

ADVANCES IN PHARMACOLOGY AND THERAPEUTICS

Proceedings of the 7th International Congress of Pharmacology, Paris 1978

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Volume 9
TOXICOLOGY

Editor:

Y. COHEN
Châtenay-Malabry



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Introduction

The scientific contributions at the 7th International Congress of Pharmacology were of considerable merit. Apart from the sessions organised in advance, more than 2,200 papers were presented, either verbally or in the form of posters, and the abundance of the latter in the congress hall is a good indication that this particular medium of communication is becoming increasingly attractive to research workers, and offers scope for discussions which combine an elaborate, thorough approach with a certain informality.

It would have been preferable to have published the entire congress proceedings within the framework of the reports. That was, however, physically impossible, and the organisers had to adopt a realistic solution by publishing only the main lectures, symposia and methodological seminars. The amount of material presented necessitated the printing of ten volumes, each volume containing congress topics regrouped according to their relevant content and subject areas. This system of division may give rise to criticism on account of its artificiality, and we readily admit that certain texts could have been placed in more than one volume. We are asking the reader to excuse this arbitrariness, which is due to the editors' personal points of view.

I draw attention to the fact that most of the symposia finish with a commentary which the chairmen had the option of including, presenting their personal opinions on one or several points. We think that such an addition will facilitate reflection, discussion, indeed even controversy.

The launching of the scientific programme for this congress began in September 1975 on returning from the last meeting in Helsinki. Long and delicate discussions took place in the Scientific Programme Committee and with the International Advisory Board. Should it be a pioneer, 'avant-garde' congress? Or one laid out like a balance-sheet? Should we restrict the congress to the traditional bounds of pharmacology, or extend the range of papers to cover the finest discipline? The choice was difficult, and the result has been a blend of the two, which each participant will have appreciated in terms of his training, his tastes, and his own research.

A certain number of options, however, were taken deliberately: wide scope was given to toxicology, from different points of view, and to clinical pharmacology, a subject much discussed yet so badly practised; the founding of two symposia devoted

to chemotherapy of parasitic diseases which are still plagues and scourges in certain parts of the world; a modest but firm overture in the field of immunopharmacology, which up until now was something of a poor relation reserved only for clinical physicians; the extension of methodological seminars, in view of the fact that new techniques are indispensable to the development of a discipline.

We have been aware since the beginning that, out of over 4,000 participants who made the journey to Paris, not one could assimilate such a huge body of knowledge. Our wish is that the reading of these reports will allow all of them to become aware of the fantastic evolution of pharmacology in the course of these latter years. If one considers pharmacology as the study of the interactions between a "substance" and a living organism, then there is no other interpretation. Nevertheless, one must admit that there exists a period for describing and analysing a pharmacological effect, and that it is only afterwards that the working mechanism can be specified; a mechanism which will permit these "substances" to be used for the dismantling and breaking down of physiological mechanisms, a process which justifies Claude BERNARD'S term, "chemical scalpel".

The reader will be able to profit equally from more down-to-earth contributions, more applied to therapeutics, and less "noble", perhaps, for the research worker. He will realise then that his work, his research and his creative genius are first and foremost in the service of Man, and will remember this statement from Louis PASTEUR:

"Let us not share the opinion of these narrow minds who scorn everything in science which does not have an immediate application, but let us not neglect the practical consequences of discovery."

I would like to renew my thanks to my colleagues in the Scientific Programme Committee and also to the members of the International Advisory Board, whose advice has been invaluable. I owe a particular thought to J J BURNS, now the past-president of IUPHAR, who granted me a support which is never discussed, and a staunch, sincere friendship. The Chairmen have effected an admirable achievement in the organisation of their proceedings, and in making a difficult choice from the most qualified speakers. The latter equally deserve our gratitude for having presented papers of such high quality, and for having submitted their manuscripts in good time.

The publisher, Robert MAXWELL, has, as always, put his kindness and efficiency at our service in order to carry out the publication of these reports. But none of it would have been possible without the work and competence of Miss IVIMY, whom I would like to thank personally.

My thanks again to the editors of the volumes who, in the middle of the holiday period, did not hesitate to work on the manuscripts in order to keep to the completion date.

Finally, a big thank you to all my collaborators, research workers, technicians and secretaries who have put their whole hearts into the service of pharmacology. They have contributed to the realisation of our hopes for this 7th International Congress, the great festival of Pharmacology. Make an appointment for the next one, in 1981, in Tokyo.

