires a complicated scientific procedure starting with careful review of all pertinent information on the chemical derived from experimental, epidemiological and/or clinical studies. It is generally agreed within the scientific community that there are four steps or components which are typically involved in carcinogenic risk assessment. The first step, which is referred to as hazard identification, entails a qualitative evaluation of data on the potential of a chemical to produce carcinogenic effects to human. The second step, expsoure assessment is the process of measuring or estimating real or hypothetical human exposure to a chemical of interest. The third step, dose-response assessment is the evaluation of both hazard and exposure information to estimate the mathematical probability that the carcinogenic potential associated with an agent will be realized in the human population under defined conditions of exposure. In the final step referred to as risk characterization, all relevant information from the first 3 steps is integrated to characterize the carcinogenic risk associated with the expected human exposure to the chemical of interest.

4. Risk assessment of classical genotoxic carcinogens

A virtually safe dose (VSD) is defined as a value corresponding to the dose level or dose range which can induce tumors at extremely low rates such as 1/100,000 or 1/1,000,000. This value can be obtained by donward extrapolation (low dose extrapolation) of animal dose-response data by use of proper mathematical models. The VSD is often used as a parameter for the assessment of human cancer risk associated with exposure to a potent carcinogen such as aflatoxin B1, bonzo(a)pyrene or dimethylnitrosamine. This kind of procedure is based on the assumption that man belongs to the animal species most sensitive to the carcinogen and, therefore, it can also be applied to the case of other strong genotoxic carcinogens either synthetic or nature-born. This assumption, however, is not necessarilly plasusible for all carcinogens designated on the basis of long-term animal tests (carcinogens according to the criteria of WHO), particularly for epigenetic or secondary carcinogens.

At present, our knowledge is still insufficient to clearly distinguish the genotoxic carcinogen and the epigenetic carcinogen. However, perhaps, different from genotoxic carcinogens, epigenetic carcinogens induce tumors in animals without any proven direct effects on DNA in target cells but through unknown secondary mechanism such as sustained effects on hormonal, metabolic or immunological functions of animals or repetitative necrosis and regeneration in the target tissues. Therefore, carcinogenic risk of these compounds can be assessed on the basis of proper mechanistic and dose-response studies on the effects of each compound secondarily leading to tumor formation.

5. Execution of risk assessment

The risk assessment is a scientific procedure to assess or infer the risk level or risk profile on the basis of existing information which includes; 1) Long-term animal test results or epidemiological evidences concerning carcinogenic potential in human; 2) Scientific data on the mechanism of action, comparative metabolism, pharmacokinetics and structure-activity relationships; 3) Biochemical or epidemiological data to estimate the level of human exposure; and 4) Experimental or epidemiological data concerning the carcinogenic potency in terms of a specific risk estimate or an upper limit on the underlying risk.

Therefore, the execution of any given risk assessment may be hampered by involvement of various uncertainties due to deficiences or critical gaps in the necessary information. On such occasions it is necessary to make assumptions on the basis of the scientific information at hand to adjust the existence of uncertainties so that the risk assessment can be completed. Accordingly plausibility of these assumptions is also regarded as a critical factor influencing the result of risk assessment. Thus, inappropriate assumptions may yield inappropriate assessment due to either over-estimation of risk or under-estimation of risk.

At present, the risk assessment is regarded as a complex mixure of currently available data and assumptions based on prevailing scientific thought. Fig.1 illustrates the execution of risk assessment on a chemical. Here, the assumptions are taking a role as the inference rules for estimation of the risk on the basis of insufficient information.

From these points of view, it is concluded that risk assessment requires 2 categories of future studies. The first one is the issue on the scientific information necessary for the assessment on a particular specified chemical which includes the methodology of data production and the system for data distribution. Toxicity test guidelines should also be scrutinized in respect of risk assessment. The second one is the problem on the inference rules or the assumptions for risk assessment which includes a series of basic research concerning the extrapolation from animal to man, from high dose to low dose, from environmental concentrations to exposure levels and from intake levels to effective levels at the target sites.

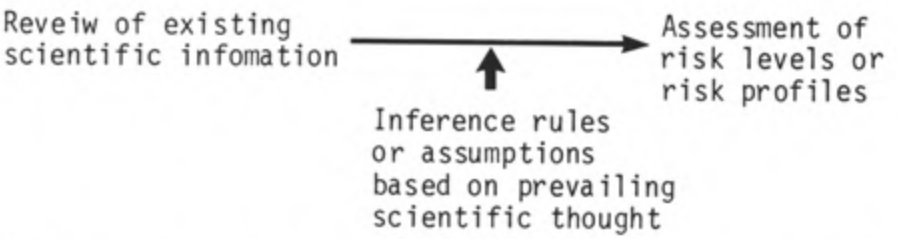


Fig.1 Process of risk assessmet on a particular specified chemical

SYMPOSIA

S10 SYMPOSIUM I SOCIAL APPLICATION OF ACCEPTABLE DOSE CONCEPT

Chairpersons: M. Ikeda (Tohoku Univ. Sch. Med.)
& K. Sasaki (Tohoku Coll. Pharmacy)

S-1-1 Exposure limits recommended for occupationally exposed workers
H. Sakurai (Keio Univ. Sch. Med.)

S-1-2 Ambient air quality standards
K. Yoshida (Kyoto Industr. Health Ass.)

S-1-3 Acceptable dose of food additives
M. Tobe (Nat'l Inst. Hyg. Sci.)

S-1-4 Tolerance of agrochemicals in food
T. Hayama (Tokyo Univ. Agri. & Tech.)

SCOPE:

It is established that a threshold generally exists in the dose-response relationship of non-carcinogenic effects of chemicals. Accordingly, the concept of the threshold has been introduced in many fields of occupational, public and environmental health in setting socially acceptable doses of potentially toxic chemicals. Proposals have been made also for the socially acceptable dose of carcinogens, e.g., "virtually sage dose". It is not yet extensively discussed, however, whether or not such doses are set in a harmonized manner so that the risk will be even in various facets of life and no specific group(s) of people have to face with unduely higher risk than others.

Four experts are invited in this symposium to report on the current concepts of national air quality standards, the occupational exposure limits recommended by Japan Association of Industrial Health, allowable daily intake of additives and pesticide residues in food. Other related items may also be presented. It is expected that, through discussion, the toxicologists from various scientific backgrounds will not only realize the common principles but understand life facet-related differences in the process of application. Mutual understanding among the toxicologists may hopefully be the first step to the achievement of coordination in practice of the "acceptable dose" concept.

S11

EXPOSURE LIMITS RECOMMENDED FOR OCCUPATIONALLY EXPOSED WORKERS

H.Sakurai (Keio Univ. Sch. Med.)

INTRODUCTION :

Japan Association of Industrial Health (JAIH) now recommends occupational exposure limits (OELs) for 140 industrial chemicals and 34 dusts (JAIH,1987). It started to publish the recommendation in 1961 (JAIH,1961) and its latest annual recommendation approved by the General Meeting of JAIH in April 1988 will appear in the forthcoming number of Jap.J. Ind.Health.

Although JAIH is a non-governmental organization, its recommendations on OEL have been extensively used as reference values for the evaluation of occupational exposure levels and guidelines for the improvement of the working environment.

American Conference of Governmental Industrial Hygienists (ACGIH), also a non-governmental organization in spite of its naming, recommends Threshold Limit Values (TLVs) for about 600 substances (ACGIH, 1987). Because of its long history of evolvement (ACGIH,1984) and inclusion of substantial number of chemicals, ACGIH's recommendation has served for the practices of industrial hygiene in many countries.

Both JAIH's OELs and ACGIH's TLVs have been worked out on the basis of the same principle and TLVs have also been utilized to supplement OELs in Japan. Of 139 chemicals for which both OELs and TLVs are recommended 85 have the same values, while 21 are higher and 33 are lower in OELs. The gaps stemmed mainly from the differences in judgement of data or additional information available at the time of revision. However, the differences in recommended values are generally not large.

DEFINITIONS AND CHARACTERISTICS :

Occupational exposure limit (OEL) is defined as the concentration below which nearly all exposed workers are expected to be free from adverse health effects if the arithmetic mean of exposure concentration for a normal 8-hour workday and a 40-hour workweek is less than this value and if physical work load is not heavy. Its several characteristics which are specified in the note to JAIH's recommendation are the following.

(1) Because man's susceptibility to hazardous chemicals varies among individuals, discomfort, aggravation of a pre-existing ill health or occurrence of an occupational disease may not, in some cases, be prevented at or below OELs.

(2) OELs are based on diverse information from industrial experience, human studies or animal experiments, and the amount and quality of the information used for establishing OELs vary substantially.

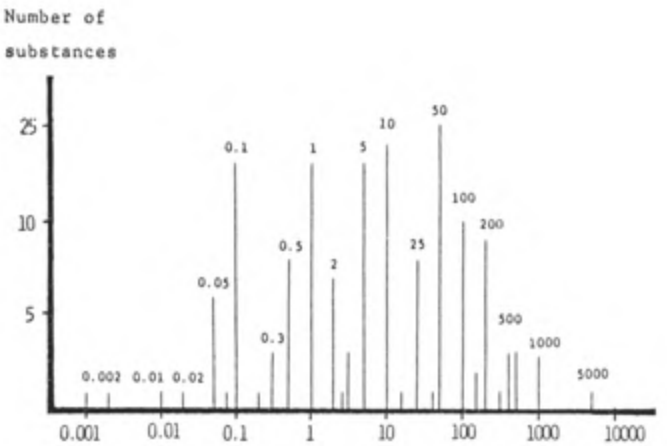
(3) The types of health effects which are taken into consideration for establishing an OEL vary from substance to substance. For some substances, basis was sought on the prevention of manifest health impairment, while for others, such effects as discomfort, irritaion, or narcosis etc. were considered.

SAFETY FACTORS:

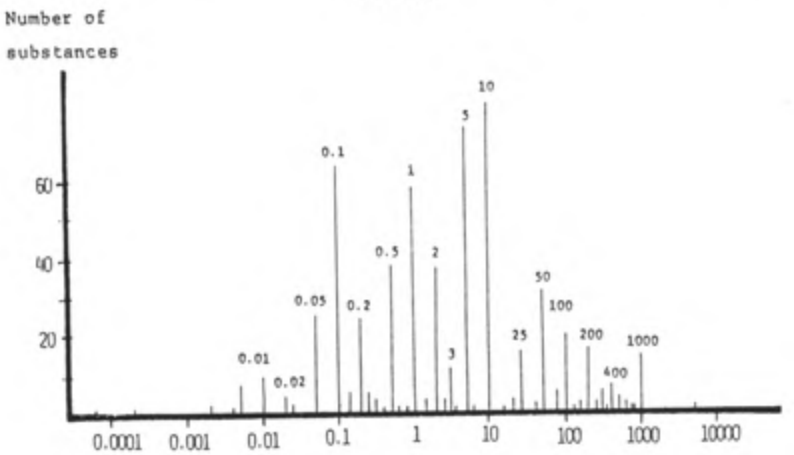
In deciding OEL values, information on exposure-effect or exposure-response relationship is essential as in any other health standard setting. However, sufficient data on such quantitative relationship are known for very few substances. For most chemicals only one set of data on the effect and the level of exposure is used to establish the OEL. It is a matter of judgement to choose a data set which is most relevant for the standard setting.

After the selection of key data, the OEL is set at the concentration that is almost the same as or lower than the exposure level of the selected data. When an OEL is decided, no specified safety factor such as 1/100 commonly used in setting standards for food additives is applied (Ikeda, 1972). Again, a judgement is needed in choosing a value which is the same as or lower than the key data.

Figs. 1 and 2 show the distribution of OEL values disreganrding the units which are either ppm or mg/m^3.
(Both ppm and mg/m^3 are assigned for gases and vapours, while only mg/m^3 for particulates.)



Fig·1 Distribution of occupational exposure limits (JAIH)



Fig·2 Distribution of TLVs (ACGIH)

It is interresting to note that most values have rounded digits of either 1, 2 or 5 in both JAIH and ACGIH recommendations. This fact implies that OELs have the precision of the order of approximately $1/2\chi \sim 2\chi$. And it also suggests that, in choosing a value that is lower than the observed effect level or no-observed-effect level, safety factors of $(1/2)^{n}$ may have been applied.

In order to clarify how much have really been adopted as safety factors, the published documentations for all OELs (JAIH) (1971-1987) have been reviewed.

Table 1 shows the distribution of the factors for 103 substances. Most others have been determined on the basis of analogy with other structurally related chemicals.

Table 1. Distribution of safety factors

Safety factor	Character of key exposure data: Observed effect level	Borderline between effect and no-effect	No-observed-effect level	Total
1	21	21	14	56
2/3～3/4	7		1	8
1/2	13		9	22
1/4～1/5	11		0	11
1/10	3		1	4
1/20	1		1	2

It is remarkable that 86 of 103 OELs (83%) have the factors between 1 and 1/2. Table 2 lists, for example, the names of substances for which slight symptoms are allowed to occur even at the levels of OELs. Most are set at the level where slight

Table 2. Substances for which OELs values are set at observed effect levels

Chemicals	Effects observed at OELs
Acrolein	Irritation
Acetone	〃
Ammonia	〃
Isopentyl alcohol	〃
Hydrogen chloride	〃
Ozone	〃
Chloroform	Nervous symptoms
Acetic acid	Irritation
Isopentyl acetate	〃
Ethyl acetate	〃
Butyl acetate	〃
Propyl acetate	〃
Pentyl acetate	〃
Dimethylamine	Histopathology
Potassium hydroxide	Irritation
Sodium hydroxide	〃
Styrene	Subjective symptoms
Nickel	Dermatitis
Dusts	Pneumoconiosis (slight)
Benzene	Hematological findings
Formaldehyde	Irritation

irritation on eyes and nose cannot be avoided. However, recent trend is toward avoiding even transient irritation to mucous membranes. The safety factor of 1/20 has been applied only for carcinogens. The choice of a safety factor from among 1～1/20 is an area of expert judgement, but main factors appear to be those shown in Table 3.

Table 3. Factors considered at the choice of safety factors.

1. Source of data, human or animal?
2. Reliability and precision of data.
3. Kinds and degree of the cretical effect.
4. Other effects observed at higher exposure.
5. Comparison with similar substances.
6. History of human usage and exposure.

CARCINOGENS :

JAIH's recommendation includes a list of establisbed and suspected carcinogens without the assignment of OEL value. But several carcinogens have been given numerical OELs. As mentioned above, those carcinogens tend to include more strict safety factors such as 1/10 and 1/20.

A characteristic feature of OELs for carcinogens is the fact that so-called low dose extrapolation has never been applied. However, it is interesting to notice that Occupational Safety and Health Administration (OSHA, 1988) of USA recently proposed tentatively Permissble Exposure Limits (PELs)for several carcinogens on the basis of low dose extrapolation by using 1/1000 as acceptable lifetime risk for working population.

CONCLUSION :

Occupational exposure limits (OELs) have been recommended for many chemical substances by a non-governmental organization, Japan Association of Industrial Health. In establishing TLVs from available data on dose-response relationship, a specified safety factor has not been introduced. However, the review of the published ducumentation on individual chemicals indicates that safety factors of the order of 1 to 1/20 have been used. It was noted that approximately 80% of OELs have safety factors of only 1 to 1/2. Clearly, OELs are set at or near the threshold. It is considered that, although OELs have many limitations, they have the merits of simplicity and pratical feasibility.

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S12

AMBIENT AIR QUALITY STANDARDS

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Abstract....Conceptual methods of setting national ambient air quality standards in Japan are discussed in comparison with case of occupational exposure limits in industrial health.

The air quality standards are set by the national government, based on recommendations of the Central Council on Countermeasures for Environmental Pollution, in conformity with the Fundamental Act on Countermeasures for Environmental Pollution. In setting the standard values, thresholds and the existence of specific groups in the people with high susceptibility to a certain pollutant are taken into account.

The standards are set following minimum effect reports in three branches of health effect studies on air pollution: animal experiments, human exposure, and epidemiology. Safe levels take into account the absence of effects, suspicion of irreversible effects, dwellings of the elderly/infant/sick, epidemiologic growth of nonspecific diseases such as asthma, and so on. Under these conditions, air quality standards become far severer than in the case of industrial health limits.

Key words threshold, air quality standards, occupational health limits, safety rate

INTRODUCTION

The history of setting ambient air quality standard in Japan is relatively short. With the episode of Yokkaichi-asthma as a turning point, the importance of standards was

realized and their setting was begun.

Ambient air is the largest in quantity among matters taken into the body. Air is taken in for 24 hours per day. Its quantity reaches about 12 cubic meters of volume, about 13 kg in weight. And it exceeds water intake (about 2 kg including water contained in foods) and food intake (dry weight about 0.5 kg).

The intake-rate of air pollutants (absorption rate or deposition rate) usually reaches about 90%, so setting standards is very important for the nation's health.

SETTING STANDARDS

Conceptual methods of setting standards are similar to the methods of setting industrial health limits, but in some important points fundamentally different.

1. Existing ambient air quality standards are fewer than the occupational health limits.

   The number of air pollutants which have national standards is far smaller than the number of threshold limits in industrial health. At the present, five pollutants, SO_2, SPM (suspended particulate matter), CO, NO_2, and photochemical oxidants, have standard values.

2. Air quality standards are governmentally determined.

   As ambient air quality standards have important effects on the nation's health, the standards are governmentally set under the Fundamental Act on Countermeasures for Environmental Pollution. In practice, the Central Council on Countermeasures for Environmental Pollution recommends actual values for the standard.

3. Actual procedure of setting the value

   Initially, research papers on three branches of the pertinent pollutant's health effects (animal experiments reports, human exposure reports, and epidemiologic findings) are collected, and thus the dose-effect/dose-response relationship is investigated. Generally, the most important and affective data are those of epidemiologic findings on the pertinent pollutant.

4. The safety rate, as we called it, is not considered as a general rule. In the case of standards, findings from the three research fields are investigated, but the epidemiologic effects which are actually observed in the residents show the lowest value in practice. It can be told that the safety rate is not necessary because standards are based on data which are actually observed in humans including those with high susceptibility.

5. Reasons for differnce between the air quality standards and occupational health limits

1) In the case of the standard, the sick, the infant, the elderly and the pregnant are contained in the applied subjects.

2) Exposure to the pollutant is continuous through 24 hours and its interruption can not be assumed.

3) Usually, epidemiologic growth of nonspecific diseases such as asthma or chronic bronchitis becomes a problem. On the other hand, in the case of occupational limit, usually specific disease such as intoxication from pertinent chemicals is the problem.

4) In the case of air pollution, in general, specific legal liability does not always exist between the polluter and the dwellers. In the occupational case, the employer's resposibility is well established.

5) In the occupational case, health monitoring systems are the legal obligation of the employer. In contrast, such an obligation does not exist in most cases of air pollution.

S13

ACCEPTABLE DOSE OF FOOD ADDITIVES

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In 1955, the Joint FAO/WHO Expert Committee on Food Additives established general principles governing the use of food additives, in which the committee stated that a reasonable basis for the evaluation of the safety of the food additives taken at a ceratain level should be obtained from critically designed animal experiments. At a meeting held in 1957, the committee recommended the guidelines on biological tests providing the data needed to establish the safety of intentional food additives for use. In the guidelines, the details of short-term and long-term toxicity tests were definitely described, in addition to the proposal of the utilization of 2 factors for the safety evaluation of food additives; they were the maximum ineffective dose (MID) and the margin of safety. In 1961, the committee proposed the concept of acceptable daily intake zones for man in order to establish the safety for use of intentional food additives in humans. This proposal meant that the idea of acceptable intake derived from the risk-benefit concept was introduced to the safety evaluation of food additives.

Maximum ineffective dose (MID)

Although the report by the committee admitted no question that the evidence obtained from humans in a proper way was the most rational for the evaluation of toxic response of humans, we should rely upon the evidence obtained in a different way because of the difficulty in performing human studies. The committee, therefore, recommended the extrapolation of data obtained from various animal species into humans. Namely, the committee proposed the determination of MID, which is a factor used for the calculation of acceptable daily intake (ADI).

MID was defined as a maximum dose inducing no demonstrable effects upon animals examined.

The fact that MID was selected as a basis of ADI calculation meant nothing, but indicated that the committee recognized the presence of dose-response relationship for food additives and also assumed the presence of a thershold. Furthermore, it seems to be generally accepted that toxic effects have thresholds, except for carcinogenicity. Therefore, I would like to demonstrate the presence of threshold based on the experience in a long-term toxicity test on methylmercury chloride in monkeys.