Jacques R BOISSIER

Chairman

Scientific Programme Committee



Dr. Elizabeth C. Miller and Dr. James A. Miller

DEDICATION

Human beings are exposed to many naturally occurring and man-made lipid soluble chemicals in food and elsewhere in the environment. The metabolism of these compounds by oxidation and subsequent conjugation makes them more water soluble and facilitates their rapid elimination in urine and/or bile. Without metabolism, lipid soluble chemicals would remain in the body for many months or even years. Although metabolism of foreign compounds to biologically inactive water soluble metabolites by oxidative enzymes is an important detoxification mechanism, the same enzymes may also metabolize foreign chemicals to highly reactive intermediates that interact with cells to cause toxicity. A better understanding of factors that influence the metabolism of chemicals may lead to the development of compounds that selectively inhibit the formation of toxic metabolites or enhance detoxification pathways. In addition, it may be possible to administer safe chemicals that react with and inactivate toxic metabolites.

The pioneering research of Dr. James A. Miller and Dr. Elizabeth C. Miller has laid the foundation for the field of reactive metabolites. Because of their outstanding research during the past 30 years, we join the organizers of the Seventh International Pharmacology Congress in dedicating this symposium on reactive metabolites and their implications for toxicology to Dr. James A. Miller and to Dr. Elizabeth C. Miller. In 1947, the Millers discovered that administration of hepatocarcinogenic aminoazo dyes to rats resulted in the covalent binding of azo dye metabolites to macromolecules (protein) in the liver; little or no covalent binding occurred in non-target tissues that were refractory towards tumorigenesis by the azo dyes. The Millers found that factors that influenced the *in vivo* binding of azo dyes to liver macromolecules also influenced carcinogenesis, and they suggested that covalent binding of azo dye metabolites to liver macromolecules was required for the dyes' carcinogenicity. In 1951, Elizabeth Miller painted benzo[a]pyrene on mouse skin and reported covalent binding of benzo[a]pyrene metabolites to skin macromolecules, and she suggested that this binding was necessary for the carcinogenicity of polycyclic aromatic hydrocarbons. In 1960, the Millers demonstrated the metabolism of 2-acetylaminofluorene by N-hydroxylation to a metabolite that was more carcinogenic than the parent molecule, and they found that the N-hydroxylated metabolite was further metabolized to highly reactive esters with even greater toxicity. The Millers have elucidated the molecular events leading to the metabolic activation of 2-acetylaminofluorene, aminoazo dyes, aflatoxin B₁ and safrole to chemically reactive metabolites that react with macromolecules in cells. These studies have led to the important unifying concept by the Millers that many carcinogenic and mutagenic chemicals undergo metabolism to reactive electrophilic intermediates that exert toxic effects by covalently binding to critical sites on DNA, RNA and protein. It is now known that the toxicity of many drugs and environmental pollutants requires metabolism to reactive electrophilic intermediates that covalently bind to cellular macromolecules. The discovery by the Millers that many chemical carcinogens must undergo metabolism to highly reactive electrophilic intermediates prior to exerting their carcinogenic effect has laid the foundation for subsequent research indicating a good relationship between the mutagenicity of chemicals (after metabolic activation) and carcinogenicity. The Millers' sustained pioneering research on the metabolic activation of chemicals during the past 30 years has shaped the development of this field. The Millers more than any other individuals can be called the parents of the field of reactive metabolites and their implications for toxicology. It is because of their many outstanding contributions that we dedicate this symposium to Dr. James A. Miller and Dr. Elizabeth C. Miller.

A. H. Conney
J. R. Gillette
F. Oesch
July, 1978
Paris

Metabolic Activation of Chemicals to Reactive Electrophiles: An Overview*

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ABSTRACT

The pharmacologic and toxic properties of most organic compounds appear to result from noncovalent, and thus reversible, interactions with cellular molecules over a wide range of binding affinities. On the other hand, the toxic manifestations of most, if not all, chemical carcinogens, many mutagens, some allergens, and some drugs appear to arise from covalent interactions *in vivo*. The covalent interactions of many chemicals may thus pose a variety of carcinogenic, mutagenic, teratogenic, allergenic, and necrogenic hazards in their use or presence in humans and their environments. Most chemical carcinogens and most drugs that yield covalent interactions *in vivo* are unreactive *per se* and must be activated metabolically to form strong electrophilic reactants. These electrophiles combine covalently with numerous nucleophilic sites in cellular molecules, especially the nucleic acids and proteins. The metabolic conversion of organic compounds to electrophilic reactants can be detected and measured in several ways. The mutagenic activities of these reactive metabolites are the basis of the most sensitive tests currently available. Examples of the metabolism of chemical carcinogens, drugs, and other compounds to reactive electrophiles will be presented at this symposium.