In the test methylmercury chloride was administered daily to the animals for 1,578 days in the longest case using 4 levels, L-1, L-2, H-1 and H-2. Although lower alkylmercury-specific neurological manifestations appeared on day 61 on average in the highest-dose group H-2 and on day 181 on average in group H-1, no such neruological signs were observed in group L 1 or L 2 until the final day of the test. Total doses as Hg of the compound up to the appearance of signs were nearly identical between 2 groups, namely, 15.9 mg/kg in group H-2 and 15.3 mg/kg in group H-1.Total doses given to the groups manifesting no symptoms were 39.6 mg/kg in group L-2 and 13.2 mg/kg in group L-1. The Hg concentrations in the hair and blood of animals of groups L-2 and L-1 decreased gradually after reaching peaks at 30 months in spite of continued administration of the compound.

Safety factor

As the committee mentioned, the order of 100 is mot commonly used as a safety margin. However, this figure has not been based on scientific evidence, but upon experiences, and a higher order is sometimes used.

Acceptable daily intake for man (ADI)

Acceptable daily intake for man (ADI) presently used was formerly expressed as acceptable daily intake 'zones' for man, which meant the concept of 'range'. That is, each figure of ADI is expressed in the range of O-x mg/kg/day, suggesting that the lower dose is always desirable for daily use of food additives.

It may be generally agreed that the determination of ADI calculated on the basis of scientific data is the most adequate procedure to our present knowledge to establish the safe use. Needless to say, the promotion of a deeper understanding and the development of technology in various scientific fields are required to increase the validity in MID which is now called no-observed effect level (NOEL), an important factor of ADI. At the same time, I look forward to seeing a new basis for more rational safety evaluation, taking the place of MID.

S14 TOLERANCE OF AGROCHEMICALS IN FOOD

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Abstract ----- The virtually safe level of the agrochemical residue in food is statistically discussed.

INTRODUCTION

Pesticides, fungicides, herbicides, animal drugs and feed additives are used to promote the productivity in agriculture. Although the chemicals have contributed greatly to the economy through stabilized high production of food and to our health through control of certain vector borne diseases, potential adverse effect on human health and on environment have been pointed out. In this review, the safety level of agrochemical residue in food is discussed briefly.

NONCARCINOGENIC CHEMICALS

A set of full toxicological data is required for legal approvement or registration of pesticides and feed additives. ADI and tolerance in each product(food) are calculated by the routine method in which one hundredth of NOAEL(no

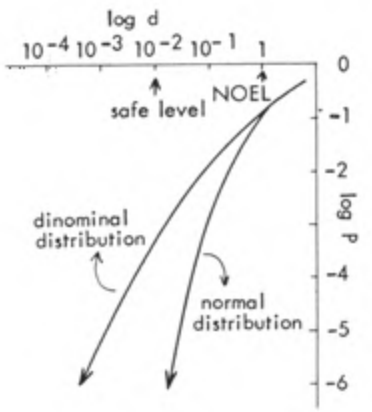

Fig 1. low dose extrapolation for threshold recognizable response (TD84/TD16 = 10, TD16 = NOAEL)

observable effect level) is usually considered as safe level.

Fig.1 shows a simulation of low dose extrapolation curves from a slow slope dose response relation(TD86/TD16=10 and TD16 is no effect level). TD16/100 will be the highest estimate of safe level for the response. If a normal distribution can apply to the dose response relation, possibility correspond to TD16/100 is about one to million. If the individual sensitivity to the response distribute dinominally, P will be about 3 to ten thousands.

Since a large number of chemicals with small amount is applied as animal drugs, a negligible residue level in food and withdrawal period after application are decided from the subchronic level toxicological data for each drug.

CARCINOGENIC CHEMICALS

The anticancer clause (Delany Clause) of US Food, Drug and Cosmetic Act provided the DES proviso which allows the use of a possible

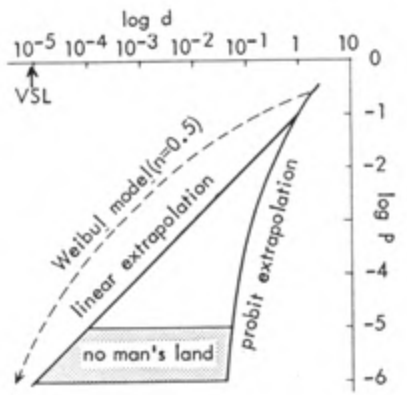

Fig 2. low dose extrapolation for carcinogen.

carcinogen as an animal drug or feed additive if no residue in food will be found by the method approved by the Secretary. This brought a dispute on the sensitivity of method for a quarter of century. FDA published the final SOM(sensitivity of method) regulation on the end of 1987. In this rule, FDA described only the principles in the regulation and transferred the controversial items to guidelines which could revise any time.

1). Any animal drug is determined as to whether it is subjected to the SOM regulation by the threshold assessment guideline.

2). On the drug come under the regulation, a virtually safe level, by which the possibility of carcinogenesis is one to million, is estimate from the chronic feeding data(fig.2).

3). A guideline for detail statistical procedure is not published yet, but upper 95 % confidence limit at lowest positive dose is suggested as the starting point for low dose extrapolation(fig.3).

4). From the tests on target animal, adequate target tissue, marker residue and regulatory assay

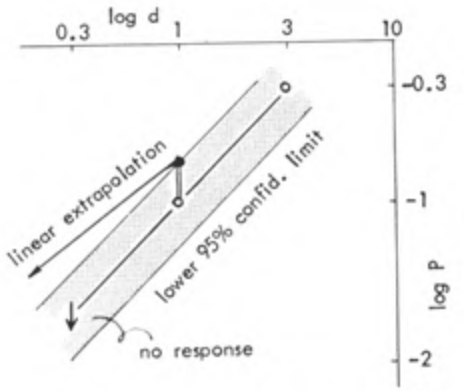

Fig 3. selection of starting point for low dose linear extrapolation. (dose;response : 3;50%, 1;10%, 0.3;0%)

method for residual detection must be established.

5). Propose a withdrawal period.

The principal points of view underlying the SOM regulation seem to be 1)do not deny existence of nonthreshold response and 2)one to million is a socially acceptable possibility.

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S20 SYMPOSIUM 2 BIOTECHNOLOGY AND ITS APPLICATION TO TOXICOLOGICAL SCIENCES

Chairpersons:
M. Watanabe (Res. Inst. Tbc Cancer, Tohoku Univ.)
& Y. Suzuki (Pharmaceut. Inst., Tohoku Univ.)

S21 The gene technology provided a key to understanding the genetic basis of human disease
M. Obinata (Res. Inst. Tbc Cancer, Tohoku Univ.)

S22 Micromanipulation of mammalian embryos
S. Sugawara(Tohoku Univ. Facult. Agricul.)

S23 Transgenic mice as systems for analyses of biological functions
M. Katsuki (Tokai Univ. Sch. Med.
& Central Inst. Exp. Animals)

S24 Protein engineering in studies on metabolism of toxic substances
Y. Imai (Inst. Protein Res., Osaka Univ.)

S25 Development of a new type of drug by using cell technology; preparation of human interferon-beta from human diploid fibroblast cultures
S. Kobayashi (Biomaterial Res. Inst., Ltd.)

SCOPE:

It is well known that toxicological sciences have been developed by making practical applications of principles in advanced general biology. With the remarkable progress in genetic and cellular technology we were awakened to new and profound needs to study the fundamental toxicological sciences.

Five excellent researchers were invited in this symposium to express their views in the recent progress of biotechnology, especially, a role of oncogene in cell differentiation and growth, DNA diagnosis in clinical medicine, a developmental biotechnology in mammals, the production of experimental animals as a model for human diseases, a role of protein engineering in research on drug metabolizing enzyme systems, a use of human diploid cells for the production of human minor constituents. It is expected that the toxicologists participated could realize the application of modern biotechnology in the research of toxicological sciences.

S21 THE GENE TECHNOLOGY PROVIDED A KEY TO UNDERSTANDING THE GENETIC BASIS OF HUMAN DISEASE

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INTRODUCTION

In this symposium, I review the recent development of gene technologies and their contribution to understanding human disease.

The gene technology was started in the early 1970s by the molecular cloning of eukaryotic genes in E.coli. Thereafter, many eukaryotic genes were cloned and their nucleotide sequences were determined. General views of genome structure and regulation of gene expression of eukayotes including human are presented. The cloned gene from one organism can be transmitted as a genetic material to another organism and its gene product can be produced if an apropriate manipulation of the gene is given. For example, human gene products can be produced in bacteria, in yeasts, in mammalian cultured cells, and even in mice as whole animals.

Development of precise analysis of gene structure made possible to examine human diseases such as inherited disease and at a molecular level.

INHERITED DISEASES

Most of inherited disease may be caused by the mutation of genes. I briefly review the genetics of human hemoglobin. Thalassemia mutation of the hemoglobin chains are well studied at the clinical and the molecular levels (Orkin and Kazazian, 1984). Developments in understading of the thalassemias at the molecular level have led to far better comprehension of the mutational lesions than of any other mammalian mutations. From the analysis of structure, function, and organization of normal and mutant

globin genes, it has become clear that mutational interference with the different steps involved in globin synthesis can reduce or abolish globin production.

Molecular diagnosis of human diseases is most accurate method and recent development of the polymerase chain reaction (PCR) method made possible to diagnose a point mutation using single hair root (Higuchi et al., 1988).

Gene therapy of the inherited diseases can be examined at the experimental animal models by transferring normal cloned gene into somatic cells such as hemopoietic stem cells.

THE GENETIC BASIS OF CANCER

The initial research of cancer was focused on identifying agents that can induce cancers in experimental animals. The agents that were found were viruses, certain chemicals and radiation. Most of retroviruses that transform cells in vitro carry oncogenes and retrovirus oncogenes (v-oncogenes) are derived from normal cell genes (c-oncogenes) (Bishop, 1987). With a deeper understanding of the mechanism of action of viruses and of chemicals and radiation as mutagens, it became apparent that the majority of agents that can induce cancer act at the level of DNA. Today it is widely accepted that cancer is a molecular disease of malfunctioning cellular genes. Recently, cloning technologies allowed the isolation of cellular genes from spontaneously arising tumors. These v- and c-oncogene proves made possible to study cancer at the level of DNA or RNA. The study of these genes and the mechanism which they transform cells provided a key to understanding the genetic basis of cancer and the growth and differentiation of mammalian cells.

From the analysis of retroviral oncogenes, we can divide oncogenes into several distinct families of related protein sequences (Table 1).

Oncogenes in the same family encode proteins with similar enzymatic activity or intracellular location, suggesting that their role in cell growth and transformation may be similar.

Table 1 ONCOGENE FAMILIES

oncogene	subcellular location	properties of function of protein
src	plasma membrane	Tyrosine-specific
abl	plasma membrane	protein kinase (TSK)
erbB	transmembrane	EGF receptor/TSK
fms	transmembrane	CSF-1 receptor/TSK
K-ras	plasma membrane	GTP binding protein
H-ras	plasma membrane	GTP binding protein
sis	secreted	PDGF derived
myc	nucleus	
myb	nucleus	
fos	nucleus	
jun	nucleus	AP-1
erbA	cytoplasm	thyroid hormone receptor

src gene family encodes phosphoprotein with protein kinase activity and may have a role for generation of natural signals for cell proliferation.

Some oncogene (erbB and fms) are derived from cellular genes that encode receptors for polypeptide growth factors. erbB is the truncated form of EGF receptor and has lost not only its EGF binding but also its normal controls, so that it constantly signals the cell to divide even in the absence of EGF.

Members of the ras gene family encode 21kdal proteins that bind guanine nucleotides and may have a function of signal transduction for growth control of the cell.

sis oncogene is derived from the cellular gene for platelet-derived growth factor.

Nuclear oncogens such as c-myc, c-fos, and jun may have functions in the control of transcription and replication. Recently jun gene product has been shown to be identical to the transcription factor (AP-1).

We have examined the role of c-myc oncogene in cell proliferation and differentiation. In murine erythroleukemia (MEL) cells which can be induced toward erythrocytes with the addition of

dimethylsulfoxide, c-myc level changes dramatically after induction. we have introduced c-myc gene under the control of human metallothionein gene into MEL cells and the effect of tranferred c-myc gene on the differentiation of MEL cells was examined (Kume et al., 1988). The results indicated that c-myc controls the commitment of differentiation of MEL cells and the tissue-specific gene expression in a strict dose-dependent and time-dependent manner. It is likely that c-myc has a deterministic role for the molecular events in the MEL cells to undergo growth or differentiation.

erbA gene product is a truncated form of thyroid hormone receptor that is a DNA binding protein and activates the transcription of specific group of genes.

In summary, oncogenes control growth of the cell and thus, many cellular growth control genes may have potentials to behave like oncogenes after genetic change.

Human cancer are thought to be the results of multiple mutations and epidemiology revealed that enviromental factors cause most cancers. Recently, human cancer genes are detected by transfection of chromosomal DNA from human tumors into mouse 3T3 cells. Most of molecularly cloned transforming genes from human tumors are the activated forms of ras gene family. Activated ras genes result from a single-base change that leads to a single-amino -acid change. Activated ras gene products have decreased GTPase activity and may constantly signal the cell to proliferate in combination with other oncogene.

Many human tumors have chromosomal abnormalities, and frequently specific abnormalities are associated with specific forms of cancer. Translocation of the c-myc oncogene to immunoglobulin gene loci was common in human B cell tumors (Burkitt's lymphoma).

Amplified oncogenes in double minutes and homogeneously staining regions are found in certain human tumors.

In retinoblastoma that is an eye tumor of children and is one of several heritable cancer, cancer is caused by loss of heterozygosity.

Mutations (deletions) at the RB-1 locus are recessive to a normal gene and only contribute to tumorigenesis when the normal allele is inactivated.

These observations all suggest that most of human cancer are generated by somatic mutations.

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S22

Micromanipulation of mammalian embryos

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Abstract:Micromanipulation of embryos provide a new and valuable biological tool for animal agriculture and medical fields. Current techniques for embryo manipulation are in practice but some of techniques is still way off for application. The present status in new biotechnology appling to animal science and medicine is reviewed and its future is also discussed.

Key Wards: embryo, gene, manipulation

Introduction

The history of agriculture is largely a history in the progress of scientific phenomena especially concerned to genetics. Recent advances in manipulating techniques of mammalian embryos involved oocytes in oogenesis invitro have resulted in opportunities for research an mechanism of gene action and its expression during development and also have made possible the transfer of cloned genes from one organism to the genome of another(Wagner and Murray 1985, Trautwein 1985).

Current technology for embryo micromanipulation and gene transfer is now available to use for improvement and making of new bred or strain in livestock species and experimental animals(Church, R.B. et al 1985, Meinecke-Tillmann 1985, Sugawara 1986)

In medical field, the techniques had been applied to making a model animals for research and treatment of genetic disorder in human and animal health.

In this paper, I will review the progress in new biotechnology in animal agriculture and mankind, and present one of our research on cell interaction between oocytes and other ovarian tissues by using chimeric animals.

Genetic(developmental)engineering and methodology in mammals.

A. Domain of embryo micromanipulation

The purpose and research area of embryo micromanipulation are given in Fig. 1 in correlation to the close related scientic fields: embryoloogy and experimental embryology.

B. Procedures for micromanipulation of mammalian embryo

The procedures for embryo manipulation consisted of the essential and basic techniques shown in Table 1. Also, a schmatic program for embryo

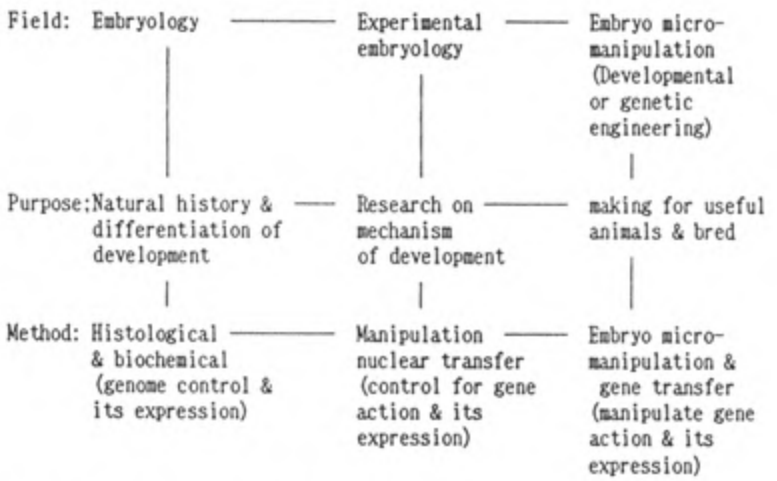


Fig 1. Correlation of embryology, experimental embryology and embryo micromanipulation.

Table 1. Procedures for embryo manipulation

A. Essential techniques
  a) Embryo micromanipulation:
    embryo splitting, embryo aggregation, blastomere separation, enucleation, nuclear transplant, gene transfer etc.
  b) Culture of the manipulated oocytes or embryos:
    until transfer of embryos to recipient. culture for recovery and judge of manipullated embryos
  c) Transfer of manipulated embryos to recipient
  d) Preservation of manipulated embryos

B. Basic techniques
  a) Embryo production:
    donor selection
    superovulation, mating system, egg recovery
  b) Estrus synchronization:
    recipients
  c) A. I. :
  d) Gene making (plasmid construction)
    DNA→cDNA, Promoter
  e) Cryopreservation of gamates

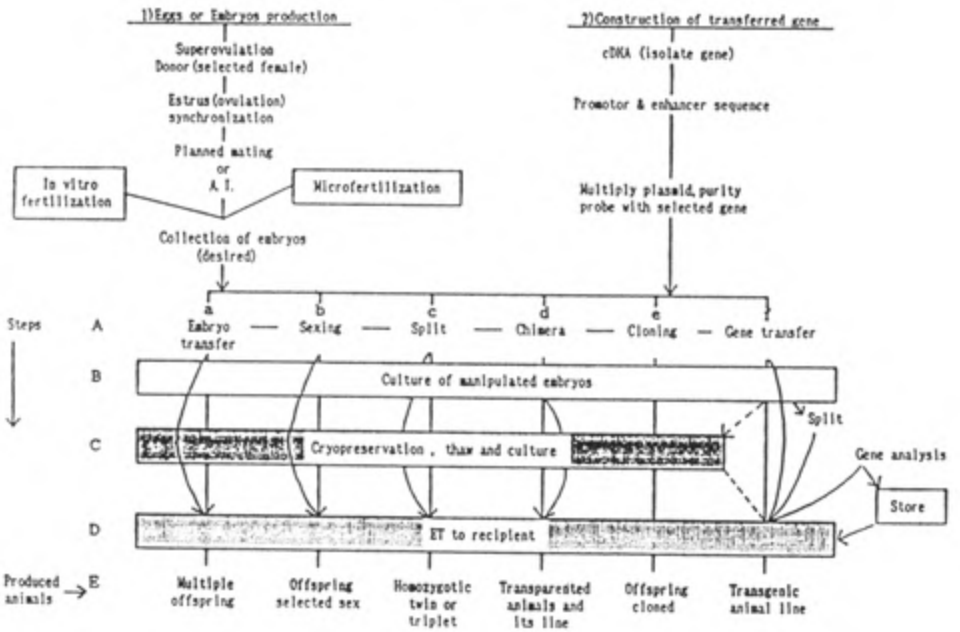
manipulation in mammals is shown in Fig 2.

Therefore, not until each steps for the treatment are proceeding completely, before genetic engineering of mammalian embryo resulted in success.

C. First successful study of embryo manipulation

First successful application of embryo manipulation in laboratory and

Fig 2. Schematic program for embryo manipulation and gene transfer in mammals



livestock species are summarized in Table 2. The routine application of these techniques in the Table is not practicable in domestic animals yet.

Application and results of embryo manipulaation.

Application of new biotechnology to animal agriculture can be expected in at least major area as follows: A)effective increase in animal production B)genetic improvement and making for new bred of livestock species C)animal health management D)manipulation of animal physiology E)improved crops and feeds.

A. Effective increase in animal production

a)Twin production. It is well known that twins or multiplets occurr naturally in several mammals such as man, catle, sheep, goat and armadillo, In domectic animals, first experiments to produce artificial monozygotic twins were done by Willadsen(1979) who devided sheep embryos at the two cellstage and morla stage, respectively. At present time, monozygotic twin ortetraplets can be produced in many of domestic animals using the morula orearly blastocysts method or the 4-cell stage deviding method (Williams, et al 1982). Twins or multiplets are economic interest in monotocous species for increase the number of offspring per ovulation. In research, monozygotic twins can be used for investigation of interaction between genotype and enviroment.

Table 2. First successful experiment of micromanipulation to embryos

	Laboraotry animals		Livestock species	
Embryo transfer	rabbit	Heape (1980)	sheep	Warwick et al (1934)
Monozygotic twins	frog	Spemann (1901)	cattle	Umbaugh (1949)
triplet	mouse, rabbit	Seidel (1959)	sheep, cattle	Willadsen (1979, 1982) Williams (1982)
Parthenogenesis	frog	Hertwig (1911)	-	
	mouse, rabbit	Illemensee (1975) Pincus (1936)	-	
Chimeras	rat	Nicholas & Hall (1842)	sheep, goat	Stranzinge et al (1983)
	rabbit, mouse	Gardner (1968) Moustafa (1972)	cattle	Brem et al (1984)
Cloning	frog	Briggs & King (1952)	sheep	Willadsen (1986)
	mouse	Illmense & Hoppe (1981)		
In vitro fertilization	rabbit	Chang (1959)	cattle	Brachett et al (1982)
Deep-freezing	mouse	Whittingham et al (1972)	cattle	Wilmut & Rowson (1973)
Sexing	rabbit	Edwards & Gardner (1967)	cattle	Hare et al (1976)
Gene transfer	mouse	Janish (1975)	swine, cattle	Hammer et al (1985)

b) In vitro fertilization. Normal offsprings born from in vitro fertilization has been reported in 8 species of mammals such as cattle, sheep, goat, monkey, human and laboratory animals (rabbit, rat and mouse). This technique is very important as any because many of embryo manipulation will utlize in vitro fertilized embryos as recipients in their application (see Fig 2). At present, normal development after in vitro fertilization of oocytes, which were collected from abattoir ovaries and matured in vitro, has been described only in cattle.

The technique was well known by its application in the certain types of infertility of humans.

c) Sex selection. For many years experiments were made to determine the sex of spermatozoa and embryos. In animal sciences, the breeder is

Table 3. Sexing by several techniques in mammals

Methods	Species
A. Sex chromatin	
a) trophoblast (blastocysts)	rabbit, cattle
B. Sex Chromosome	
a) blastomere (beyond 4-cell stage)	
b) trophoblast	rabbit, cattle
C. HY antigen	
a) Rabbit antibody vs spleen or antigen	mouse, cattle, swine
b) autoantibody vs spleen or testis antigen	rat, mouse
D. Separation of XY spermatozoa	
a) Serial density gradient of percoll	human
(i) F-body (ii) Hamster egg: male pronuclus	cattle
b) Electrophoresis	cattle
	Swine

interested in shfting the sex ratio according to the need. There has been success in separating x and Y-bearing spermatozoa of a number of mammals, for example, human, cattle, pig, mouse and rats. However, at present the technology (percoll method) is not practical for collecting the number of sperm used with routine artificial insemination and in vitro fertilization. Sex selection of embryos can be done by biopsying embryos and examining sex chromatin or chromosome or by identifying male embryos with an antibody to the male-specific molecule (H-Y antigen) (White et al, 1987).

d) cryopresevation of gamate and embryos: In vitro storage of sperm has been extensively used for artificial insemination program in a number of species, for example cattle, sheep, goat and humans. Freeze storage of female gamates and embryos is less well advanced, but the embryos developed to compact morula and early blastocysts beyond can be stored at low temperature in liquid nitrogen. This technique was first successfully demonstrated by Whittengham, et al (1972) in mouse and established by him and other groups for domestic and laboratory animals. Therefore, frozen embryos circumvent the problem of synchronization of recipients for they can be used at any time. Currently, this method was applied to storage of human embryo according to the program of in vitro fertilization. In animal agriculture, valuable genotypes may be stored permanently and used for animal production.

e) Chimera. Chimeric embryos and individials can be produced either by fusing two or more embryos at 8-cell stage to make an aggregation chimera or by injecting one or more totipotent cells into the inner cell mass of a blastocyst (Papaioannou & Dietherlen-Lievre, 1984).

In current techniques, the injected cells should be from another embryos and from a cell line derived from a teratocarcinoma or a stem cells maintained in cell culture in laboratory. Usually, a genome of the injected cells have partially expressed in host embryos, but, frequently the introduced cells become incorporated fully into all tissue of the developing chemeric embryo including the germ line. As yet it is not possible to combine various genotypes in relative contributions as desired. But, this technique could be used for the making of a model animal and the treatment of genetic desiease in human and livestocks.

B. Genetic improvement of livestock species

Selection or phenotype of animals including sire has resulted in significant improvement of domestic animals over the past decades. The use of A. I. and other breeding techniques have allowed to increase markedly in refinement of evaluating individual animals.

Recombinant DNA technology in combination with new biotechnology should allow further refinements in evaluation pherhaps by the use of restriction fragment length polymorphisms desired. A necessary prerequisite for this should be to identify linkage between restriction fragment of gene and production traits. Because production traits are controlled primarily by multiple genes, this may be a difficult task(Rando and Masina 1985).

The possibility of improving livestock species by the direct gene transfer to early embryos suggested in the experiemtns that is the first reports of successful gene transfer to mice.

Metablothionine-human growth hormone(MThGH) fusion gene have been transfered to male pronucleus of the fertilized mouse ovum. This cloned DNA sequences have been shown to be integrated into the chromosomes, and are inherited as Mendelian traits. Offspring that inherit the donor DNA have also been shown to grow larger. Hammer et al(1986) reported the production of transgenic rabbit, sheep and pig which retained MThGH fusion gene and shown that hGH levels in plasm of several pigs increased between birth and 90 d of age, but did not stimulate postnatal somatic growth rates.

C. Applications in animal health management

In animal industry, economic losses due to animal morbidity and death are significant. In the developed counties, total economic losses due to decreased production being estimated to about 20% of the direct gross income. Advances in imunobiotechnology can be expected to have a significant impact in the area of animal health managemtnt. Improved kits using monoclonal antibodies are being developed to detect and treat disease conditions. In furture, it is possible to make a bred or strain inserted gene for resistance in the germ line of viral disease(Kobbe, 1985).

D. Manipulation of animal physiology

Materials produced by eukaryotic genes in bacteria will apply to research in determination of physiological mechanisms and controls involved

in such processes as milk production and reproduction.

Recent studies with recombinantly derived bovine and procine growth hormone have been accumulating and indicating that these hormone can stimulate milk production or growth rate in livestock animals.

E. Improved crops and feeds for animal production

Significant advances in plant breeding and crop improvement are expected by applying biotechnology. Application of engineered microbes also are predicted to results in significant utilization of feeds and feed technology. Such advances will impact significantly on animal agriculture (Coffman, 1983).

Future in new biotechnology

Technical problelms with gene transfer to livestock species can be expected to be overcome within next 10 years. Progress in the area of recombinant DNA and gene transfer technology has been more rapid than originally expected. Animal and medicals science research and application of the future can be expected to incorporate the new biotechnology of recombinat gene and related technologies.

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S23 Transgenic Mice as Systems for Analyses of Biological Functions

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Abstract: Recent advances in biotechnologies have made it possible to manipulate mammalian embryos. This technology in combination with the recombinant DNA technology provides embryos with new and purpose oriented genetic information. Subsequent embryo transfer to the pseudopregnant females also makes these manipulated embryos develop to term as transgenic animals. Transgenes integrated in the transgenic animals are usually transmitted to their offspring according to the Mendel's Law. Therefore, once we obtained a particular transgenic animal, we could rear them as a strain non-existent in nature. In this report, methods of producing transgenic mouse and a few examples which were applied to the analyses of biological functions are mentioned.

Key words: embryo manipulation, transgenic mouse
recombinant DNA, gene transfer

Progress in biotechnology has resulted in the techniques of embryo manipulation by which mysteries of biological functions are being revealed through in vitro fertilization, in vitro culture of embryos, various kind of manipulation methods and embryo transfer techniques.

On the other hand, recombinant DNA technologies have made it possible to isolate a particular gene DNA from any kinds of animal, plant, bacteria, virus etc. However, even if we could obtain the complete base sequence of human chromosomes which will be realized in near future, we need an adequate system to analyze the biological functions of the particular cloned DNA. Today, thanks to the remarkable progress of these biotecnologies, embryo manipulation and recombinant DNA techniques, we are able to introduce cloned DNA to the mouse zygotes and rear them

to develop to term as transgenic mice.

The method how to produce transgenic mice is as shown in Fig.1.

1) Zygotes (Fertilized eggs) were obtained by natural or in vitro fertilization.
2) Zygotes were cultured in vitro and the DNA solution was injected into male pronucleus of mouse zygotes.
3) Manipulated zygotes were transferred to the oviducts of pseudopregnant females.
4) The candidates of the transgenic mice were born 20 days after transfer.
5) The segments of the mouse tails were subjected to the DNA blot analyses after weaning and the transgenic mice were determined.

Only when all these processes were carried out in perfection, the transgenic mice might be obtained. We could carry out injections into more than 500 eggs a day. About 75% of manipulated eggs were survived to the embryo transfer and about 15% of transferred zygotes developed to term as pups. about 20 to 30% of pups obtained were transgenics. Therefore, we could obtain about 15 transgenic mice a day. This score was sufficient to analyze the biological functions of a particular gene.

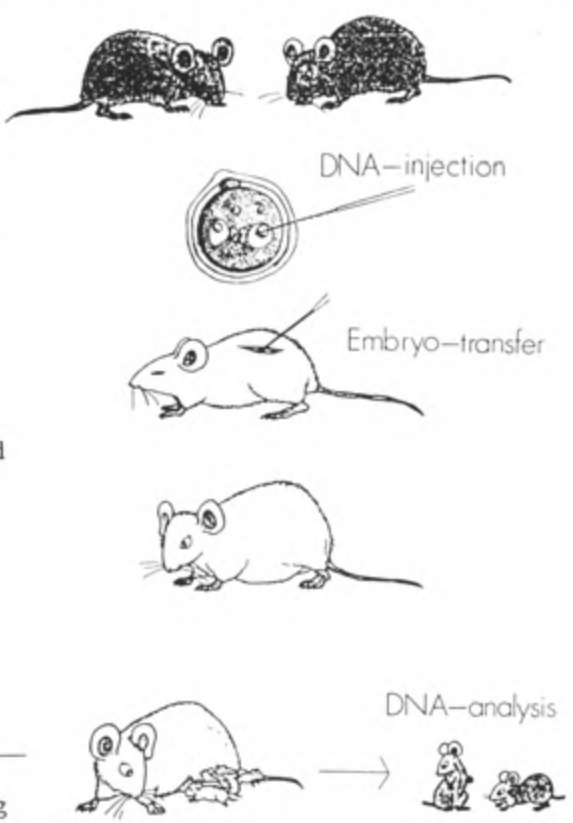

Fig.1
Method of producing transgenic mouse

ONCOGENE TRANSGENIC MOUSE

Many oncogenes were isolated using mouse cell line, NIH3T3, when it was transformed by DNA transfection. But, it is impossible to determine whether and how a particular oncogene is involved in the tumorigenesis in vivo. We attempted to produce transgenic mice with activated human c-Ha-ras gene, but failed to obtain living transgenic mice. In other word, we could obtain only non-transgenic mice, although more than 1,000 eggs were introduced with these DNAs. However, when we detected the transgenes in the mid-gestation stage embryos, about 25% of them were transgenics. We finally found that the activated human c-Ha-ras gene made the embryos to embryonal carcinoma, so that they died during early developmental stages.

When transgenic mice with human proto c-ha-ras gene were produced, we also found that almost all transgenic mice were too weak to survive to the weaning stage. Two of them were survived. One died 4 months after birth by spleen cell tumor and its offspring died of the same manifestation. The other was suffered from adenomas in the Harder gland. All these phenomena suggested that the deregulated expression of proto c-Ha-ras might be the causes of tissue specific tumorigenesis.

GENE THERAPY

Transgenic mice were used for the gene therapeutic experiment. There is a mutant, shiverer, which lacks myelin basic protein gene. The typical phenotype of the shiverer is a tremor behavior whenever it intends to move.

We isolated myelin basic protein (MBP) as well as the MBP cDNA. We constructed MBP cDNA expression vecter DNA with MBP promoter gene and injected it to the shiverer zygotes. 116 pups were obtained. Surprisingly, 5 out of 116 pups were recovered from tremor behavior. All these mice were transgenic. MBP, myelin formation and behavior were cured by the expression of the introduced transgenes.

As in the case of this experiment, a particular biological function could be analysed by the introduction of a particular gene(s) and DNA prepared for injection was manipulable to its own purposes.

Transgenic mice will be produced and delivered by the order of investigators as standard mouse strains in the very near future.

S24

PROTEIN ENGINEERING IN STUDIES ON METABOLISM OF TOXIC SUBSTANCES

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Abstract--- Expression of cytochrome P-450s, drug metabolizing enzymes, from their coding sequences in heterologous cells were developed and the enzymatically active P-450s were synthesized. cDNAs of various chimera or mutant P-450s were constructed and expressed. Thus, regions or residues important for the function of cytochrome P-450 were analyzed.

Key words: protein engineering, cytochrome P-450, drug metabolism

INTRODUCTION

A number of toxic substances such as drugs, carcinogens and environmental pollutants are metabolized by hepatic microsomal monooxygenase system containing cytochrome P-450, a large family of a heme-thiolate protein. Properties of cytochrome P-450 has been studied extensively at the microsomal and protein levels, but a little has been disclosed on the structural and functional relationships of this unique hemoprotein. Recently, a number of cDNA and genomic clones for different forms of cytochrome P-450 have been isolated and their sequences analyzed (reviewed in Nebert and Gonzalez, 1987, reviewed in Gotoh and Fujii-Kuriyama, 1988). Thus, attempts to explore the structural basis for the function of cytochrome P-450 have now been undertaken utilizing the techniques of protein engineering. In this review, outline of such approaches and, as an example, our studies on P-450(ω-1) will be described.

EXPRESSION OF CYTOCHROME P-450 IN HETEROLOGOUS CELLS

As the first step of protein engineering of cytochrome P-450 expression of functional proteins from their coding sequences in heterologous cells were undertaken. Thus, four forms of hepatic cytochrome P-450 were synthesized in the transformed yeast cells under the control of yeast promoters (Oeda et al., 1985, Shimizu et al., 1986, Imai, 1987, 1988). Several forms of the hepatic and adrenal cytochromes were expressed in the cultured momkey kidney cells utilizing virus vectors (Battuta et al., 1987, Zuber et al., 1988).

Table I. Expression of P-450s in heterologous cells.

Expression vector/host (promoter)	Cytochrome P-450 expressed
pAAH5/yeast AH22	P-450c, P-450d [rat]
(Alcohol dehydrogenase)	P-450(ω-1), P-450(16α)[rabbit]
pAM82/yeast AH22	P-450d [rat]
(Acid phosphatase)	
pCD/COS-1 cell	
(SV40 early region)	P-450$_{17\alpha}$, P-450$_{C21}$, P-450$_{scc}$ [bovine]
pKCR H2/COS cell	
(SV40 early region)	P-450d [rat], P-450(scc)[bovine]
pSC-11/vaccinia virus-infected CV-1 cell	
(vaccinea virus)	P_1-450, P_3-450 [mouse]

PROTEIN ENGINEERING OF CYTOCHROME P-450

Sakaki et al. (1987) showed from analysis of chimeras of P-450c and P-450d that the central and the carboxy-terminal one-third regions of both P-450s play important roles in substrate-binding and electron transport, respectively. Murakami et al. (1986) constructed the P-450c/NADPH-cytochrome P-450 reductase fused enzyme. Shimizu et al. (1988) described from the site-directed mutagenesis study that cystein in the HR2 region is the axial ligand to the heme iron and that conserved hydrophobic amino acids near the heme binding sites are essential to holding and/or

incorporation of the heme in the active site. It was reported, utilizing chimeras of cytochrome P-450 and secretory proteins, that the memrane-anchor signal is located at a short amino-terminal segment of microsomal cytochrome P-450 (Sakaguchi et al., 1987, Szczesna-Skorupa et al., 1988).

FUNCTIONAL REGIONS OF P-450(ω-1)

The deduced primary structure of P-450(ω-1) is 81% similar to that of P-450(16α) (Imai et al., 1988a). Chimeras of the both P-450s were synthesized in yeast cells transformed with plasmids constructed for expression of chimeric P-450 cDNAs and their properties were examined. As will be seen from Fig. 1, the region spanning about 50 residues (211-261) is essential to the substrate (laurate and caprate) binding for P-450(ω-1) (Imai, 1988) and, in addition to the sequence enough to bind the substrates, the

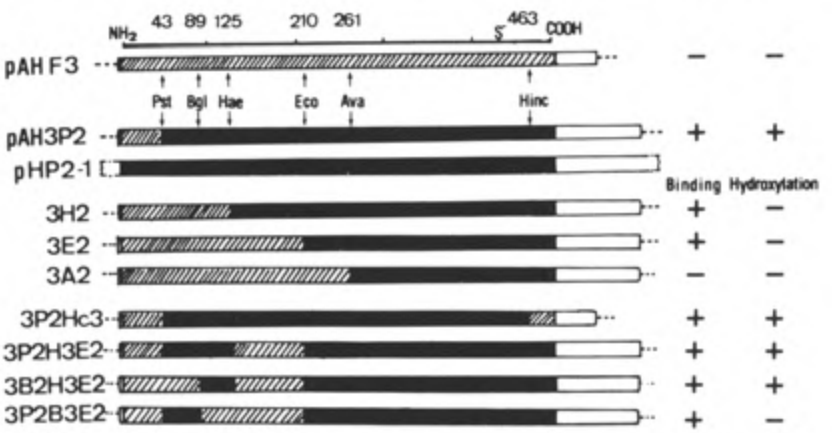

Fig. 1. Structure of the constructed expression plasmids for chimeric P-450s and properties of P-450s purified from the transformed yeast.

The filled and hatched boxes indicate the coding regions of cDNAs for P-450(ω-1) and P-450(16α), respectively; the open boxes indicate the noncoding region of cDNA inserts. The vertical arrows indicate restriction enzyme sites (Ava, AvaI; Bgl, BglII introduced by site-directed mutagenesis; Eco, EcoRI; Hinc, HincII; Hae, HaeII; Pst, PstI). Figures on the line at the top indicate the amino acid residue numbers of P-450 protein. Binding, laurate-induced spectral change; Hydroxylation, laurate (ω-1)-hydroxylase activity.

segment of about 35 residues (89-124) is necessary for the hydroxylase activity (Imai et al., 1988b) (Fig. 2). The segment of the carboxy-terminal 28 residues of P-450(ω-1) appears to be independent of the substrate specificity of P-450(ω-1) (Imai et al., 1988b) (Fig. 1 and 2).

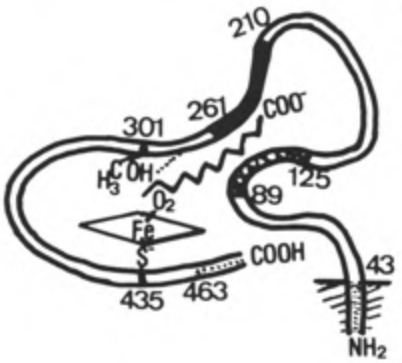

Fig. 2. A model for structure-function relationships of P-450 (ω-1).
Figures on the line (P-450 protein) indicate the amino acid residue numbers of P-450(ω-1). The filled and hatched lines indicate the region necessary for the binding of the fatty acid substrates and that necessary for the hydroxylase activity in addition to the sequence enough to the binding of the fatty acids, respectively.

THR-301 OF P-450(ω-1) AND SUBSTRATE SPECIFICITY

Threonine-252 of P-450cam is highly conserved in all P-450s and located at the distal heme surface trans to the thiolate ligand (Poulos et al., 1987). The corresponding residue of P-450(ω-1), Thr-301, was substituted by Val, Ser, Ala or His via site-directed mutagenesis (Imai and Nakamura, 1988a, 1988b) (Table II). The substrate-induced spectral change was observable in the Val- and Ser-mutants

Table II. Interaction of fatty acids with Thr-301 mutants of P-450(ω-1).

Amino acid at position 301	Substrate binding (Spectral change)			Hydroxylation			
	Cap	Lau	Myr	Cap (ω-1)	Cap (ω)	Lau (ω-1)	Myr
Thr (ACA)[a]	+	+	±[b]	++	±	++	−
Val (GTA)	+	+	+	−	−	+	−
Ser (TCA)	+	+	+	++	+	+	−
Ala (GCA)	−	−	−	n.e.		−	n.e.
His (CAC)	−	−	−	−	−	−	n.e.

[a], Wild-type; [b], partial change; n.e., not examined.

but not in the His- or Ala-mutant. These mutants were also devoid of the hydroxylase activity. On the other hand, the substrate specificity of P-450(ω-1) was altered by substitution of Val or Ser for Thr-301. These findings indicate that residues (or atoms) at the γ-position of the amino acid-301 is important to the substrate interaction.