INTRODUCTION

A very great variety of structures is found among the approximately four million known synthetic and naturally occurring organic compounds of low molecular weight (<1000). Not surprisingly these structures produce a very wide range of effects in living systems. A few serve as nutrients, some are essential to life, many are useful as drugs, and all of them are toxic in sufficient dosage. Although a great mass of information on the toxic and pharmacologic properties of organic compounds has accumulated, the development of knowledge on the mechanisms of action of these agents, especially at the molecular level, has been limited by the chemical, physical, and, especially, the metabolic complexities of living systems. The toxic and pharmacologic effects of organic compounds are generally the results of noncovalent, and thus reversible, interactions with cellular molecules. However, studies in the

*This lecture was also presented in part on the occasion of the 1978 Founders Award of the Chemical Industry Institute of Toxicology, Research Triangle Park, NC, U.S.A.

past few decades have shown that the toxic manifestations of chemical carcinogens, many mutagens, some allergens, and some drugs arise from covalent interactions of these compounds or their metabolites with critical cellular molecules. The possible occurrence of similar reactions in vivo with many other toxic substances is obvious.

Covalent interactions of foreign molecules with cellular constituents in living cells have come to imply that the reactive forms of these agents are most probably electrophilic in nature (1,2). That is, these reactive forms seek to combine with available electrons in nucleophiles. Water, the major component of cells, is a weak nucleophile. The major nucleophiles in cells are components of the informational macromolecules, the proteins and nucleic acids. Numerous weak to strong nucleophilic centers (at certain N, S, and O atoms) are present in these biopolymers, but they contain only weakly electrophilic centers, such as carbonyl groups and double bonds α , β to carbonyl groups. Covalent bond-making and bond-breaking occurs continuously in normal cellular metabolism. These events take place under tight and ordered control at the active centers of enzymes, so that the physiological electrophiles and nucleophiles formed and joined in these reactions do not enter into random reactions with each other elsewhere in the cellular milieu. Cells make use of several low molecular weight potential strong electrophiles in many enzyme-catalyzed reactions, e.g., acyl CoA's, *S*-adenosylmethionine, adenosine triphosphate, 3'-phosphoadenosine-5'-phosphosulfate. These cofactors react with their nucleophilic targets at the proper enzymatic sites. In contrast, foreign molecules that are electrophiles or are metabolized to electrophiles react covalently with cellular molecules in relatively nonspecific and generally non-enzymatic reactions in vivo. These principles first became evident in studies with chemical carcinogens.

CHEMICAL CARCINOGENS AND CARCINOGENESIS

Carcinogenesis results in seemingly irreversible changes in the phenotypes of normal cells in relation to the control of their growth. Carcinogens of any kind must therefore interact directly or indirectly with critical informational macromolecules that control cell replication. The metabolism of chemical carcinogens, especially to reactive forms, and the interactions of chemical carcinogens and their metabolites with macromolecules during tumor formation have provided valuable leads to the nature of the initiation of carcinogenesis, but the identification of the critical interactions and macromolecules has not yet been achieved in any instance. These general aspects of chemical carcinogenesis are outlined in Fig. 1.

It was first noted about 30 years ago that residues of two carcinogens (an aminoazo dye and a carcinogenic polycyclic aromatic hydrocarbon) bound tightly in vivo to protein in tissues that later developed tumors (1). These interactions were characterized as covalent by criteria which today are not regarded as adequate. Tight binding in vivo of these and many other carcinogens to both proteins and nucleic acids in tissues susceptible to tumor formation has since been observed in many laboratories (1). More definitive proof of the covalent natures of these bound forms became available as proximate and ultimate forms of the carcinogens were recognized. This information was first developed for the versatile carcinogen 2-acetylaminofluorene. Thus, in 1960 *N*-hydroxy-2-acetylaminofluorene was recognized as a nonreactive, proximate carcinogenic metabolite, and subsequent work showed that the major ultimate reactive metabolite of this carcinogen in the rat liver was the sulfuric acid ester of *N*-hydroxy-2-acetylaminofluorene (1). This ester is a highly electrophilic reactant in vitro toward proteins and nucleic acids. The major products formed in these reactions were characterized and shown to be identical with the covalent adducts formed in vivo when the parent or the proximate carcinogen is administered. This approach, i.e., the characterization of the proximate and ultimate reactive metabolites of chemical carcinogens and comparison of the structures of adducts formed in vitro from the ultimate reactive metabolites