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S25 Development of a new type of drug by using cell technology ; preparation of human interferon-beta from human diploid fibroblast cultures

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Abstract ---- Several strains of human fibroblast were identified as good producers of human interferon-beta (HuIFN-β), amoung DIP-2 cell was one of the best. We have developed an improved microcarrier culture system for both the mass culture of such cells and the large scale HuIFN-β production. A routine pilot plant, and successively a large plant, have been accomplished for preparation, purification and preclinical or clinical trials of HuIFN-β. The purified and lyophilized HuIFN-β was assayed for its safety for clinical use under the regulation of the National Institute of Health of Japan.

Using this HuIFN- preparation, the clinical trials on various viral diseases and malignant tumors were started from the middle of 1979. More recently, the Ministry of Health and Welfare approved this HuIFN-β as a new drug against melanoma, glioblastoma and chronic active B type hepatitis.

Key words HuIFN-β, human diploid fibroblast, microcarrier culture system

INTRODUCTION

HuIFNs are being prepared on a large-scale for clinical evaluation (Stewart II, 1979). So far, at least three types of human tissue-drived cells have been used for preparing large amounts of HuIFNs. These are buffy coat leukocytes (Mogensen and Cantell, 1977), diploid fibroblast strains (Horoszewicz et al.,1978) and lymphoblastoid cell lines (Adams et al., 1975).

A large supply of fresh human leukocytes is difficult to obtain in common laboratories. On the other hand, the human diploid fibroblast strains can be readily prepared from neonatal foreskins among other tissues (Havell and

Vicek, 1972). Further, these appear to be suitable substrates for preparing safe HuIFN for clinical use since these are normal diploid cells (Hayflick, 1973). However, large-scale production system for HuIFN-β using the diploid fibroblast strains have seldom been reported (Horoszewicz et al., 1978; Billiau et al., 1973). One major reason is that these anchorage dependent cells can grow only in monolayer culture conditions and their growth rates are slow in comparison with those of other established cell lines.

In our laboratory, we have developed an improved microcarrier culture system for the mass culture, as reported by Giard and Fleischaker (1980). We have accomplished routine pilot-plant for preparation, purification and preclinical trials of HuIFN-β. And, nowadays, a large plant was constructed for large-scale production of HuIFN-β for clinical use.

EXPERIMENTAL PROCEDURES

Cell culture ---- Several hundred strains of human diploid fibroblasts have been drived from various kinds of tissues such as neonatal foreskin, amnion, embryonic tissues among others in our laboratory. Some strains have been selected as good sourses for HuIFN-β production in accord with our criteria for cell selection. Stocks of the cells at the 6th population doubling level (PDL), 14th PDL and 22nd PDL were kept in frozen ampoules in liquid nitrogen (2×10^6 cells/ampoule). The growth medium for both stock and mass cultures was Eagle's minimum essential medium (MEM; Nissui Seiyaku Co., Japan) supplemented mainly with 5% precolostrum newborn calf serum (PNCS; Mitsubishi Kasei Institute for Life Science, Japan).

An ampoule of the cells at the 22nd PDL is routinely used to supply the starting cells for large-scale HuIFN-β production in our laboratory. The starting cells are grown to about the 42nd PDL by using several hundred liters of the microcarrier culture vessel. At this stage, sufficient numbers of the cells (over 10^{10} cells) can be obtained to produce HuIFN-β.

HuIFN- production and purification ---- HuIFN-β was superinduced by treating the cells with poly I: poly C (Yamasa Shoyu Co., Japan), cycloheximide (CH: Sigma Co., USA) and actinomycin D (Act-D: Mackor Co., Israel). The superinduction method used was similar to the one reported

by Havell and Vilcek (1972), Billiau et al. (1973) and Horoszewicz et al. (1978) except for the use of serum free medium inthe whole processes of HuIFN-β production.

When preparing HuIFN-β for clinical use, the following problems must be considered; removal of pyrogenic substances, removal of calf serum proteins used for cell culture, elimination of all the chemicals which were employed in preparation steps, preventation of denaturation of IFN molecule, easy handling of large volumes of crude material , making up salt-free preparation and highly purified one with good recovery. With these requirements in minds, we examined various chromatographic systems including ion-exchange-hydrophobic adsorption-, metal chelate- and molecular sieve chromatographies for purification of HuIFN-β . As the results of these trials, a relatively simple procedure was developed for large scale purification of HuIFN-β (Hosoi and Ozawa, 1980). Using this procedure, over a thousand liters of the crude preparation were concentrated and purified to more than 10^8 international reference units (IU)/mg protein of specific activity per preparation cycle. The lyophilized HuIFN-β is the final preparation for clinical trials and is stored at 4° C until its use.

IFN assay and other tests ---- HuIFN-β was determined by a semimicro method based on the inhibition of cytopathic effect of vesicular stomatitis virus (VSV: New Jersey sero type; CPE_{50} method) (Kobayashi et al., 1985). The IFN titers were expressed in terms of the international reference HuIFN-β (G-023-902-527) obtained from the National Institute of Health, Bethesda, Maryland, USA.

Karyotypic analysis of the cells was carried out according to the method of Furuyama and Chiyo (1977). Protein concentration was mainly determined by the method of Sedmak and Grossberg (1977).

RESULTS AND DISCUSSION

Selection of cell strains for HuIFN-β production ---- We designated nine characteristics for selection of the cell strains as good producers of HuIFN-β. These are as follows; growth rate, cell density, split ratio, life span , IFN production, serum requirement, durability, stability in liquid nitrogen and diploidy. And five lankings were set up to evaluate the response of the cells. It has been

difficult to find cell strains which show the ranking A for all characteristics, although a few hundred cell strains have been derived from the human tissues. However, several cell strains demonstrated the A abilities in most items and the B abilities as to a few. These cell strains were selected as the candidate cells for the production of HuIFN-β. Ampoule stocks of these cells at 6th PDL were kept in liquid nitrogen as the primary cell stocks.

DIP-2 cell which has been derived from neonatal foreskin as one of the best candidate cell strains showed the abilities of ranking A in seven items and ranking B in two , respectively. This strain has typical fibroblast morphology and grow well to form a uniform monolayer. And the in vitro life span was approximetely 60 PDL. DIP-2 cell had no tumorigenicity in nude mouse and were free of detectable adventitious agents such as viruses, mycoplasma, bacteria and fungi. So we decided to use this cell strain as the primary source for large-scale preparation of HuIFN-β for clinical use.

Properties of HuIFN-β ---- SDS-PAGE analysis on the purified HuIFN-β preparation revealed the antiviral activity associated with a single band which had molecular weight of aroud 23, 000 daltons. The purified and lyophilized HuIFN-β preparation was stable in a common refrigerator (4°-7° C) for over a year. For clinical trials, the lyophilized preparation is reconstituted in the physiological saline and has been used as for subcutaneous, intramuscular, intrathecal and intraveneous injections. The ability of injection was fairly stable at 4° C for over a month, although a remarkable loss of activity occurred at 25° C incubation within a couple of weeks.

Quality control and safety of HuIFN- for clinical trials ---- The final HuIFN-β preparation was evaluated following the guideline of National Institute of Health of Japan on Safety, pyrogenicity, potency, contaminants and other factors. This guigeline is very similar to that issued by the Food and Drug Administration (FDA) of USA in 1981.

The final preparation showed negative results for various contaminants such as poly I: poly C, Act-D, CH and bovine serum albumin. The preparation also easily passed the tests for general safety, pyrogenicity and sterility.

These quality control and safety tests have been routinely examined for each lot in the final container in both our laboratory and the Natinal Institute of Health in Japan.

Discussion and summary ---- We have tried to develope an improved microcarrier culture system for the anchoraged depended cells such as human diploid fibroblasts. The system was also able to be applied, with easy handling, for the production of HuIFN-β in a large-scale from the above fibroblast cell strains. It become easy to prepare several hundred liters of the crude HuIFN-β (over 10^{10} IU of IFN titer) from the fibroblast cell strains at one time in the pilot plant using this culture system. And the highly purified and safety tested HuIFN-β for pre-clinical or clinical evaluations has been prepared on a large-scale . The development of this system in a big-plant, nowadays, enables us to prepare enough amounts of HuIFN-β preparation for adequate clinical evaluations.

Each lot of our final HuIFN-β preparations for clinical use has passed the quality control and safety tests under the regulation of the National Institute of Health of Japan for use the IFN research groups for clinical trials organized by the Ministry of Health and Welfare of Japan. That is, we have completed all the quality control and safety tests of HuIFN-β from the cell culture through the production and purification of HuIFN-β with the support of the Japanese government. Since the middle of 1979, the above IFN research groups have started phase I or phase II studies using our HuIFN-β preparations with various viral and neoplastic diseases such as viral warts, herpetic keratitis, chronic active B type virus hepatitis, melanoma, medulloblastoma, glioblastoma, lymphoma, leukemia and others.

In April of 1985, the Japanese goverment (the Ministry of Health and Welfare) approved our HuIFN-β as a new anti-tumor drug against melanoma and glioblastoma. Moreover, in September of 1986, the government approved this IFN as a new anti-viral drug against chronic active B type hepatitis.

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FREE COMMUNICATIONS IN HALL A

A01 IN VITRO STUDY ON CELL AGING WITH FOOD CHEMICALS

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Previously, we found that food additives allylisothiocyanate, cinnamaldehyde and aspartame(AS) induced shortening of lifespan in human diploid cells HAIN-55, accompanied by the aged cell markers i.e., decrease in saturation density in monolayer cell culture(SD) and in plating efficiency(PE), and increase in heat lability of glucose-6-phosphate dehydrogenase(G6PD). The observed effect of AS, a non-mutagenic dipeptide is suggestive of incorporation of AS as amino acid analog into cellular proteins. In this respect, present study was done to explore potential causative agents of cell aging among non-protein amino acid analogs of food plants.

HAIN-55 cells were treated once with non-toxic, effective dose of either AS, 2-aminoisobutylic acid(AIB) or canavanine(CAN), or treated successively with one tenth the amount of each chemical. During the process, changes in the aged cell markers were determined and were compared with those of non-treated cells. Remarkable decrease in SD and PE, increase in heat labile G6PD and sister chromatid exchenge, and subsequent cell degeneration were observed in the cells exposed successively to AS or AIB at earlier passage generations than the control cells. The reactivity of 2-deoxy-glucose-6-phosphate to G6PD increased in the AS- or AIB-treated cells in parallel with the increase in heat lability of G6PD.

A02 INDUCTION OF HEPATIC AND RENAL METALLOTHIONEIN BY X-IRRADIATION

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Metallothionein (MT) mRNAs were determined in tissues of rat following whole-body X-irradiation. The tissue samples were analyzed for MT-mRNA levels using a human MT-II RNA probe. When compared with control rats, marked elevations in liver and kidney MT mRNA levels and slight elevations in brain and spleen MT mRNA levels were observed in irradiated rats 72 hr after irradiation(20 Gy). However lung, heart, and testis MT mRNA levels did not show an increase 72 hr after irradiation(20Gy). Time course experiments indicated that hepatic and renal MT mRNAs increased significantly compared to control values at 3 or 6 h after high and low dose irradiation(20 Gy and 2 Gy). In rats exposed to high dose irradiaton, the hepatic and renal MT mRNA levels decreased obviously at 18 hr, and also showed an increae at 72 hr after irradiation. In rats exposed to low dose irradiation, the hepatic and renal MT mRNA levels decayed to control values by 18 hr after irradiation. These data indicate X-ray treatment produces an in vivo induction of MT mRNA in irradiated rats.

A03 FUNDAMENTAL STUDY ON THE HEPATOTOXIC EVALUATION USING BILE ACIDS

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We studied a species difference of bile acid(BA) composition in bile of dog, rat and mouse by means of HPLC. Muricholic acids were dominant in rat and mouse but not existing in dog. CA and DCA were dominant in dog but these conjugate types were taurine unlike glycine in human. In chronic liver injury rats treated with CCl_4(50% olive oil soln. 1ml/kg, s.c.) for 12 weeks, changes of BA concentration and compositon in bile were the same as serum, but the degree of these changes could not exceed one of serum enzymes. In acute liver injury dogs treated with galactosamine(100mg/kg, i.p.) for 2 days, the changes of BA in serum has showed the similar changes to some serum enzymes(GOT, GPT, γ-GTP) but not to ALP and bilirubin. In bile of these dogs, the content of BA had not difference from normal dogs but the increase of CDCA ratio and the decrease of CA ratio were observed in these dogs.

A04 EFFECTS OF METYRAPONE ON THE CYTOCHROME P450 MEDIATED DRUG METABOLIZING ACTIVITIES IN THE PRIMARY CULTURES OF RAT HEPATOCYTES

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We studied the effects of metyrapone on the maintenance of cytochrome P450 and drug metabolizing activities in the cultured hepatocytes.

Hepatocytes, prepared from male Sprague-Dawley rats were cultured on collagen-coated dishes. The cultures were maintained at 37 °C in William's E medium with the addition of 10% fetal calf serum, 10^{-9}M insulin, 10^{-6}M dexamethasone.

Protein concentration, cytochrome P450 contents, benzo(a)pyrene hydroxylation, ethoxycoumarine-O-deethylation, and propoxycoumarine-O-depropylation of cultured hepatocytes were declined depending on the culture time. These declines were suppressed by the addition of metyrapone to the culture medium. Furthermore, the apparent induction of benzo(a)pyrene hydroxylation activity occurred in the presence of metyrapone. However, the decline of methoxycoumarine-O-demethylation, aminopyrine-N-demethylation, and estradiol-2-hydroxylation were not affected by metyrapone.

Immunochemical detection of cytochrome P450 subspecies revealed that cytochrome P450d (high spin form of P448) induced by metyrapone as well as P450c. However, metyrapone had little effect on the maintenance of cytochrome P450b and P450e.

These results suggest that metyrapone may act as an 3-methylcholanthrene-like inducer in the primary cultures of rat hepatocytes.

A05 IMMUNOHISTOCHEMICAL STUDY OF CYTOCHROME P-450 IN THE LIVERS

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Enlargement of hepatocytes is frequently found in the toxicological study of drugs, the fact is sometimes characterized by an increase in smooth endoplasmic reticulum. This change has generally been considered to be due to an increase in cytochrome P-450 contents. In the present study, distribution of cytochrome P-450 in the hepatocytes of animals treated with different kinds of drugs was examined immunohistochemically. 'Finger print' which is produced by repeated administration of drugs such as phenobarbital was also examined by the same procedures. Method; Phenobarbital was administered to mice, rats and beagles, and amitriptyline, imipramine, chlorpromazine, spironolactone and β-naphtoflavone to rats. The formaline-fixed livers of the animals were paraffin-embedded, sectioned and examined immunohistochemically, using a rabbit anti-rat cytochrome P-450b antibody (P-450b antibody). Results; The response of hepatocytes to P-450b antibody was negative in untreated animals. It was remarkably positive in rats treated with phenobarbital, amitriptyline, imipramine, chlorpromazine, and in mice treated with phenobarbital. The finding that some of 'Finger print' showed positive responses to P-450b antibody suggests that the function of 'Finger print' changes periodically.

A06 DEVELOPMENT OF AMINE OXIDASE IN RAT PLASMA AFTER HEPATOTOXIN ALLYL FORMATE ADMINISTRATION

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Development of amine oxidase in plasma of rats were investigated after pretreatment with the perilobular hepatotoxin allyl formate (AF). Amine oxidase activities in plasma of rats pretreated with AF elevated with 1 μM Benzylamine (Bz), 100 μM Bz, 10 μM β-phenylethylamine (β-PEA) and 100 μM 5-HT as substrates. But the complete inhibition of amine oxidase activity with 5-HT and β-PEA were not observed by MAO inhibitors (clorgyline and deprenyl). The deamination of 1 μM Bz was not inhibited at high concentrations of MAO inhibitors, while was inhibited at low concentrations of phenelzine and semicarbazide. While, Bz (100 μM) deamination was highly sensitive with these inhibitors, but was inhibited by KCN. After gel filtration of plasma of rats given with AF by Shepharose 6B, three distinct peaks of amine oxidase activities were observed with 10 μM β-PEA, 1 μM Bz and 100 μM Bz as substrates. These results indicate that amine oxidases in plasma of rat administered with AF may mainly consist of MAO, CRAO and PAO.

A07 EFFECTS OF SEVERAL VEHICLES ON VARIOUS PARAMETERS FOR TOXICITY STUDY IN RATS

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Control animals are usually given a vehicle that is used for a test compound in toxicological studies. This experiment was undertaken to see some possible influences of repeated treatments of several vehicles which are commonly used in such studies.

Six-week-old SD rats received physiological saline intravenously, or 1% methylcellulose, 0.5% CMC, Tween 80 or olive oil by gavage for 4 weeks. Non-treated animals served as a control group. General observation, clinical laboratory tests and pathological examination were made together with several functinal tests including electroretinogram, ECG and blood pressure.

Slight but significant changes such as a reduction of body weights gain, anemia, higher WBC, enhanced blood coagulation, were seen in saline injection group, probably reflecting to stressful condition of intravenous treatment in a restraining cage. In addition, physiological fluctuations of ERG and A/G ratio were noticed in this group. Increase in food convertion efficiency and an appearance of lipid droplets in the liver cells were observed in oil treated rats. In oil treated group several changes were also seen in ECG, GOT, GPT, and BUN, but the values were within the normal limits. No significant changes occured in other vehicles groups. The above findings are important to differentiate between the effect of a vehicle and that of a test compound.

A08 COMPARISON OF VARIABILITY IN EMBRYONIC GROWTH AND DEVELOPMENT AMONG DIFFERENT SETTINGS OF MATING IN MICE

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It is assumed that the overnight mating would produce more heterogeneity in embryonic development among the litters of pregnant mice than the short-period mating. So, we compared the interlitter and intralitter variability in embryonic growth and limb development among the different settings of mating. The ICR females were paired with a male either continuously throughout the day or overnight or during 2 hours in the morning at light period(0800-2000). Mice were killed by cervical dislocation at 0000(midnight) on day 12 or at 1000 on day 17 of gestation (plug day = dg 0). On dg 17, the interlitter variability of fetal body weight was smallest in the group with short-period mating. On dg 12, the interlitter and intralitter variability in embryonic body weight was smaller in the short-period mating group than the overnight-mating group. Also, on dg 12, the embryonic growth (body weight) and digit formation in limb buds were more advanced in male embryos than in female embryos. From these findings, for the reproductive toxicity testing protocols a short-period-mating schedule is advised and the incidence of developmental anomalies should be analyzed separately for male and female fetuses for the purpose of increasing test sensitivity.

A09 THE USE OF RAT WHOLE EMBRYO CULTURE AS THE ALTERNATIVES IN REPRODUCTIVE TOXICITY TEST

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The difficulty as the alternative for animal test in vitro is that rat whole embryo culture requires much amount of rat serum although having many advantages as in vitro screeneing system. Previously we reported that rat embryos were able to be cultured by the use of pig serum, but in which embryos showed less growth and tissue differentiation than those in rat serum. In the present study, in order to find the critical components contained in rat serum for the embryo development, embryos were cultured from gestational day 11 for 48 hours with used once(1FS) or twice(2FS) culture medium which were filtrated by millipore filter (0.8 + 0.45um) to remove the cloudiness of medium. As regards blood circulation of embryo or yolk sac, protein contents and somite number, embryos cultured in 1FS showed almost same as those cultured in fresh rat serum(FRS). However, embryos of 2FS group showed much less developments than those of 1FS or FRS groups and also showed some abnomalities. These results suggested that minimum dose of critical components necessary for embryo development might be contained in 1FS but not in 2FS.

A10 THE INVESTIGATION OF THE PERIODICAL ULTRASONIC DIAGNOSIS ON THE TOXICITY STUDY IN DOGS

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In order to establish an uninfluential and periodical method for detecting patho-morphologically hepatic damages, ultrasonic and histopathlogical diagnoses (biopsy) of livers were investigated and compaired monthly in dogs to which carbon tetrachloride had been treated orally for 3 months. On blood chemical analyses, very high plasma GOT and GPT activity, and high total bilirubin concentrations were evident after 1 month. On the clinical observation, extensive venous collateral channeles were noted on the abdominal wall and jaudice were showed after 2 months. On the biopsy of the liver, necrosis and fibrosis of hepatic cells were showed after 1 month, and these changes were proceeded by repeating treatment. On the ultrasonic diagnosis, diffuse hyperechoes were showed after 1 month. In the serious cases with jaudice and/or extensive venous collateral channels, the unclearness of hepatic inner structure, the irregular surface and the figure like nodules were seen. These results indicate that slightly histopathological changes could not be caught on the ultrasonic diagnosis, but the progress of hepatic damages were found on the ultrasonic diagnosis as similar to the histopathological one.

A11 Hepatic foamy cell in Fischer 344 rats: Its age-related morphological properties.

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On long-term studies using Fischer 344 rats, foamy cell of the liver (hepatic foamy cell, HFC) was detected in both sexes ranging from 20 to 111 weeks of age. This study reports age-related morphological properties of HFC in Fischer 344 rats.

Fischer 344 rats were supplied from Charles River Japan. The rats used in the present study were control animals (50 males and 50 females) from five different toxicological studies with different durations; the ages examined were 20, 32, 58, 84, and 111 weeks old. Tissue samples of the liver were fixed, embedded in paraffin and stained with hematoxylin-eosin. Some sections were stained with Sudan III, PAS, diastase PAS, Alcian blue, and Berlin blue. Number and size of HFC in Glisson's sheath area and sinusoidal area were measured by a pattern analyzer. Enzymatic cholesterol staining using 4-chloro-1-naphthol was also performed. Selected samples were prepared for electron microscopy.

Number and size of HFC were increased with age, and the both indicators in female were greater than those in male. The number of HFC in Glisson's sheath area was higher than that of sinusoidal area. The HFC was positive with staining of Sudan III, PAS, and diastase PAS, and cholesterol. Staining with Berlin blue was weakly positive, and Alcian blue was negative. At electron microscopic level HFC had numerous cholesterin crystals in many secondary lysosomes.

These findings suggest that HFC is a type of macrophage which accumulated some kind of lipids, mainly cholesterol in lysosomes.

A12 EVALUATION AND ITS MECHANISM OF DIARRHEA INDUCTION BY FLUORO-PYRIMIDINE DERIVATIVES

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Toxicological effects on hematopoietic, immuno-responsible and reproductive organs have been generally induced by administration of fluoropyrimidine derivative in the experimental animals. Besides these effects, we examined the diarrhea induction caused by several fluoropyrimidine derivatives in mice, rats, dogs and monkeys.

[Material and Method] Two to twenty folds doses of clinical application of fluoropyrimidines (5-FU, Furtulon, Tegafur, UFT) were employed for 2-4 week toxicity study in mice, rats, dogs and monkeys. Diarrhea incidence was checked by our grading system of fecal status and histopathological evaluations.

[Results] Hematopoietic and immunoresponsible changes were observed in all animal species, but no obvious diarrhea induction was detected in mice, rats, and dogs. Only monkeys showed the severe loose passage and diarrhea. The grading method for fecal status well corresponded to histopathological changes, that is, atrophy in the mucosa and regenerated epithelial cells in the small and large intestines.

A13 DEVELOPMENTAL PROCESS OF MDP-LYS(L18) INDUCED ARTHRITIS IN RATS

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N^2-(N-acetylmuramyl-L-alanyl-D-isoglutamyl)-N^6-stearoyl-L-lysine[MDP-Lys(L18)] is reported to have adjuvant activity and to indue arthritis in rats. We examined developmental process of the arthritis in male Slc:SD rats receiving sc injections of MDP-Lys(L18), 4 mg/kg/day, for 14 days in the present study.

Swelling of the tarsal joint appeared from Day 8 and continued up to Day 15. Histologically, synovial cells with vesicular cytoplasms were arranged in several layers, accompanying by very slight infiltration of neutrophils, 6 hr after a single dosage. That was followed by desquamation of synovial cells and infiltration of neutrophils into dilated joint space 24 hr later. The degree of these changes was reduced on Day 4, but increased again on Day 8. Infiltration of mononuclear cells was added in the synovium. On Day 15, the synovium showed remarkable edema, fibrin deposition and infiltration of many mononuclear cells and macrophages. Hematological examination revealed increased number of neutrophils in the blood 6 hr after 1, 7 and 14 injections. Moreover, monocytes and lymphocytes increased in number after 7 and 14 administrations, respectively. On the other hand, IL-1 was produced by rat macrophages stimulated by MDP-Lys(L18) in vitro.

From the above results, immune system through cytokines including IL-1 may be related to development of MDP-Lys(L18) induced arthritis in rats.

A14 SUBACUTE TOXICITY OF (-)15-DEOXYSPERGUALIN IN THE BALB/c MICE
1. Hematological study

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Deoxyspergualin (DSP) was previously shown to have a potent immunosuppressive action. However, possible side effects due to the drug have to be thoroughly investigated and its safety margin has to be determined prior to its clinical use. Accordingly, we studied subacute toxicity of DSP. Six-wk-old femaleBALB/c mice were given either PBS, 50μl, or DSP, 0.5, 2.5 or 5.0 mg/kg daily for 3 months. Their body wt was measured biweekly, blood drawn to study the effect of DSP on WBC and on the levels of SGPT and SGOT, and various organs taken at the time of sacrifice were weighed.

Slight decrease in body wt was observed in 2.5mg group. On the other hand, because of the reduction in body wt reaching to 35%, 5mg group of mice were sacrificed on day 30. Leukopenia, anemia and thrombocytosis were observed in 2.5 and 5.0 mg of mice. Increase in SGPT level was observed in 5.0 mg group. Wet wt of various organs such as heart, lung, liver, spleen and kidney significantly decreased in 5.0 mg group($p<0.05$). Spleen wt, in particular, showed 72% reduction from 120 to 30 mg after 3 months of DSP treatment. From the above results, it may be presumed that the optimal dose of DSP in mice is less than 5.0 mg/kg/day. DSP may preferentially act on hematopoietic and lymphoid organs.

A15 SUBACUTE TOXICITY OF (-) 15-DEOXYSPERGUALIN ON THE BALB/c MICE:2 pathological study

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Although immunosuppressive potency of (-) 15-deoxyspergualin (15-Dsp) is already reported, there is little discussion on its side effect and safety. Accordingly, we have investigated on the subacute toxicity of 15-Dsp. Materials and methods:BALB/c mice were injected 15-Dsp 0.5-5.0mg/Kg i.p. for 6 weeks to several months. Their heart, lungs, liver, spleen, kidneys, intestine and bone marrow were weighted and light microscopically examined. Spleen was studied with immunoperoxidase technique using monoclonal anti-Thy 1 and anti-B 220 antibodies. Result:Mice treated with 0.5mg or 2.5mg showed slight decrease in their spleen weight at both 57th and 93rd days. On the other hand, mice administered with 5mg showed marked decrease at the 57th and 93rd days. The histrogical examination of the spleen revealed atrophy of the white pulp, hypocellurallity and fibrosis of the red pulp. Bone marrow was hypocellular and the sinus dilated. Intestinal epithelium was degenerated, detached and infiltrated with mononuclear cells, Conclusion:Spleen, bone marrow and intestine, whose cells were repeatedly dividing, were preferentially affected by the drug.

A16 EFFECT OF ANTICANCER AGENTS ON LYMPHOCYTE FUNCTIONS

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It is generally accepted that anticancer agents induce disorder of hematopoietic organs as one of their serious toxicities followed by a significant decrease of white blood cells. At the present time we investigated on the in vivo and in vitro effects of adriamycin (ADM) and cisplatin (CDDP) on lymphocyte functions and obtained the following results.

1) PHA- and LPS-induced blastogenic reactions decreased significantly in the lymphocytes prepared from the peripheral blood of rats treated with 2mg/kg iv of ADM and CDDP. However, there were no changes in number of white blood cell and lymphocyte, histopathology of bone marrow, spleen, thymus, and lymphonodus and other toxicological parameters in the treated rats.

2) An in vitro addition of 0.1μM ADM and 3.3μM CDDP inhibited PHA- and LPS-induced blastogenic reactions in the cultured lymphocytes of rat, mouse and human. There were no significant species differences in the sensitivity of lymphocytes to ADM and CDDP among rat , mouse and human.

3) The in vitro toxic concentration of ADM and CDDP against PHA- and LPS-induced blastogenic reactions in cultured rat lymphocytes was approximately equal to the pressumed blood concentration of ADM and CDDP in rats given the in vivo toxic dose.

These results suggest that the blood level of 0.1 μM ADM and 3.3 μM CDDP is immunotoxic concentration in clinical treatment of patients. It is concluded from these results that the blastogenic reaction of T cell by PHA and B cell by LPS is more sensitive and valuable to anticancer agents than other in vivo toxicological parameters.

A17 SUPPRESSION OF IN VIVO CLASTOGENICITY BY GINSENG

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This study was initiated to investigate the suppressive effect of ginseng on the genetic abnormalities caused by chemical carcinogens. The method used was mouse bone marrow micronucleus test. The effect of ginseng was manifested by describing the decrease of micronucleated polychromatic erythrocytes 24 hours after the challenge of carcinogens. The ginseng used was Korean red ginseng extract with 35% moisture. The extract was diluted with water at the ratio of 1 : 1 by weight for the oral administration. Single oral dosing of ginseng (0.19g/10g body weight) on 7 days, 3 days, 1 day, 2 hour and 0 hour before challenging cyclophosphamide did not suppress the clastogenicity. However, multiple administration (2 to 7 times) during the periods above showed significant suppression of the clastogenicity of cyclophosphamide; 58% suppression with 7 administrations in 3 days. Difference among treatments was not significant. The clastogenicity of urethane and EMS was not suppressed by ginseng. The extent of the suppression by ginseng was comaparable to that reported by carrot juice. However, it was suggested that the action mechanism might be different.

A18 TOXICOLOGICAL STUDY ON RATS FED HALOPERIDOL THROUGHOUT THEIR LIFE-SPAN

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The effect of long-term administration of haloperidol (HPL) on the general conditon and locomotor activity in male and female rats was investigated. HPL pellets were given in daily dosage of 1.0mg/kg throughout each rat's life. The death curves of males and females in HPL-treated groups were not significantly different from the control animals. Blepharitis was observed in three males in all females of HPL-treated groups. However, the symptom appeared naturally in the latter stage of the administration period. The body weight gain of HPL-trated males decreased during the administration period, but increased in females in the early stage and then decreased after the middle of the period. The food consumption of both sexes of HPL-treated groups was similar to the control animals, but water consumption of both sexes was less than the control groups. Locomotor activity of both sexes significantly declined in HPL-treated groups and no tolerance developed. The study indicates that long-term administration of HPL at the above dosage dose not influence the mortality of rats.

A19 EFFECTS OF 1-METHYL-5-THIOTETRAZOLE ON TESTICULAR PROTEINS IN JUVENILE RATS

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In our previous histological studies, the testicular toxicity induced by 1-methyl-5-thiotetrazole (MTT) in juvenile rats was suggested to result from inhibited formation of the blood-testis-barrier (BTB) in the seminiferous epithelium. In order to confirm this hypothesis, testicular cytosol was prepared and the protein content was analyzed by means of gel electrophoresis (GE) study and binding assay with 14C-testosterone (TS).

SD male rats were subcutaneously administered with MTT at a dose of 300 mg/kg/day from 7 to 35 days old. The testes were removed, homogenized with 0.32M sucrose and centrifuged at 10,000g for 20 min. The resulting supernatant, testicular cytosol, was used in the studies. First, protein of the MTT-treated cytosol was compared with that of the control by SDSPAGE. The band of Mol. wt. at 80k which was suggested to be androgen binding protein (ABP) decreased and the protein bound to TS decreased in the MTT-treated cytosol. Two-dimensional GE study revealed that the content of transferrin (TF)(Mol. wt. is about 80k.) decreased in the MTT-treated cytosol. It was confirmed that the incomplete formation of BTB caused major components such as ABP and TF to decrease in the MTT-treated testis.

A20 STUDY ON THE DEPRESSED BODY WEIGHT GAIN INDUCED BY 2, 2'-METHYLENE-bis(4-ETHYL-6-tert-BUTYLPHENOL)(MBEBP) IN RAT

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We previously reported depressed body weight gains and decreased T-GLY of serum in rats fed MBEBP, an antioxidant, at dietary levels of 0, 0.2, 1.0 or 5.0%. Moreover, the depressed body weight gains were more evident in the 1.0% group than in the 5.0% group in male rats. The present study was undertaken to explain those MBEBP-induced effects. Male Wistar rats were fed diets containing 0.2, 1.0 or 5.0% MBEBP for 4 weeks. Body weight gains, lipid peroxide(LPO), glutathione(GSH) and ascorbic acid(AsA) levels in the liver and MBEBP contents in various tissues were measured. Effects of MBEBP on LPO in microsomes of rat liver and on oxygen uptake of rat liver mitochondria were also examined.

Depressed body weight gains were again more evident in the 1.0% group than in the 5.0% group. LPO levels in the liver were slightly increased in the 1.0% group. GSH levels were decreased in the 0.2, 1.0 and 5.0% groups. AsA levels were increased in the 0.2 and 5.0% groups. The distribution of MBEBP in the intestinal tract was clearly higher in the 5.0% group than in the 1.0% group. On the other hand, in the adipose tissue and liver, it was slightly higher in the 1.0% group than in the 5.0% group. MBEBP did not increase LPO levels in microsomes of rat liver in vitro. MBEBP showed uncoupling effect on liver mitochondria.

The results indicate that the cause of depressed body gain was not due to the antioxidant action of MBEBP, but was due to the uncoupling effect of this compound.

A21 ACUTE EFFECTS OF MATERNAL ADMINISTRATION OF ETHANOL ON BRAIN DEVELOPMENT IN MOUSE FETUSES

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Pregnant C57BL/6CrSlc mice were treated with two ip injections of 25% ethanol 4 hrs apart (3.97 g/kg x2) on day 10 or 13 of gestation. Treated females were placed in an incubator at 27°C atmospheric temperature in order to avoid excessive hypothermia after the ethanol injection. Fetuses were obtained from mothers at varying intervals ranging from 3 to 48 hrs after the second injection, and the fetal brain was examined in histological sections.

In day-10 group, fetal mortality after the ethanol treatment began to increase during 9 and 12 hrs and gradually reached about 48% at 48 hrs, while in day-13 group, the mortality increased sharply during 6 to 9 hrs and reached over 50% at 9 hrs. The incidence of pyknotic cells in the ventricular zone of telencephalon in day-10 group, attained its peak (7.5%) at 12 hrs and decreased afterwards. In day-13 group, the incidence of pyknotic cells was very low and peaked at 0.7% (slightly higher than the control level) at 24 hrs.

These results indicated that cytotoxic effects of ethanol on the fetal brain in day-10 fetuses were more marked than those in day-13 ones. The effects in day-13 fetuses might have been masked by the fetal death occurring shortly after the treatment.

A22 EFFECTS OF CLIOQUINOL ON LIPOPEROXIDATION IN NEONATAL RAT SUPERIOR CERVICAL GANGLION AND VAGAL GANGLION

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Previously we reported that clioquinol(Cf) abolishs NGF-induced stimulation of RNA synthesis in neonatal rat superior cervical ganglion(SCG). On the other hand, Yagi et al. reported that Fe^{3+}-Cf chelate stimulates lipoperoxidation in retinal neuroblast and degenarates the cells. Then, in this paper, we tested the lipoperoxidation in neonatal rat SCG and vagal ganglion(VG) to compare that with the abolition of NGF-induced stimulation of RNA synthesis.

Ten μM Cf did not change the level of lipoperoxide(LP) in the ganglion even in the addition of 0.1mM $FeCl_3$. 8-Hydroxyquinoline(8HQ) alone did not change the level of LP in the ganglion even at 0.2mM, but increased that level by incubation of ganglion together with 0.2mM $FeCl_3$. Ten μM Cf and 0.2mM 8HQ did not significantly inhibit RNA synthesis in SCG, but completely abolished NGF-induced stimulation of RNA synthesis. 8HQ(0.2mM)-$FeCl_3$(0.2mM) mixture completely inhibited RNA synthesis in the ganglion, either in the presence or in the absence of NGF. These results suggest that the lipoperoxidation by Cf is not related with the abolition of NGF-induced stimulation of RNA synthesis.

A23 SEX DIFFERENCE IN EOSINOPHILIC BODY INDUCTION IN PROXIMAL TUBULES OF RAT KIDNEY FOLLOWING POTASSIUM BROMATE ADMINISTRATION

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We have reported that potassium bromate($KBrO_3$) induces eosinophilic bodies(EB) in the proximal tubules of the rat kidney and male rats show greater sensitivity to $KBrO_3$ than female rats. To clarify the mechanism of the sex difference, we studied the effects of gonadoectomy and male sex hormone on the EB induction by $KBrO_3$ in the proximal tubules of male and female rats. 600ppm $KBrO_3$ solution was given in drinking water to gonadoectomized, gonadoectomized and testosterone(TS)-injected(20mg/kg SC, 3 times weekly), or sham operated male and female Crj:CD rats for 4 weeks. In males, EB induction was drastically decreased by castration, but was comparable to sham operated rats in castrated and TS-injected rats. On the contrary, in females, EB induction was increased slightly by ovariectomy and was comparable to sham operated male rats in overiectomized and TS injected rats.

We also studied the effect of cysteine on the EB induction by $KBrO_3$ in order to find out the possible mechanism. Male rats were given 600ppm $KBrO_3$ in drinking water for 4 weeks and they were fed 0%, 2% or 5% of cysteine containing diet simultaneously. In cysteine treated rats, $KBrO_3$-induced EB in the proximal tubules decreased in dose-dependent-fashion.

These results indicate that the sex difference in EB induction by $KBrO_3$ is closely related to male sex hormone, and the non-protein sulfhydryl compound affects the toxic action of $KBrO_3$ in the rat kidney.

A24 COMPARISON OF GLOMERULONEPHRITIS BY ADMINISTRATION OF METHYLCELLULOSE IN WISTAR RATS

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By using Wistar rats, the effect on the kidney of the administration, oraly (PO), intravenously (IV), intrasubcutaneously (SC) and intraperitonealy (IP) of methylcellulose solution for 20 days at the rate of 6 times per week, was examined physiologically, hematologically, biologically as well as pathologically on animals. From the results, the following conclusion was obtained: The IV and IPinjections of methylcellulose causes glomerulonephritis. This is accompanied by hypertension, urinaly-volume, -protein and -occult blood, RBC, Ht, Hb and THC in hematological examination and Ca, Na, K, Cl, IP, BUN, total-protein, total-cholestrol and CRE in serum biological examination. Microscopically the former also showed glomerular enlargement and crescent formation, glomerular and afferent arteriolar hyalinization, abundant hyaline and granular casts, hyaline cuffing of tubular elements, and frequently sclerosis and hyalinization of intrarenal vessels the wall of which often contained red cells. In several instances there was hemorrhage into Bowman's space. Thickening of indivilual glomerular capillary loops and even focal fibrinoid necrosis was also evident. Possible mechanisms which might be involved in the genesis of hypertension and the vascular lesions are explored.

A25 RENAL GLOMERULAR ALTERATIONS IN RATS TREATED WITH NEUTRAL SODIUM PHOSPHATE

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It has been shown that pathological lesions are induced by inorganic phosphate in the rat kidney and the form and distribution of lesions are varied with the experimental procedure. This study was designed to investigate the initial stages of renal glomerular damage in rats treated intravenously with 0.5 M neutral sodium phosphate solution. Twenty-five male Wistar rats of 10 weeks of age were divided into 5 groups of 5 animals each. Each group of 5 rats received a daily dose of 5 ml/kg of body weight for 1,3,7,10 or 14 days. After the last dose, urine protein contents were estimated and then the rats were sacrificed and necropsied. Microscopic findings - Calcium deposits were first noted in both the basement membranes (BM) of the glomerular capillaries (GC) and the parietal layer of Bowman's capsule (BC) after 3 days. These changes became more pronounced on 7 day, and also slight calcium deposition occurred thereafter in the proximal convoluted tubules. Moreover, thickening of BM of the GC swelling of the epithelial cells of the BC, and hyaline droplets or casts in the proximal convoluted tubules were found after 7 days. Ultrastructural findings - Edema of the epithelial cells and partial adhesions between the podocyte processes were observed in the glomerulus after the initial dose. In the mesangial cells and BM of GC as well as in the visceral and parietal layers and capsular spaces of BC, calcium deposition were found after 3 days. Urine protein contents were slightly increased after the initial dose and became more pronounced thereafter. It was assumed from these results that proximal convoluted tubular lesions might be proceeded by renal glomerular alterations under the present experimental conditions.

A26 COMPARATIVE RENAL TOXICITY STUDY OF AMINOGLYCOSIDE(AGS) ANTIBIOTICS -IN VIVO AND IN VITRO STUDY-

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Renal toxicity of a new AGS, isepamicin(ISP), was examined by <u>in vivo</u> and <u>in vitro</u> in comparison with tobramycin(TOB) and amikacin(AMK). Method; <u>A. in vivo study</u>: 1)SD rats(250g, ♀, 5 rats/group). 2)TOB(4, 10, 20, 40mg/kg), AMK and ISP(15, 40, 80, 160mg/kg) treated i.p., for 4 and 10 days with a BID schedule. Results; Kidney weight and BUN in treatment groups were not different from control. Sphingomyelinase activities of renal cortex decreased by AGS treatment but Phospholipids(PL) increased contrary. These degrees were most extent in TOB, and ISP was almost equal with AMK. ISP showed the half values of AMK in incorporation of ^{3}H-thymidine and in content of urinary PL. In equilibrium dialysis, Kd to liposome(Chol.:PC:SM:PI=5.5:4:4:3) of ISP, AMK and TOB showed 24μM, 10μM and 7μM, respectively.