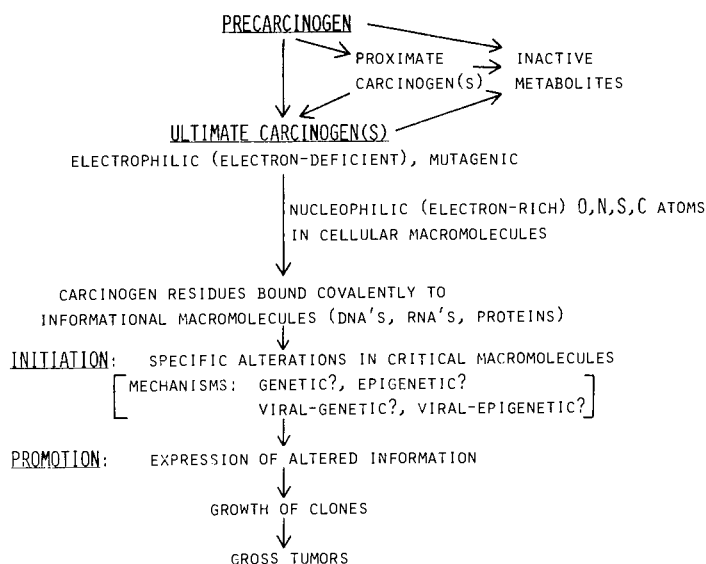


Fig. 1. The possible steps involved in carcinogenesis by chemicals.

with those derived *in vivo* from the parent carcinogens, has now been used in the characterization of the covalently bound forms of a variety of chemical carcinogens. In all cases studied to date the reactive forms of chemical carcinogens are electrophiles. Chemical carcinogens are structurally very diverse, and the majority of these agents require metabolism to generate these reactive electrophilic forms. Numerous nucleophilic sites for covalent binding of these reactive electrophiles to proteins and nucleic acids have been found both *in vivo* and *in vitro*. In addition to the multiple bound forms of some chemical carcinogens that occur *in vivo*, some chemical carcinogens may also be metabolized to more than one electrophilic form.

The early attempts to correlate the carcinogenicity and mutagenicity of chemicals did not take into account the metabolism of chemical carcinogens to reactive mutagenic forms *in vivo*. By 1971 (3) it was evident that a relatively close association between these two biological activities existed for the reactive electrophilic forms of chemical carcinogens. With the use of sensitive strains of microorganisms and fortified liver microsomes for the metabolism of test chemicals to electrophiles, 90-95% of chemical carcinogens are mutagenic and most of their noncarcinogenic analogs have little or no mutagenicity (4). Tests based on these observations appear promising for the detection of potential carcinogenicity among the many man-made and naturally occurring chemicals in the environment.

Appreciation of the complexity of carcinogenic processes is essential to an understanding of studies on the critical targets and the molecular mechanisms of chemical carcinogenesis. While the electrophilic forms of chemical carcinogens appear to be involved in the initiation of carcinogenesis, it is likely, but less certain, that electrophilic metabolites may also participate in the promotion phase; the same or different metabolites of a complete carcinogen may be involved in these two stages. Some chemicals appear to possess essentially only initiating activity for some tissues. Similarly, some substances possess only promoting activity and have little, if any, initiating activity.

The initiation phase of chemical carcinogenesis is now commonly regarded as involving mutations in DNA. This mechanism is consistent with the rapidity with which the initiation step is achieved and its persistence. While covalent interactions of the promoter or a metabolite thereof with cellular constituents may be involved in promotion, there are currently few data to explain the pleiotropic responses elicited by phorbol myristate acetate in mouse skin or cell cultures or on the actual mechanisms involved in promotion (5). Similarly, there are no unequivocal data with regard to the role of carcinogenic viral information in chemical carcinogenesis. At present both genetic and epigenetic mechanisms, with and without the participation of viral information, must be considered in studies on the molecular mechanisms of the initiation and promotion phases of chemical carcinogenesis.