A27 RELATIONSHIP BETWEEN URINARY SEDIMENT AND HISTOPATHOLOGICAL FINDING OF KIDNEY AFTER ADMINISTRATION OF KANAMYCIN IN RAT

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Kanamycin was administered to 6 week-old male and female S.D. rats daily for 14 days at level of 1000mg/kg intramuscularly. 3-hrs-urine were collected everyday throughout the administration period and urinalysis and observation of sediment were made. And on 1,3,5,7 and 14 day, rats were sacrificed for histopathological examination.

The number of epithelial cells and damaged cells increased at 24 hrs after 1st administration. But at the end of administration period, only a few damaged cells were seen in sediment. Histopathologically, vacuolizaion, necrosis and hyaline droplet were enhanced chronologically.

These results suggest that sediment has to be examined daily or at appropriate intervals in case of renal toxic substance.

A28 METHODOLOGICAL EXAMINATION ON WEIGHING OF RAT THYROIDS

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Effects of fixation on thyroidal weight changes were estimated for the purpose of validating the protocol that rat thyroid will be weighed after fixation with trachea in order to prevent the histological artifact.

(1) Thyroids of 8 SD rats per group were weighed at some different times after fixed with 2.5 % glutaraldehyde solution (GA), buffered neutral formalin solution (F) or 1.5 % glutaraldehyde plus 0.9 % formaldehyde solution (GA+F). Weight of thyroids fixed with GA showed no remarkable changes, while those fixed with F or GA+F were linearly increased from 0.5 to 4 hours after start of fixation and remained unchanged thereafter.

(2) Twenty SD rats for each dose were orally administered with an antithyroidal compound (methylthiouracil,MTU) at the doses of 20, 40 and 80 mg/kg for 10 days. Weight changes were compared between the thyroids of 10 rats for each dose weighed before fixation with F and those of another 10 rats weighed after fixation. Between " pre-fixative" and "post-fixative weighing groups", there was no significant difference in the dose-dependency of thyroidal weight increases induced by MTU. Thyroids from "post-fixative groups" showed only few artifacts on their histological preparations, while those from "pre-fixative groups" did serious artifacts.

A29 KANAMYCIN INDUCED AUDITORY DISTURBANCE IN DOGS
(Electrophysiological methods)

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Aminoglicoside antibiotics have been known to cause auditory disturbances in man and animals. We administered kanamycin (KM) to dogs to induce auditory disturbance, and followed up the sequence of disorder by recording the changes of Auditory Brainstem Response (ABR). KM was administered subcutaneously to 4-months-old male beagle dogs at daily doses of 250 and 500 mg/kg for 26 and 13 days, respectively. The ABR was recorded in a quiet and darkened room under nonanesthetized condition at different time intervals, during and up to 12 days after the kanamycin-treatment. For recording the ABR, small steel clip electrodes were placed at 3 sites on the head; the vertex (positive(+)), edge of the tragus of the ear (negative(-)), and pinna of the contralateral ear (ground). After administration of 500 mg/kg KM for 13 days to a dog, the ABR disappeared completely, simultaneously with loss of pinna reflex. Even after eight days' recovery periods, the disorder of ABR remained unchanged. Administrations of 250 mg/kg KM for 18 days decreased amplitude and prolonged implicit time in the ABR, to a greater extent in the right ear, in which the ABR disappeared completely on 26 days of KM administration.

A30 MYELOSUPPRESSIVE EFFECTS OF ANTICANCER PLATINUM COMPLEXES IN MICE

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The myelosuppressive effects of platinum complexes(PC) cause little parallel decrease of white blood cell(WBC) counts in the peripheral blood of mice. Thus, mice are not considered to be useful models for blood toxicity of PC. This study examined whether the mouse could become an effective animal model by introducing bone marrow cellularity as a parameter to predict the myelosuppressive effects. A simple method for counting nucleated bone marrow cells(NBMC) was established. On day 0, 7-week-old male CDF_1 mice received a single intravenous dose of cisplatin, carboplatin, iproplatin, oxaliplatin, 254-S or No. 237 at 25%, 50%, or 100% of its LD_{10}. The mice were sacrificed 2, 4, 7 and 14 days after dosing and the NBMC and WBC and neutrophils of the tail and vena cava blood were counted. A marked dose-related reduction of NBMC was observed at all time points except at 14 days in all groups, with minimum values on day 4 or 7. At 14 days, the values recovered to nearly the control levels in all groups. Decreases of WBC and neutrophils were observed only on day 4 or 7 in oxaliplatin and 254-S groups. The other four drugs led to no obvious reduction. These results indicate that the mouse can be a useful model for predicting blood toxicity of PC when NBMC is used as a parameter.

A31 PROBLEMS ON THE PREPARING OF BONE MARROW FILMS ON SLIDES.

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SD rat and ICR mice were used. Rat bone marrow cells were obtained from femurs under ether anesthesia, suspended in autologous serum, a part of which was used to prepare wedged smears for May-Giemsa stain, and a part was diluted with 0.2 % nigrosin for dead cell counting. The beginning area of the smear contained more lymphocytes and less damaged cells than the terminal area. Damaged cells at the biginning were 11.6 % and those at the end were 18.3 %, while nigrosin stainable cells were less than 10 %. BM cells from mice, stained with nigrosin beforehand and resuspended in serum, were submitted to smear procedure. The percentile of nigrosin stainable cells at the beginning and at the end equaled to that in the suspension.

It was concluded that lymphocytes and other fragile cells were damaged during spreading procedure on slides.

A32 A NEW METHOD FOR BONE MARROW EXAMINATION IN RATS

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We established a new method for counting of nucleated bone marrow cells (NBMC) and applied it on examination of the bone marrow in rats receiving a single dosage of phenylhydrazine (PHZ), sc, or adriamycin (ADM), iv.

Bone marrow tissues were removed from the femur, weighed, suspended in a mixture of rat serum and Medium RPMI-1640 (1:1) using 22 and 25G needles and syringes, filtrated with Nylon mesh -200 and counted for NBMC with Coulter Counter. Values obtained were expressed as X10^{4}cells/mg bone marrow. Subsequently, the suspensions were centrifuged, and smear tests were performed using Wright-Giemsa stain. Persentages of myelocytes (M), erythrocytes (E), and others were measured and each cell density was calculated. NBMC density was 233 X10^4/mg in a male 4-week old rat.

Results were as follows: marrow cells were well preserved in the suspension at room temperature for 24 hr, there was no differences in NBMC density among the femur, tibia and humerus; NBMC in the femurs were decreased with aging, and the values were correlated with morphometric ones (% of hematopoietic tissues in the marrow). Moreover, PHZ increased NBMC and E values in rats, and ADM dereased NBMC with very low values of both M and E.

From the above results, our method is thought to be useful for bone marrow examination in rats.

A33 OTOTOXICOLOGICAL EVALUATION OF SENSORY HAIRS IN CORTI ORGAN BY USING SCANNING ELECTRON MICROSCOPY

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For the evaluation of ototoxicity in exploratory safety studies, scanning electron microscopical (EM) and audiometric estimations have been carried out by using Kanamycin treated guinea pigs. [Material and Method] Guinea pigs intramuscularly treated with 500 mg/kg/day of Kanamycin for two weeks were used as model animals. Pinna-reflex was measured by audiometer (200-2,000Hz, 32-60db). After fixation with Kanovsky solution by transcardiac vital perfusion, the cochlea was cut off, and stapes, round window, and the surface bone of scala vestibuli and scala media were peeled out carefully under the stereoscope. Following re-fixation, inner and outer sensory hairs in Corti organ were observed by scanning EM.

[Results] Sensory hairs in non-treat animals exhibited the clear construction with ordinary lining. However, deformation or destruction of these sensory hairs was observed in the animals treated with 500 mg/kg/day of Kanamycin.

These changes were paralleled with the reaction of pinna-reflex by using audiometer and the morphological changes of inner and outer hair cells.

A34 AN ATTEMPT TO APPLY AN AUTOMATIC RECORDING SYSTEM OF LOCOMOTOR ACTIVITY AND DRINKING BEHAVIOR IN A TOXICOLOGIC STUDY
(1) EFFECT OF STREPTOZOTOCIN ON BEHAVIOR IN RATS

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We assembled a non-invasive system for continuously recording locomotor activity and drinking behavior of rats in their home cages. In this system, locomotor activity and drinking behavior were measured with a photoelectric cell and a drinkometer, respectively. The counts of the locomotor activity and drinking behavior were printed out at 3-hr intervals by an automatic printer.

To test the reliability and applicability of the system, locomotor activity and drinking behavior in rats with diabetes induced by streptozotocin (STZ, 50mg/kg, iv) were investigated in light and dark phases alternating every 12-hr for 35 days. In the control rats, both locomotor activity and drinking behavior showed a typical nocturnal pattern. In the diabetic rats, total counts of drinking behavior in 24-hr were 8 times higher than those in the control for 31-35 days after the STZ-injection. However, neither total counts of locomotor activity in 24-hr nor the circadian rhythms of locomotor activity and drinking behavior were changed.

These findings indicate that this system for measuring locomotor activity and drinking behavior and analyzing their rhythms and amplitudes is useful to evaluate the effects of drugs on the behavior of rats in toxicologic studies.

A35 A MEDIUM-TERM MULTIPLE ORGANS BIOASSAYS FOR CARCINOGENESIS MODIFIERS

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The modifying effects of 14 different carcinogens on the induction of neoplastic lesions in their respective targets organs were tested using multi-organ initiation model with 3 different carcinogens. Male, 6-week-old F344 rat were divided into 14 groups (total 295 rats). Rats were consecutively injected with DHPN (1000 mg/kg x2, i.p.), EHEN (1500 mg/kg x2, i.g.) and DMAB (75 mg/kg x2, s.c.) for initiation, then treated with 2-AAF (0.01%, D), Clofibrate (1.0%, D), DDPM (0.1%, D), Ethionine (0.25%, D), 3'-Me-DAB (0.06%, D), Phenobarbital (PB) (0.05%, D), B(a)P (0.02%, D), BBN (0.1%, W), BHA (1.0%, D), Catechol (0.8%, D), DMBA (0.01%, D), 3-MC (0.02%, D), MNNG (0.005%, W), Caprolactam (1.0%, D). Rats were killed at week 16. Test chemicals enhanced the incidence of neoplastic lesions in their targets organs, liver with 2-AAF, Ethionine, 3'-Me-DAB and DMBA; thyroid and liver with DDPM and PB; urinary bladder with BBN; forestomach with BHA and Catechol; lung and liver with 3-MC. The results indicate that combined initiator protocol followed by administration of test chemicals can be used for the medium-term bioassay for carcinogens and modifying agents with unknown target organ.

A36 BIEL WATER MAZE LEARNING DEFICITS AND HIPPOCAMPAL INJURY

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The learning task most commonly used for testing new drugs in Japan has been a Biel water maze (path A). However, there have been no path A studies using animals with a known site of brain injury. This study was undertaken to determine whether poor maze performance was associated with hippocampal injury using the path A, mirror image of path A (MIA), and path B (in which the start and goal positions were reversed against MIA). SD rats were trained for 3 days, given orally 9 mg/kg of trimethyltin (TMT), and tested for path A performance 4 hr and 1, 3, 5, 6, 7, 8, 11, and 32 days after TMT treatment and for MIA and path B performance at 33-35 and 36-38 days. In TMT rats, errors and swimming time required on path A were much more than in the controls at 3 days and the errors at 11 and 32 days were the same in number. TMT animals showed poor performance also on MIA and path B. The results suggest that path A, MIA, and path B are likely to be tools for determining impairment of learning and memory caused by hippocampal injury.

A37 ACID HEMATEIN TEST USED FOR DETECTING DRUG-INDUCED LIPIDOSIS.

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Drug-induced lipidosis is ultrastructurally characterized by occurrence of myeloid bodies, which are probably due to the formation of lipid-drug complexes. Histochemically, the acid hematein test is used for detecting lipidosis. In order to clarify the differences of the staining, three techniques of the acid hematein test, Baker's method(1946), the simplified method devised by Hori(1963) and the modified method by AFIP(1968), were compared. Liver tissues were obtained from rats orally given 4,4-Diethyl-aminoethoxy-hexestrol. One of the differences in procedure, between Baker's original technique and modified methods, is the way of dichromate treatment. The dark blue staining granules were clearly observed in cytoplasm of liver cells and stellate cells. The condition of the staining corresponded to the ultrastructural figure. The positive reaction disappeared after pyridine extraction test. In cases of modified methods fine granular materials were diffusely seen with positive reaction of mitochondrial lipids. The results indicate that simultaneous oxidation, chromation and fixation of lipids by means of dichromate before freezing might have been a important process, and particularly suitable for demonstrating the presence of myeloid bodies. In second experiments we also examined what effects of the period of 20% neutral buffered formalin fixation might have on the staining of Baker's method. The reaction products were observed with satisfactory results after six months, although the color intensity was weaker.

A38 ANTINEOPLASTIC AGENT-INDUCED EMESIS IN THE FERRET

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The antineoplastic agents cause severe emesis in man which should result in treatment refusal. Animal models for emetic activity have been limited to the dog and cat. In this study, the ferret was evaluated as a smaller animal model for determing the emetic activity and for testing antiemetic agents.

Adult, castrated, male Fitch ferrets weighing 1-1.5 kg were supplied by Marshall Res.(NY). They were individually housed and were given cat chow and water. Cisplatin caused emesis at the doses of 7 and 10 mg/kg, i.p., respectively. The emetic episodes induced by cisplatin began 100 minutes after administration. The profile of emesis induced by cyclophosphamide (200 mg/kg, i.p.) in the ferret was similar to that of cisplatin but more rapid in onset.

GR38032F, a highly selective antagonist at 5-HT_3 receptor, produced a marked antiemetic activity for the emesis induced by cisplatin and cyclophosphamide in ferrets. Ferret is a useful species in emetic testing and may be valuable in evaluating antiemetic drugs.

A39 DEVELOPMENT OF A MEDIUM-TERM BIOASSAY MODEL FOR BLADDER CARCINOGENS AND PROMOTERS

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To establish a medium-term bioassay model for bladder carcinogens and promoters, an investigation of the effects of uracil stone associated epithelial cell proliferation on test chemical administration was conducted. Male F344 rats, aged 6 wks, were divided into 6 groups of 20 rats each. Animals were given 0.05% BBN in the drinking water (W) for the initial 4 wks, and then treated with the bladder carcinogens or promoters (groups 1-5), DBN (0.05% W), MNU (50 mg/kg, 1/wk, ip), 3,3'-DCB (0.3% in diet (D)), sodium o-phenylphenate (SOPP, 2% D) or sodium saccharin (SS, 5% D), or no test chemical (group 6) for 5 wks. For the next 3 wks, they received 3% uracil diet and then were returned to test chemical supplement until sacrifice at week 20. The bladders were quantitatiavely evaluated for development of putative preneoplastic papillary or nodular hyperplasic (PNH) lesions and papillomas (PPL). Incidence and number of PNH in groups 1-5 were significantly increased as compared with control group 6 value. In addition PPL in groups 1 (DBN), 4 (SOPP) and 5 (SS) were significatnly elevated. These results suggest that the model may be very useful for early detection of bladder carcinogens and promoters, and for comparative evaluation of their potential.

FREE COMMUNICATIONS IN HALL B

B01 TOXICITY AND FATE IN BRAIN OF TRIBUTYLTIN CHLORIDE IN CHICKEN

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As shown in the next paper, tributyltin chloride (TBT) was more potent than trimethyltin chloride in inhibiting cholinergic parameters of chicken brain *in vitro*. In the present experiment, toxicity of TBT, and fate of TBT and its metabolites were examined in male white Leghorn (9 to 10 weeks old). The oral LD_{50} was 125 mg/kg. A method with a HPLC for determining the amount of TBT and its metabolites was devised to detect 0.05 μg/ml and recover 72 to 79%. Birds were administered with 40 mg/kg of TBT and killed daily to collect brains and blood for 5 days. The highest content of TBT, monobutyltin (MBT) and inorganic tin (Sn) in the brain were 16.9, 15.2 and 15.4 nmol/g brain which were observed 1, 3 and 5 days after administration, respectively. The amount of dibutyltin (DBT) remained low. Biological half life of TBT in the brain was 1.4 days. While the content of TBT reached 6.6 nmol/g blood 2 days after administration, those of DBT, MBT and Sn remained low (<2.5 nmol/g) for 5 days. The highest TBT (16.9 nmol/g≒1.69 μM) observed in the brain may be enough to influence the central cholinergic system of chicken *in vivo*.

B02 EFFECTS OF ORGANOTINS ON CHOLINERGIC SYSTEM IN CHICKEN BRAIN *IN VITRO*

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The effects of two organotins, trimethyltin chloride (TMT) and tributyltin chloride (TBT) on the cholinergic system in the chicken brain were investigated *in vitro*. Both compounds, at concentrations less than 10^{-4} M, had almost no effect on acetylcholinesterase activity in brain homogenate. On the other hand, TMT and TBT inhibited noncompetitively choline acetyltransferase activity with a Ki value of 1.5 and 0.99x 10^{-5} M and a high-affinity choline uptake with a Ki of 35 and 2.5 x 10^{-6} M, respectively. These inhibitory effects were not reversed in the presence of cystein. TMT and TBT inhibited a low-affinity choline uptake with a Ki of 2.6 and 1.25 x 10^{-4} M. Although both compounds inhibited ACh release from brain slices, only 10^{-4} M TBT suppressed the ACh synthesis. TBT at concentrations of 10^{-5} and 10^{-4} M but not TMT inhibited the [^{3}H]-QNB binding which is an indicator for muscarinic ACh receptor. These results suggest that TBT and TMT influence the cholinergic system suppressively.

B03 THE METABOLISM OF BENZENE IN MAN

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Time-weighted average breath zone benzene level and benzene metabolites in shift-end urine was measured in 152 benzene-exposed workers in the second half of a working week. The benzene level was measured utilizing diffusive samplers, whereas 5 urinary metabolites (i.e., phenol, catechol, quinol, 1,2,4-benzenetriol and t,t-muconic acid) by either GC or HPLC. A significant relationship ($P<0.01$ for each) could be achieved between the two parameters. With 3 assumptions that 15 L/min of air will be inhaled, that the up-take ratio of benzene in the lungs is 50% and that 1 ml/min of urine will be excreted, cross-sectional absorption - excretion balance could be figured out. Thus, benzene absorbed will be excreted as phenol (by 13.2%), catechol (1.6%), quinol (10.2%), 1,2,4-benzenetriol (0.47%) and t,t-muconic acid (1.9%). To investigate the pathway of 1,2,4-benzenetriol formation, 50 mg/kg each of phenol, catechol and quinol was injected i.p. to rats and 24-hr urine was collected for metabolite analyses. 1,2,4-Benzenetriol was detected most abundantly after quinol administration, and less so after phenol injection, but not detected when catechol was given. It is most likely therefore that 1,2,4-benzenetriol is formed by stepwise hydroxylation of benzene via phenol and quinol.

B04 SPECIES AND STRAIN DIFFERENCE IN THE EFFECTS OF VARIOUS INDUCERS ON HEPATIC MICROSOMAL CARBOXYLESTERASE ACTIVITIES IN INBRED STRAINS OF RATS AND MICE.

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Difference in the effects of *trans*-stilbene oxide (TSO), kaneclor 500(PCB) and 3-methylcholanthrene(3-MC) on hepatic microsomal carboxylesterase(CEase) activities in inbred strains of rats(Fischer 344, Lewis) and mice(C57BL, DBA) was studied. Intraperitoneal administration of TSO to both strains of rats and mice increased the activities of isocarboxazid(ISOC) hydrolase. When PCB was treated to both strains of mice, the activities of ISOC, p-nitrophenyl acetate and butanilicaine hydrolase were increased. Conversely, CEase activities in both strains of rats were not affected. On the other hand, administration of 3-MC to both strains of rats and mice, only ISOC hydrolase activity was increased in C57BL strain of mice. In conclusion, we have found that the CEase activity in inbred strains of rats and mice are induced to different extents by TSO, PCB and 3-MC.