We have earlier referred to the possibility that tight non-covalent binding by some compounds might also initiate carcinogenesis. Preliminary data (6) suggest that adriamycin may be carcinogenic and mutagenic in this manner. However, this compound also forms free radicals, and these electrophilic species may in turn induce free radicals in tissue macromolecules in a hit-and-run mechanism that does not leave bound residues of the carcinogen.

CHEMICAL MUTAGENS

Most of the known chemical mutagens are, like the chemical carcinogens, either electrophilic per se or are metabolized to electrophiles to exhibit mutagenic activity (3). These electrophilic agents thus act as mutagens through covalent binding to DNA. However, the members of the numerically small groups of base analog mutagens and non-binding frameshift mutagens are not electrophiles and do not act as mutagens through covalent binding to DNA. The base analog mutagens are incorporated into DNA, and the non-binding frameshift mutagens appear to interact non-covalently with DNA in an intercalative manner.

CHEMICAL ALLERGENS AND DRUGS

Evidence exists that some small molecules can provoke allergic reactions through covalent binding to tissue proteins (7). Antibodies to these hapten-protein complexes appear responsible for the allergic responses. Many chemical allergens in their reactive forms are acylating, diazonium coupling, or arylating agents. These electrophiles appear to react primarily with amino groups in proteins to form the hapten-protein complexes, but other sites of binding have not been excluded. Thus, the allergenic factors responsible for penicillin allergies in both man and animals appear to be penicilloyl conjugates with proteins. However, covalent binding of compounds to macromolecules in vivo does not always lead to the formation of antibodies and the generation of allergic responses (8). Some drugs (acetaminophen, furosemide, isoniazide, etc.) are metabolized in vivo to reactive electrophiles that bind covalently to tissue proteins (8). Necrogenic responses appear to be the immediate effects of these reactions. In some cases the degree of protein-binding and the toxicity depend on the level of reduced glutathione, which is a protective nucleophile. The structures of some of the covalent bound forms of these drugs with tissue proteins have been partially determined. Whether or not nucleic acids react in vivo with the reactive electrophilic metabolites of these drugs has not yet been determined. The possible mutagenicity and carcinogenicity of these drugs at high dosages also require more attention.

RECENT DATA ON SOME CHEMICAL CARCINOGENS

The broad outlines of the metabolism of several structurally dissimilar chemical

carcinogens to reactive, mutagenic, and carcinogenic electrophiles have been established, but much more detail about the interactions of chemical carcinogens with tissue macromolecules in relation to carcinogenesis remains to be determined.

Aromatic Amines, Amides, and Nitro Compounds

In all cases the carcinogenicities of aromatic amines, amides, and nitro compounds now appear to depend on their conversion to N-hydroxy derivatives in vivo (9). The ultimate carcinogenic metabolites have not been elucidated in most cases, and the activation reactions may differ with the aryl substituents, tissues, and species. The most studied carcinogen of this group is 2-acetylaminofluorene (1,2,10,11), which is N-hydroxylated by a NADPH- and cytochrome P-450-dependent mixed function oxidase(s) in rat liver. Rats that develop high incidences of liver tumors possess more hepatic 3'-phosphoadenosine-5'-phosphosulfate-dependent sulfotransferase that sulfonates N-hydroxy-2-acetylaminofluorene to N-sulfonyl-2-acetylaminofluorene than do resistant animals. This potent mutagenic electrophile appears to be the major ultimate carcinogenic metabolite for the rat liver. However, conversion of N-hydroxy-2-acetylaminofluorene to electrophilic reactants also occurs by at least 3 other metabolic reactions. Presumably, one or more of these electrophilic products is involved in the initiation of carcinogenesis by N-hydroxy-2-acetylaminofluorene at non-hepatic sites that lack sulfotransferase activity. The hepatocarcinogen N-methyl-4-aminoazobenzene is activated similarly by N-hydroxylation of the amine and sulfonation of the N-hydroxy derivative. However, in this case the N-hydroxylation is catalyzed by a flavoprotein that does not require cytochrome P-450 and the sulfonation appears to depend on a different sulfotransferase from that which uses N-hydroxy-2-acetylaminofluorene. 4-Nitroquinoline-1-oxide is reduced to the corresponding hydroxylamine by liver and other tissues, and the activation of the latter compound can be accomplished by the seryl-tRNA transferase-dependent formation of the seryl ester (12).

Recent studies (see 13-15) have provided evidence that the nitrenium ions formed on protonation of N-hydroxy-2-naphthylamine and N-hydroxy-4-aminobiphenyl may be ultimate carcinogens for the induction of urinary bladder tumors in the dog and human (Fig. 2). These hydroxylamines and N-hydroxy-1-naphthylamine are N-glucuronidated by a glucuronyl transferase system(s) in the hepatic endoplasmic reticulum and the glucuronides are apparently carried via the blood to the kidney for excretion. Many dog and human urines are sufficiently acidic to hydrolyze the N-glucuronides and to protonate the resulting hydroxylamines for reaction with DNA. These findings are consistent with the quantitative correlation between the carcinogenicities of 1- and 2-naphthylamine and 4-aminobiphenyl in the dog bladder and the urinary excretions of the corresponding N-hydroxy amines. Furthermore, bladder carcinomas were induced in dogs by the instillation of N-hydroxy-2-naphthylamine, but not 2-naphthylamine, into the bladders of dogs. The very weak carcinogenic activity of 1-naphthylamine for the urinary bladder is apparently a consequence of its very low degree of N-hydroxylation in vivo. N-Hydroxy-1-naphthylamine is much more carcinogenic than N-hydroxy-2-naphthylamine or N-hydroxy-4-aminobiphenyl at sites of injection in rodents. The latter finding is consistent with the much greater reactivity of N-hydroxy-1-naphthylamine with DNA. The reaction of N-hydroxy-1-naphthylamine with DNA at pH 5 results in the substitution of the ⁶O atom of the guanyl residues with either the nitrogen or C-2 of the hydroxylamine. This substitution would be expected to result in miscoding of the DNA.

Aflatoxin B₁

Aflatoxin B₁ is the most potent known hepatocarcinogen for the rat and probably plays a role in the etiology of some human hepatic cancers in Africa and the Far

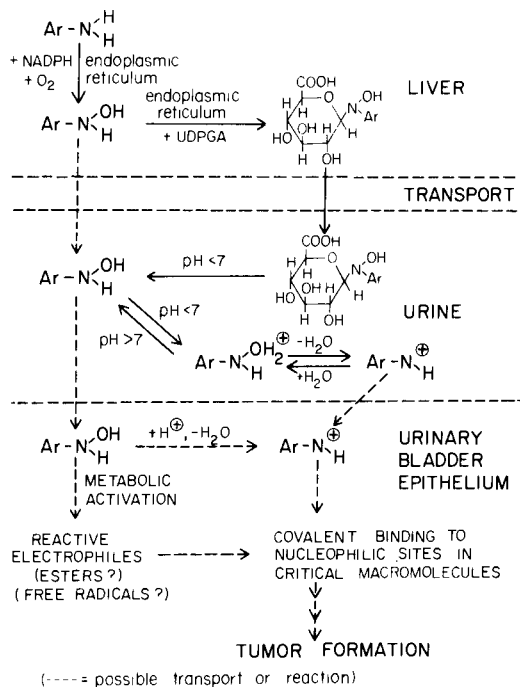


Fig. 2. The formation and transport of metabolites of arylamines for the induction of urinary bladder cancer in the dog and human.

East. The toxicity, carcinogenicity, and mutagenicity of aflatoxin B_1 appear to depend on its 2,3-double bond, and an NADPH-dependent mixed function oxidase system is required for the conversion of this carcinogen to a toxic, mutagenic, and electrophilic derivative (Fig. 3).

Administration of aflatoxin B_1 to rats yields covalent adducts of the carcinogen with hepatic nucleic acids and proteins and similar adducts are formed on incubation of aflatoxin B_1 with NADPH-fortified hepatic microsomes from humans, rats, or other rodents. 2,3-Dihydro-2-(guan-7-yl)-3-hydroxy aflatoxin B_1 is the major acid degradation product of both the *in vivo*- and *in vitro*-formed DNA and rRNA-aflatoxin B_1 adducts (16,17). During the extraction of the nucleic acids with phenol the imidazole ring of the guanine residue of this adduct is susceptible to hydrolysis, and some of the guan-7-yl derivative is isolated as 2,3-dihydro-2-(N⁵-formyl-2,5,6-triamino-4-oxypyrimidin-N⁵-yl)-3-hydroxy aflatoxin B_1 . The latter derivative is very labile to weak acid and is apparently the precursor of the major share of the dihydrodiol obtained in hydrolysates of the nucleic acid adducts. No data are yet available to specifically associate the above adduct with the initiation of carcinogenesis. Attempts to synthesize aflatoxin B_1 -2,3-oxide for study have so far not been successful. However, the model compound aflatoxin B_1 -2,3-dichloride (Fig. 3), showed strong carcinogenicity and is a very potent mutagen without metabolic activation (18).