B05 METABOLISM AND EXCRETION OF ORALLY AND INTRAPERITONEALLY ADMINISTERED TRIMETHYLARSINE OXIDE IN THE HAMSTER

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The preset study was made to elucidate themetabolism and excretion of trimethylarsine oxide(TMAO) in experimental animals. The hamsters were administered 10 mg of TMAO(as As) per kg orally once only. The urinary excretion of trimethylarsenic compound in urine during the first 12 hrs after the administration of TMAO amounted to 66.0±12.7% of the administered dose, and that during the first 24 hrs, to 86.9±11.1%. It was found that the principal excretory route of TMAO is the urinary excretion thought the kidneys, and that TMAO is an arsenic compound eliminated extremely rapidly. The unchanged form of TMAO proved neither to be demethylated nor to be converted to arsenobetaine. The TMAO concentration in the whole blood and that in the plasma reached a peak at an hour after the administration of TMAO, and then decreased rapidly, respectively. trmethylarsenic was the one and only chemical species of arsenic detected in the liver after the administration of TMAO. It was shown, on the other hand, that TMAO is partly reduced in vivo and that only trimethylarsine was detected the expired air.

B06 **DISTRIBUTION OF LEPTOPHOS AND ITS CHANGE IN NERVOUS AND MUSCULAR TISSUES OF HENS AFTER A SINGLE *IV* INJECTION**

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The level of leptophops (MBCP), an organophosphate which caused OPIDN, was determined in plasma, nervous, muscular and other tissues of hens at 1, 6 and 24 hr after a single *iv* injection at the rate of 30mg/kg. Myoglobin (Mb) level in muscular tissues was measured.

MBCP decreased very rapidly in plasma, cerebrum and cerebellum, but decreased very slowly in sciatic nerve. Spinal cord showed intermediate manner. Most of all muscles in lower limbs, which were red muscles, took in high level of MBCP and decreased it slowly, in contrast with low uptake and rapid reduction of MBCP in white muscles of upper limbs. MBCP level in muscular tissues showed significant correlation with Mb level of each muscle. It was suggested that Mb may take part in the maintenance of MBCP in muscle. Gizzard contained high level of myoglobin, but not so high level of MBCP.

B07 PARAQUAT TOXICITY OF SKIN INCISIONS IN RABBITS

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Paraquat toxicity induced by skin incision was examined using rabbits as our experimental subjects. Seventy female, 10 month old, JW-NIBS rabbits(2.0 to 3.0 kg) were divided into 4 experimental groups. Group I(n=10), our control group, was administered only saline. Group II(n=20) was subjected to skin incision and saline administration. Group III(n=20) underwent both skin incision and paraquat administration. Group IV(n=20) received paraquat treatment only. Thirty minutes after the skin incisions were completed at 4 sites on the rabbits' backs, 25 mg of paraquat/kg or 2.5 ml of saline/kg was subcutaneously administered for a period of 2 days. Samples for the determination of serum ceruloplasmin concentrations, for plasma fibronectin levels and the collagen content of the lungs and skin were obtained on days 3, 5, 10, 20 and 32. During this experimental period, 11 rabbits from Group III and 12 rabbits from Group IV expired due to acute paraquat poisoning. The serum ceruloplasmin levels for Group III were significantly increased on days 3 and 5, compared to Group I. However, Group IV also demonstrated elevated serum ceruloplasmin levels, but they were determined not to be statistically significant, compared to Group I, on days 3 and 5. The plasma fibronectin concentrations were similarly increased for Groups III and IV on days 3 and 5, but also were not statistically significant compared to Group I. The lung hydroxyproline content of the rabbits that expired from acute paraquat poisoning was determined to be lower than Group I.

B08 CHRONIC TOXICITY STUDIES ON TBM, DBCM AND BDCM IN WISTAR RATS

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The volatile chloro compounds produced by chlorination of tap water have been a great concern as new environmental pllutants, since chloroform was found to cause cancer. In this study, a microcapsulation technique, newly developed for volatile compounds, was employed to ascertain whether tribromomethane (TBM), dibromochloromethane(DBCM) and bromodichloromethane(BDCM) cause disorders in the body as sources of environmental pollution. Microcapsulated trihalomethane(THM) were continuously administered to rats by being mixed with the diet for 24 months. The concentrations of each THM in the diet are shown in the Table.

THM	Concentration (%)		
	L	M	H
Control			
TBM	0.04	0.16	0.65
DBMC	0.022	0.088	0.35
BDCM	0.014	0.055	0.22

As a result, suppression of the body weight gain, decrease of TG and ChE activities in the serum were generally observed in high and middle dose groups of THM-treated rats. But no change was found in the mortality and hematological findings. Histological changes were found only in the liver by administeration of THM, such as discoloration and rough surface in gross findings and fatty degeneration, microgranuloma, bile duct proliferation and cholangiofibrosis by histological examination. As neoplastic lesions, cholangiofibromas were significantly increased in H group of female rats fed BDCM.

B09 CHRONIC TOXICITY STUDY OF p-sec-BUTYLPHENOL IN RATS

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The chronic toxicity of p-sec-butylphenol(BP), used as a raw material of phenol resin, paint, etc. was investigated. Five-week-old male and female slc:wistar rats were fed BP in the diet at levels of 0, 1000, 3000, 10000 and 30000 ppm for 6, 12, 18 and 24 months. Body weight was depressed in both sexes receiving over 10000 ppm from 1st week. As a general symptom, diarrhea were observed in both sexes receiving 30000 ppm at 1st week and the number increased gradually. A few cases of alopecia in abdomen were also observed in the same groups from 6th week. Biochemically, BUN increased in all BP-treated groups from 6th month. Kidney weight increased in both sexes receiving 30000 ppm from 6th month. By histological examination, hyperplasia, keratosis and hyperkeratosis of mucosal epithelium in the esophagus and forestomach were found in both sexes receiving over 10000 ppm from 6th month. In the kidney, degeneration of proximal tubules, collecting tubules and pelvis were found. Degeneration of renal tubules was accompanied with dilatation, swelling, calcification and desquamation of epithelium with secretion. Nephritis, fibrosis, cyst and polycystic changes were recognized in 10000 ppm and 30000 ppm groups from 12th month. Considerable changes were not found in nephron and Bowman's capsules.

In conclusion, under present experimental conditions, the terget organs of BP by chronic administration were esophagus, forestomach and kidney.

B10 ELEVATION OF SOME URINARY ENZYME ACTIVITIES IN RATS ADMINISTERED WITH AROMATIC NITRO-AMINO COMPOUNDS

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Nephrotoxicities of some aromatic nitro-amino compounds were evaluated by some urinary enzyme activities and renal microscopic changes. Male Fischer 344 rats were intraperitoneally injected with aniline, p-aminophenol (PAP), p-acetylaminophenol (PAAP), p-chloroaniline (PCA), p-nitrochlorobenzene (PNCB), p-anisidine (PAN), or p-nitroaniline (PNA) at 1.0 mmol/kg. In PAP-administered rats, necrosis of renal tubular epitherial cell was caused, and urinary N-acetyl-β-D-glucosaminidase (NAG) and γ-glutamyltranspeptidase (γ-GTP) activities were remarkably elevated. In PAN-administered rats, swelling of the tubular epitherial cell and significant elevation of the urinary NAG activity were caused. In PCA-administered rats, significant elevation of the urinary NAG and γ-GTP activities were caused. However, the urinary enzyme activities of the other rats administered with aniline, PNCB, PAAP, or PNA did not show significant changes compared to those of the control rats. These results indicate that PAP is a strong nephrotoxic substance, and that nephrotoxicities of PAN and PCA may exceed PAAP which has been known to cause a renal damage.

B11 EFFECTS OF PRENATAL EXPOSURE TO STYRENE ON THE NEURO-BEHAVIORAL DEVELOPMENT IN RATS

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Styrene was evaluated for possible functionaleffects in the offspring of rats exposed during gestation. Pregnant Wistar rats were exposed to 0, 50 or 300 ppm styrene for 6 hours/day during days 7 to 21 of gestation. Several neuro behavioral tests detected differencesfrom controls in the offspring from dams exposed to styrene during gestation. Even exposure to relatively low concentrations of styrenedelayed some physiologicaldevelopments such asthe righting reflex and auditorystartle reflex,in additionto causing disturbances of the neuromotorcoordination function (Rota-Rod performance) and learning acquisition(CRF). Furthermore, large doses (300 ppm) led to subtle changesin emotional behavior and increases in spontaneousactivitiesin addition to the delay of neurobehavioral developments such as pivoting,and to negative geotaxis.Analyses of neurochemical studiesshowed dose-dependent decrease of neuro-transmittersof newborn offsprings of styrene exposed rats.

B12 NEUROFILAMENT DEGRADATION IN THE NERVOUS SYSTEM OF RATS INTOXICATED WITH ACRYLAMIDE AND RELATED COMPOUNDS

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Degradation of neurofilament(NF) proteins by Ca^{2+}-activated neutral protease(CANP) was studied in the nervous system of rats treated with neurotoxic or non-neurotoxic compounds. In the tibial nerve, the degradation of NF 68K was depressed by five neurotoxic compounds: acrylamide, N-hydroxymethylacrylamide, N-isopropylacrylamide, methacrylamide and 2,5-hexanedione. A non-neurotoxic compound, diacetone acrylamide, did not show any effect on the degradation. An immunoblot analysis confirmed the reduction in the degradation and revealed a difference in the degradation pattern between the control and acrylamide-treated rats. In the spinal cord, the degradation of the three subunits of NF was depressed in animals treated with acrylamide. Although the exact mechanism of the reduction in the degradation of NF is not yet known, the present results suggest that an inhibitory effect on CANP activity might be relevant to the mechanism of neurotoxic action of acrylamide derivatives.

B13 EFFECT OF LEAD ON CELLULAR IRON METABOLISM

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When human erythroleukemia cells (K562) were cultured in the presence of 50 and 100 μM Pb^{2+} for 48 h, iron uptake from transferrin by these cells reduced to 60 and 40 % of that of control cells, respectively. From Scatchard analysis of transferrin binding to the cells, transferrin binding sites on the cell surface were found to be decreased in Pb-treated cells, while binding affinity was not affected. This was confirmed by immunoprecipitation of cell-surface receptors with a monoclonal antibody (5E9) to human transferrin receptor. However, no difference in the total amount of transferrin receptors was found between control- and Pb-treated K562 cells. Furthermore, neither biosynthesis nor degradation of transferrin receptors was affected by Pb-treatment. The kinetic study of transferrin recycling in the cells showed that transferrin was exocytosed with slower rate in Pb-treated cells than in control cells. Whereas, endocytosis of transferrin was similar in both cells. These results suggested that reduced iron uptake in Pb-treated cells was attributed to the decrease in cell-surface transferrin receptors. It was possibly resulted from localization of transferrin receptors to cell interior. The successive recycling processes with reduced exocytic rate could cause the localization of the receptors.

B14 VARIATION OF PORPHYRIN-RELATED COMPOUNDS IN MICE AND RATS EXPOSED TO LEAD

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The urinary excretion of δ-aminolevulinic acid (ALA) and coproporphyrin (CP) isomers was investigated in mice exposed to lead in the drinking water (200 and 500 ppm) for 14 or 30 days, and the results were compared with those obtained from rats exposed to lead under the same exposure condition. The result indicated that the level of urinary ALA excretion in lead-exposed mice was much higher than that in lead-exposed rats. On the other hand, it was indicated that the increasing level of urinary CP excretion was higher in rats than in mice, contrary to expectation. The reason for the discrepancy between urinary excretion of ALA and that of CP in rats and mice exposed to lead is, however, unknown.
The increase of erythrocyte protoporphyrin was not observed in both mice and rats exposed to lead.
The inhibition of δ-aminolevulinic acid dehydratase (ALAD) activity was much greater in erythrocytes than in liver, in the mice exposed to lead.

B15 EFFECT OF LECITHIN ON ABSORPTION OF INORGANIC MERCURY AND METHYLMERCURY

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The effect of lecithin (PC) on the rat small intestinal absorption or transdermal absorption of inorganic mercury and methylmercury was investigated. Inorganic mercury or methylmercury in 20% ethanol was prepared with and without PC. Samples including 36 nmol of mercury with or without 0.6mg of PC were injected in a piece of small intestinal tract or were applied on the back skin of rat. The small intestinal absorption of inorganic mercury was much higher in the mercury with PC than that of without PC for 3 hrs after the injection, 0.93% and 0.6%, respectively. The small intestinal absorption of methylmercury also was higher in the mercury with PC than that of without PC, 5.16% and 2.9%, respectively. On the other hand, the transdermal absorption of inorganic mercury and methylmercury also were increased by addition of PC, 61.1% to 78.3% and 44.2% to 74.1%, respectively. When the liberated SH was determined by DTNB test, the addition of PC inhibited significantly the release of thiols from a small intestine which was increased the time dependently by methylmercury. Liposomes prepared with PC can be widely applied to the various forms of drugs, however, if toxic substances would be applied with PC its absorption or toxicity might be changed. It was suggested that the toxicity of chemicals are affected by coexistence substances.

B16 MERCURY UPTAKE AND DISTRIBUTION IN FETUSES OF GUINEA PIGS IN LATE GESTATION AFTER *in utero* EXPOSURE TO MERCURY VAPOR

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Organ mercury distribution in neonates and fetuses after *in utero* exposure to mercury vapor (Hg^0) was different from that of the dams of these animals. In the neonates and fetuses, the highest mercury concentration was found in liver, and the brain mercury concentration was much lower than that of the dams. Gel chromatography revealed that a substantial portion of the mercury in the fetal liver was associated with metallothionein(MT)-like protein. Ethanol (Et) treatment prior to Hg^0 exposure caused the elevation of the fetal brain mercury concentration up to the maternal level in one group of litters. However, in another group of litters, pretreated with Et, brain mercury concentration was as low as that of the fetuses without Et pretreatment. Hepatic MT in the fetuses in the group with the elevated brain mercury concentration was much lower than in the group with the non-elevated brain mercury concentration. Our investigation suggested the involvement of fetal hepatic metallothionein in the uptake and distribution of mercury in guinea pigs thus exposed to mercury vapor *in utero*.

B17 EFFECTS OF MODERATE RIBOFLAVIN DEFICIENCY ON SELENITE TOXICITY IN MICE.

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Selenodiglutathione(GSSeSG) was more toxic than an equimolor dose of selenite(SS). GSSeSG, the initial product in SS metabolic pathway, may be accumulated by inhibition of glutathione-oxidoreductase(GR). Hence, we examined the SS toxicity in mice under the condition of riboflavin, the cofactor of GR, deficiency.

ICR mice maintained on a diet devoid of riboflavin had a significant decrease in GR activity after 2 weeks of feeding ($p<0.01$). When SS was injected with 30 μ mol /kg, but not with 60 μ mol/kg, body weight gain (%) of riboflavin deficient mice was significantly less than that of pair-fed control mice. Lethality due to SS injection (70 μ mol/kg) was significantly enhanced in riboflavin deficient mice after 3 weeks of feeding. More enhanced elevation of plasma enzyme activities (GOT,GPT,LDH,CK) in riboflavin deficient than in pair-fed mice was observed for GOT and CK one week after SS injection at the dose of 30 μ mol/kg, and for all the enzymes examined at the dose of 60 μ mol/kg.

B18 *ZINC STIMULATES SPONTANEOUS TRANSMITTER RELEASE AT MOUSE NEUROMUSCULAR JUNCTIONS*

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Zinc ions (Zn^{2+}) were examined for the effect on the frequency of miniature end-plate potentials (MEPPs) at the mouse neuromuscular junctions in presence or absence of $[Ca^{2+}]_o$. Zn^{2+} (10-100 uM) markedly elevated the frequency of MEPPs. This effect was time- and concentration dependent but not depended on $[Ca^{2+}]_o$. In a Ca^{2+}-free solution, the effect of Zn^{2+} was transient and required a latent period for several minutes. Low temperature (24°C) lengthened this period and suppressed the maximum effect of Zn^{2+}. The effect of Zn^{2+} was partially antagonized by dantrolene sodium or by neomycin. Both agents also reduced the effect of $[Ca^{2+}]_o$ on MEPPs in depolarizing solution. Cadmium and 2,3-bisphosphoglycerate also elevated the frequency of MEPPs by a manner independent on $[Ca^{2+}]_o$, whereas the latter was much less potent. Zn^{2+}, cadmium and 2,3-bisphosphoglycerate have been shown to inhibit inositol 1,4,5-triphosphate 5-phosphatase, thereby increase $[Ca^{2+}]_i$ by releasing from a nonmitochondrial storage site. Present results provide an evidence for an intracellular store of Ca^{2+} playing a role in the release of transmitter at the motor nerve terminal. The release of Ca^{2+} from the storage site may be coupled with metabolism of phosphatidylinositol.

B19 **DELAYED NEUROTOXICITY OF TRIPHENYL PHOSPHITE IN HENS - DISTRIBUTION OF TRIPHENYL PHOSPHITE -**

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The organophosphorus compound, triphenyl phosphite (TPPI) which is widely used in industry as an anti-oxidant caused ataxia in chickens 8-14 days after a single po or iv administration. Plasma TPPI concentrations decreased rapidly with a biological half-life of less than 60 min., whereas a large amount of TPPI localized in fatty tissue and remained there for long time. Among the neural tissues, the sciatic nerve had the highest concentration followed by the spinal cord, cerebellum and cerebrum. The red adductor magunus muscle contained about 10 times as much TPPI as did the white gastrocnemius muscles 6 hours after iv administration. Since the red muscles of chickens are mainly located in the legs, the high affinity of TPPI for red muscles may be associated with the toxicity.

B20 Centrilobular emphysema and anthracosis in human lung

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To investigate the relationship between centrilobular emphysema and anthracosis in human lung, histological examination of emphysema and elemental analysis of dust accumulated in the lung were made on 97 autopsy cases. Degree of centrilobular emphysema was histologically estimated and assigned a score ranging from 0 (normal) to 3 (most severely damaged case), and averaged on each case (EM-Score). Lung dust was separated from another part of each lung by high-speed centrifugation after dissolving tissue by alkali solution, and elemental analysis was performed by wavelength-dispersive X-ray fluorescence analyzer. As a result, degree of centrilobular emphysema significantly correlated with age, smoking index, and dust content in the lung ($P=0.01$), but observed correlation with age seemed to be secondary effects of smoking and anthracosis. Significant correlations were also found between degree of emphysema and tissue concentrations of Si, Al, Fe, Cr and Mn in the dust ($P=0.01$), and coefficient on Cr was still significant after effects of age and smoking were eliminated ($P=0.05$). These elements could probably be attributed to inhalation of particulate matter in atmosphere or cigarette smoke.

321 EFFECT OF PARTICLE SIZE ON INHALATION TOXICITY OF FENTHION

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Rats were exposed for 6hr to fenthion (MPP) mists of two different particle size distributions. Mass median aerodynamic diameters (σg) for the larger and the smaller mist were 6 μm (2.1) and 1 μm (1.4), respectively. The LC50 for the smaller mist (2.8 mg/ℓ) was 3 times of that for the larger mist (0.9 mg/ℓ). The signs of toxicity of rats exposed to the larger mist were more persistent compared with the smaller mist. During the exposure to MPP (0.6 mg/ℓ), the larger mist caused similar inhibition of erythrocyte AChE as the smaller mist, but the inhibition was much more persistent. This slower recovery was also noted in the brain AChE of rats exposed to the larger mist. The area under the plasma MPP concentration-time curve during the 6hr exposure and subsequent 18hr was 1.3 times greater for the smaller mist than the larger mist. Rats orally given MPP (60 mg/kg) showed much lower plasma concentration of MPP and stronger and more persistent AChE inhibition of erythrocytes compared with rats intravenously given MPP (20 mg/kg). These results may indicate that the larger particles of MPP deposited on the NP region are preferentially metabolized to the active formes before entering the systemic circulation via the route similar to that after the oral administration, resulting in a persistent and strong AChE inhibition, while the smaller particles rather directly enter the systemic circulation from the lung.

B22 EFFECTS OF LONG TERM ADMINISTRATION OF PHOSPHATES ON THE LIVER, KIDNEYS,MUSCLES, BONE, AND INORGANIC ELEMENTS IN THE PLASMA, URINE, FECES OF MICE

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In recet years phosphate has been used for many purposes and in extended areas. From the viewpoint of preventive medicine, we have felt necessity of long term observations and we started in 1984.

Sodium metaphosphate, sod. polyphosphate, and sod. pyrophosphate were mixed in the ratio 7:1.5:1.5 making 0, 0.5,1.0,2.0 and 5.0% mixtures. ICR and AKR strain mice were fed starting at the age of 28 days to 450 or 680 days.

Plasma P,and $1,25(OH)_2D_3$ level and urine P,Na,Fe level of treated gloup were significantly higher than control gloup. Urine Cl and Ca level of treated gloup were significantly lower than control gloup.

Thinning of cortex of bone and marrow trabeculas, osteoporosis, calcinosis in Achilles tendon, osteophyte in soft X-ray photograph of tibia. Muscle fiber diameters diminished in M.soleus. Thinning of cells in the outer layer of knee joint carcilage decrease in or loss of the number of cells, bone proliferation in subchondral bone osteophyte, and osteporosis. Swelling and shrinkage of glomerular capillaries,proliferation of mesangial cells, swelling, thinning, and desquamation of tubular epithelium, interstitial tissue inflammation in kidneys. These effects accorded with accumulated amount of phosphates taken in.

B23 EFFECTS OF BIFEMELANE ON LIPID PEROXEDATION IN RAT LIVER

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Effects of Bifemelane (BF) on lipid peroxidation and hepatic drug metabolizing enzyme activities were examined in rats. Lipid peroxidation in liver homogenate was significantly inhibited after oral administration of 100mg/kg BF for 3 days and 30mg/kg BF for 4 weeks. NADPH-dependent lipid peroxidation in liver microsomes was inhibited after administration of 100mg/kg BF for 3 days. BF remarkably inhibited NADPH-dependent lipid peroxidation at $10^{-6} \sim 10^{-4}$M , in vitro. In case of homogenate almost similar results were obtained. BF had no effect on the content of cytochrome P-450 and cytochrome b_5 and the aivity of aminopyrine N-demethylase. But BF inhibited the activity of NADPH-cytochrome c reductase, in vivo and in vitro. NADPH-cytochrome c reductase is mediated the major enzymatic system of NADPH-dependent lipid peroxidation. From these results, NADPH-dependent lipid peroxidation in vivo may depend on the low level of NADPH-cytochrome c reductase.

B24 DRUG-INDUCED LOSS OF CONSTITUTIVE CYTOCHROME P-450 IN RAT LIVERS AND THE MECHANISM

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In different from other forms of cytochrome P-450, a main constitutive P-450-male in rat livers is decreased by the treatment of various chemicals. Competition at the enzyme active site and irreversible binding to heme moiety have been shown as the mechanism of the drug-induced reduction of cytochrome P-450. However, our recent studies on the growth hormone-dependent regulation of P-450-male suggest the possible involvement of pituitary on the drug-induced reduction of P-450-male in rat livers. Therefore, the change in the level of P-450-male protein and mRNA in normal and pituitary hormone-depleted (hypophysectomized) rats were measured to assess the mechanism of the drug-induced loss of P-450-male. The results obtained indicate that phenobarbital and 3-methylcholanthrene, decrease hepatic content of P-450-male mainly through the modulation of the growth hormone-mediated process, and also suggest that growth hormone acts at pre-and post-transcriptional steps of P-450-male synthesis from non-coordinate changes in P-450-male protein and mRNA.

B25 HEPATOTOXICITY OF TOCP IN RATS

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Although the delayed neurotoxicity test using TOCP (tri-ortho-cresyl phosphate) is sometimes hampered by severe liver damage. The hepatotoxicity of this chemical has been unclear. In the present stuey groups of 4 or 6 F344 male rats were given a single intubation of 1,000 mg/kg (1/3 of LD50) of TOCP, killed by decapitation at 1, 6, 24, 72, and 168 hrs after treatment and the liver was excised to examine the liver weights, microsomal P-450 and protein contents, cytosol GSH contents, and histology. The liver weights began to increase at 6 hrs and showed the maximal value, 150% of the control, at 72 hrs. Cytosol GSH contents revealed a striking elevation, 290% of the control, at 24 hrs after a slight depression at 1 hr. Microsomal P-450 levels showed a continuing decrease to reach 58% of the control at 24 hrs and recovered at 72 hrs. On histology, the specific changes appeared first as dilatation of ER at 6 hrs and progressed to zonal necrosis at 24 hrs. These results might suggest that increased free radical production in ER can be the substantial cause of the hepatotoxicity of TOCP.

B26 TOXICOLOGICAL CHANGES IN PHENOBARBITAL-TREATED BEAGLE DOGS

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Hepatic hypertrophy following the induction of drug metabolizing enzymes is one of the common findings in toxicity studies. Beagle dogs were divided into four groups, each composed of 2 males and 2 females, and were orally administered with 0,5,20,60mg/kg of phenobarbital-sodium(PB) for one year. We selected antipyrine as a model drug to measure the extent of enzyme induction, and administered intravenously to determine its clearance. Changes of kinetic parameters (clearance,disappearance rate constant,half-life) obtained from antipyrine test were well correlated with the findings as follows:

(1)increase of plasma alkalinephosphatase and decrease of plasma albumin in PB-treated groups
(2)acceleration of PB metabolism in PB-treated groups
(3)increase of protein content, cytochrome P-450 content and activity of aminopyrine-N-demethylase in hepatic microsomal fraction in PB-treated groups
(4)hypertrophy of hepatocyte and proliferation of smooth surfaced endoplasmic reticulum in PB-treated group in electron microscopy.

B27 REPRODUCTION (SEG.II) STUDY OF SCUTELLARIA RADIX IN RAT

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Reproduction (Seg. II) studies were carried out in SPF Sprague-Dawley female rats by p.o. dosing of water extract concentrates of Scutellariae Radix at 0.25 (group I), 12.49 (group II) and 24.98 (group III) g/kg/day from day 7 to day 17 of gestation. Two-thirds of females in each group were sacrificed on day 20 of gestation and their fetuses were examined. Remaining dams were allowed to litter naturally, and postnatal development of the offspring was observed. Incidence of lumbar rib increased significantly with dose-response at 0.25 (15.03%), 12.49 (21.68%) and 24.98 (49.50%) g/kg/day. One case of brain extrusion occurred in group II, and lack of tail in group I. Incidence of abnormal urinary system (dilatation of ureter mainly) increased with dose-response, but the rate in group III was comparable to that in group II. There was no significant difference between control and treated groups in maternal body weight, intake of diet and water, efficiency of diet, hematology, resorption and dead fetus, corpus luteum, eyelid seperation, emergence of abdominal hair and incisor, traction test values, sex organ function and growth in fetuses.

B28 EFFECT OF DRUG ON THE GLUTATHIONE DEPLETING RAT HEPATOCYTES COCULTURED WITH MESENCHYMAL CELL

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The activity of the hepatic microsomal cytochrome P-450 linked drug metabolising system frequently parallels the toxicity, and the hepatic glutathione(GSH) plays in detoxication mechanism for xenobiotic toxicity. However, rapid loss of P-450 and high levels of GSH are observed in primary hepatocytes culture. An in vitro test system using hepatocytes cocultured with mesenchymal cell(3T3 fibroblasts) has developed for maintenance of P-450. And the cytotoxicity of acetaminophen(APAP) was investigated using the coculture system with GSH depleting hepatocytes from rat treated with phorone. The hepatocytes isolated by the collagenase perfusion from rat treated with phorone(250mg/kg) were cocultured with 3T3 and incubated in medium containing 1, 4 and 16mM of APAP for 18hrs at 37℃ under 95%air:5%CO_2. There was no effect of APAP in hepatocytetes alone culture, however the cytotoxicity was observed in hepatocytetes with 3T3 and depletion of GSH enhanced cytotoxicity.

B29 EFFECTS OF POISONOUS MUSHROOM EXTRACTS AND α-AMANITIN ON ISOLATED RAT HEPATOCYTES

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The effects of poisonous mushrooms, *Amanita abrupta*, *A. virosa*, *A. volvata*, *A. gymnopus*, *A. flavipes*, and *Morchella esculenta* were studied. The extract was prepared by boiling for 15 min. with 3 vol. water and by filtration after cooling. The cell viability was not affected by any extract in the present experimental condition but the glutathione content was reduced significantly by the extracts of *A. abrupta*, *A. virosa*, and *A. gymnopus*. The cytosolic Ca^{2+} concentration in the cell was reduced by the extracts of *A. abrupta* and *A. virosa* which were highly hepatotoxic. The fact that the reduction of cytosolic Ca^{2+} and the uptake of ^{45}Ca were dose-dependent suggested the increase of Ca release by α-amanitin. α-Amanitin decreased the incorporation of inositol to phosphatidylinositol and that of choline to phosphatidylcholine. These results suggested that the isolated hepatocytes are useful for the test of poisonous mushrooms and that α-amanitin influences the intracellular transmitters.

B30 EFFECT OF AFLATOXIN B_1 ON ISOLATED RAT HEPATOCYTES

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Effect of aflatoxin B_1 (AFB_1) was investigated on cytosolic Ca^{2+} level, ^{45}Ca uptake, and phospholipid turnover by using the hepatocytes freshly isolated from male Wistar rat. Cell viability, GSH content, and cytosolic Ca^{2+} level were determined by using the release of lactic dehydrogenase, DTNB method, and fura2/AM. ^{45}Ca uptake and ^{3}H-inositol and ^{14}C-choline into phospholipid were determined by following general methods. AFB_1 did not affect on cell viability during 4 h incubation and slightly decreased at 5h. GSH content was also slightly decreased time-dependently by 10 μM of AFB_1. On the other hand, AFB_1 strongly decreased cytosolic Ca^{2+} level to 37% of control 5 h after incubation. ^{45}Ca uptake was stimulated by 1 and 10 μM of AFB_1 to 1.2- and 1.4-fold of control, respectively. The incorporation of ^{3}H-inositol and ^{14}C-choline were significantly inhibited at 1 and 5 h after incubation with AFB_1. From these results which AFB_1 decreased the cytosolic Ca^{2+} level and phospholipid turnover, it was suggested that AFB_1 affected on the release and consumption of intracellular Ca^{2+}. ^{45}Ca uptake, however, was stimulated by AFB_1.

These results indicate that AFB_1 influences the intracellular transmitter, Ca^{2+}, and that the mechanism of action of AFB_1 is probably responsible for the intracellular Ca^{2+} movement.

B31 DETECTION OF HEPATOCARCINOGENIC POTENTIAL OF 5 CHEMICALS BY A MEDIUM-TERM BIOASSAY SYSTEM

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We developed a medium-term (8 weeks) bioassay system using male F344 rats for the detection of carcinogenicity of test chemicals. Rats were initially given a single dose (200 mg/kg) of diethylnitrosamine (DEN) i.p. and starting 2 weeks later were treated with test compounds for 6 weeks and then sacrificed, all rats being subjected to two-third partial hepatectomy at week 3. Glutathione S-transferase P form positive foci were quantitatively evaluated using an image analyzer with respect to difference from DEN alone group. HC-Blue No. 1 (hair dye) was positive, but HC-Blue No. 2 (hair dye), Ginseng (extract from Korean Red Ginseng), curcumin and dihydroguaiaretic acid (DHGA) (natural anti-oxidant) gave negative results. The results obtained by this system showed a clear correlation with known carcinogenicity tests that HC-Blue No. 1 is carcinogenic and all others are not. Therefore this system is practically applicable for the detection of environmental hepatocarcinogen.

B32 EFFECTS OF PREDNISOLONE ON HEPATOCYTIC MITOCHONDRIA IN RATS

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The effects of prednisolone treatment on hepatocytic mitochondria was investigated in rats. Prednisolone was subcutaneously administered to 6 week-old Sprague-Dawley male rats at the dose level of 15 mg/kg for 14 days.

Morphometric analysis revealed that individual mitochondrial size was increased and the number of mitochondria per cell was decreased significantly. Whereas, total mitochondrial area per cell was unchanged. On electron microscopy, mitochondrial matrix was more opaque and crista was more developed than that of the control. These changes were observed in the same degree in both peripheral and central zones of the hepatic lobules.

Mitochondrial protein per gram liver was less than one half of that of the control. A slight increase in the content of cytochrome b and slight decrease in cytochrome c per milligram protein were observed.

On the basis of these results, it was suggested that prednisolone induced not only an increase of mitochondrial size but also changes in it's function.

B33 *OUABAIN INHIBITS MUSCLE TWITCHES IN PRESENCE OF VERATRIDINE*

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Muscle twitches were evoked in mouse diaphragms in the presence of d-tubocurarine. Effects of ouabain (OUA) were examined on twitches, action potentials and O_2 consumption in the presence and absence of veratridine (VRT). OUA reduced twitches only in the presence of VRT, it did not affect the twitch amplitude. The interactions between OUA and VRT depended on the presence of $[K^+]_o$. Removal of $[Ca^{2+}]_o$ accelerated the potentiating effect of VRT and the antagonizing effect of OUA. Caffeine further potentiated the twitches which had been attenuated by OUA in the presence of VRT. OUA combined with VRT consistently decreased resting potentials, amplitude of action potentials and of overshoot potentials thus prolonged time to peak and duration of the action potentials. Twitch potentiation by tetraethylammonium, 4-aminopyridine, and caffeine was insensitive to OUA or removal of $[K^+]_o$. OUA also reduced O_2 consumption. The results suggest that twitches in the presence of VRT link with activation of Na^+-K^+-ATPase; accumulation of Na^+ inside the muscle fibres may impair the excitation-contraction coupling in this case. This uncoupling may not include the caffeine-sensitive process.

B34 THE EFFECT OF ASCORBIC ACID ON CISPLATIN-INDUCED CYTOTOXICITY IN CULTURED RENAL EPITHELIAL (NRK-52E) CELLS

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Cisplatin (CDDP) is effective in the treatment of many types of cancers. The most important adverse effect of this drug is nephrotoxicity. The toxic effect of CDDP is attenuated by treatment of rats with antioxidants. In this study, we investigated the effect of CDDP on cell growth, enzyme release to the medium, and electron micrographs in rat renal epithelial cell line, NRK-52E. We also examined if ascorbic acid affected these cytotoxic indices caused by CDDP. The treatment of NRK-52E cells with CDDP (1×10^{-6} M) inhibited cell growth and increased the release of LDH. Mitochondrial swelling was induced by CDDP. Ascorbic acid ameliorated the increase in the enzyme release and the structural changes in mitochondria caused by CDDP without affecting the decrease in cell growth by the anticancer agent. These results suggest that the cultured cell line, NRK-52E, is useful for investigating CDDP-induced nephrotoxicity. The nature of protective effet of ascorbic acid on CDDP-induced cytotoxicity remained unsolved in this study.

B35 CEPHALORIDINE NEPHROTOXICITY: CHANGES IN GLUTATHIONE CONTENTS AND ENZYME ACTIVITIES SCAVENGING OXYGEN RADICALS. Yoshihiro SUZUKI and Jun-ichi SUDO (Dept. of Toxicol. and Clin. Phamacol., Fac. of Pharm. Sci., Higashi-Nippon-Gakuen Univ., Ishikari-Tobetsu, Hokkaido, 061-02)

To elucidate protective mechanisms responsible for cephaloridine nephrotoxicity, changes in renal formation of malonaldehyde, contents of GSH and GSSG, and activities of superoxide dismutase, catalase and GSH peroxidase, were investigated in kidney of rats that received single i.v. injections of cephaloridine in doses of 100 and 1,000 mg/kg body weight. In the 100 mg/kg group, the above items determined remained within the control level. In the 1,000 mg/kg group, renal formation of malonaldehyde rose from the 2nd hour to the 2nd day, and more highly from the 3rd to the 7th day. GSH content fell from the 1st to the 2nd hour. The above three enzyme acitvities were deminished later than the 12th hour. These results suggested that the increment in malonaldehyde formation in the early stage might be explained by the falls of renal contents of endogenous antioxidants including GSH etc., and that those in the late stage related enzymatically to the declines in the activities of superoxide dismutase, catalase and GSH peroxidase.

B36 STUDIES ON THE MECHANISM OF NEPHROTOXICITY OF AMINOGLYCOSIDE ANTIBIOTIC V THE ANTIBIOTIC-INDUCED ABNORMALITY OF LIPID METABOLISM IN CULTURED RENAL EPITHELIAL CELLS
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We have previously reported that aminoglycoside antibiotics cause an appearance of myeloid bodies and increase content of phospholipids, especially phosphatidylinositol in cultured LLC-PK$_1$ cells of pig kidney proximal cell line. Furthermore, an addition of aminoglycoside antibiotics into the cell homogenate caused a dose-dependent inhibition of phosphatidylinositol-specific phospholipase C activity. This study was performed to investigate on the relation between the intracellular accumulation of gentamicin(GM) and the change of lipid metabolism in cultured LLC-PK$_1$ cells. GM was accumulated dose-dependently in LLC-PK$_1$ cells during cultivation after an addition of 1 and 5 mM GM into the culture medium. The distribution content of GM was 2 times more in the brush border membrane(BBM) fraction and 5-10 times more in the lysosomal fraction than that in the whole cell homogenate fraction. Total phospholipid content increased in the lysosomal fraction only at the concentration of 1mM GM but did dose-dependently in the BBM fraction at both concentrations of 1 and 5mM GM. GM also inhibited neutral phospholipase A activity in the cell homogenate fraction and neutral phospholipase C activity in the cell homogenate and BBM fraction but activated both activities of acidic phospholipase A and C in the lysosomal fraction. These results suggest that the specific accumulation of GM into brush borders and lysosomes induces the change of phospholipase A and C activity followed by the abnormal change of lipid metabolism in the brush borders and lysosomes in cultured LLC-PK$_1$ cells treated with GM.

B37 EFFECT OF OCHRATOXIN A ON INTRACELLULAR ATP TURNOVER IN ISOLATED NEPHRON SEGMENTS

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In order to verify the nephrotoxic site, potency and mechanism of ochratoxin A (OCTA), we measured cellular ATP contents in nine nephron segments incubated with or without OCTA in vitro. Intrcellular ATP contents of nephron segments isolated under stereomicroscopic observation after treatment of renal slices with 0.1% collagenase were measured by the microchemiluminescence method. OCTA decreased cellular ATP contents in a dose-dependent manner. Dose-response study of OCTA showed that minimal concentration of OCTA to decrease ATP significantly was at 10^{-8}M in the second portion of proximal tubule (S_2) ($p<0.05$), and $5x10^{-4}$M in medullary collecting tubule (MCT) ($p<0.01$). Among nine nephron segments, OCTA at $5x10^{-5}$M significantly decreased cellular ATP contents only in S_2 and S_3 ($p<0.01$). ATP synthesis in isolated mitochondria from renal cortex was significantly inhibited by 10^{-6}M OCTA ($p<0.05$). Probenecid ($4x10^{-4}$M) and diltiazem (10^{-7}-10^{-5}M) protected ATP decrease by 10^{-4}M OCTA. These results suggest that OCTA might enter the plasma membrane in S_2 andS_3 through the organic anion transport pathway, and inhibit the mitochondrial oxidative phosphrylation. This newly established method would be applicable for evaluation of intrarenal toxic site and potency by various chemical compounds.

B38 COMPARISON OF TOXICITY OF p-DCB IN MALE AND FEMALE RATS

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We reported previously that differences were observed between male and female rats in the biochemical and histopathological abnormalities and the organ distribution of p-DCB. Recently it is suggested that major urinary protein of male rat is related to the mechanism of action of p-DCB.

In the present study, immature male and female rats and also castrated and ovariectomized rats were given intragastric administration of p-DCB (300 mg/kg) twice at 12 hours intervals to compare the toxicity. Moreover the urine collected from p-DCB treated rats (500 mg/kg) was analyzed to measure the levels of the urinary protein. As a result, there was no difference in organ distribution of p-DCB between male rats and castrated rats. The p-DCB concentrations in the kidney and fatty tissues of ovariectomized rats were significantly higher than those of female rats. Histologically, the appearance of eosinophilic body was noteworthy in the kidney of both treated male rats and castrated rats. No droplets were observed in female rats , and in immature and castrated control rats. Low molecular protein was detected in the urine of treated and control male rats.

B39 THE QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS APPROACH TO PRIMARY EYE IRRITATION IN RABBITS

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A quantitative structure-activity (QSA) model was developed to assess a relation of structural features of organic compounds and primary eye irritation in rabbits. The test compounds were 70 including salicylic acid derivatives (11), organophosphorus (5), purines (6), alcohols (6), amino acids (5),benzene derivatives (8), and others (29). The intensity of eye irritation was classified on the basis of the recovery time of corneal and conjunctival damages. Thirty five descriptors were used to describe the molecules. To correlate eye irritation ratings with the descriptors, a QSA model was developed by the adaptive least-squares method.

In this study, a three-class discrimination was made as follows; class I included 16 compounds which induced the damages recovered within 24 hr; class II included 34 compounds which induced the damages persisted for more than 24 hr but recovered within 21 days; class III included 20 compounds which induced the damages not recovered within 21 days. The discriminant function included 12 descriptors. The accuracy in classifying the compounds was 90% in the recognition and 76% in the leave-one-out prediction.

These results suggest that structure-activity relationships analysis can contribute to predict the primary eye irritation of untested compounds.

AUTHOR INDEX

Abbreviations;
Ann; Free communication in Hall A
Bnn; Free communication in Hall B
CH; Chairperson
PLn; Plenary lectures
Snn; Symposium