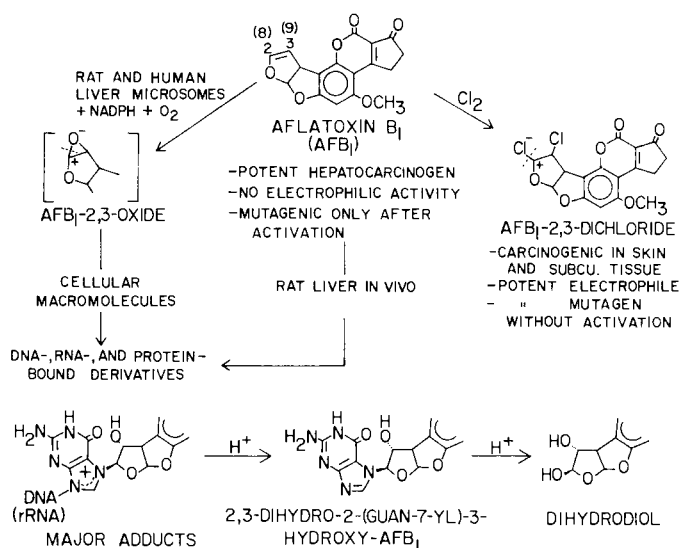


Fig. 3. Activation of aflatoxin B₁ by liver endoplasmic reticulum and the products formed on acid hydrolysis of the nucleic acid adducts.

Safrole and Related Allylic Benzenes

Numerous allylic benzene derivatives occur in the essential oils from a wide variety of plants. The most studied is safrole (1-allyl-3,4-methylenedioxybenzene) (19), which is a weak hepatocarcinogen for rats and mice. Estragole (1-allyl-4-methoxybenzene) has a similar level of hepatocarcinogenicity to that of safrole on administration to preweanling mice (see 13).

Safrole and estragole are 1'-hydroxylated by rat and mouse liver both *in vivo* and *in vitro*, and both 1'-hydroxy derivatives are stronger hepatocarcinogens than the parent compounds (see 9). Esterification of the 1'-hydroxy derivatives with acetic anhydride yields in each case an electrophilic ester, and 1'-acetoxysafrole is more carcinogenic than safrole or 1'-hydroxysafrole at sites of application. There is no evidence for the metabolic formation of these acetic acid esters, but rat and mouse liver cytosols, when fortified with 3'-phosphoadenosine 5'-phosphosulfate, synthesize small amounts of the strong electrophile 1'-sulfonoxysafrole. The release of small amounts of 3'-methylmercaptoisosafole from the liver protein of rats and mice given [2',3'-³H]1'-hydroxysafrole provided evidence for the formation of a reactive ester of 1'-hydroxysafrole *in vivo*; reaction of 1'-acetoxysafrole with methionine yields a sulfonium derivative that decomposes to 3'-methylmercaptoisosafole.

Metabolic 2',3'-epoxidation of safrole and estragole and of their 1'-hydroxy metabolites occurs in rat liver cells or with NADPH-fortified rat or mouse liver microsomes (20,21). NADPH-fortified mouse liver microsomes also 1'-hydroxylate the 2',3'-epoxides. Kinetic data indicate that the major formation of the 1'-hydroxy-2',3'-epoxy derivatives occurs via hydroxylation followed by epoxidation.

The 2',3'-epoxides of safrole and estragole and their 1'-hydroxy derivatives, as

well as the 2',3'-epoxide of eugenol (1-allyl-4-hydroxy-3-methoxybenzene), are quite mutagenic for *Salmonella typhimurium* TA100 and TA1535 and are electrophilic toward guanosine (21,22). 1'-Acetoxysafrole and 1'-acetoxystrogoles also show mutagenic activity for *S. typhimurium* TA100 and TA1535 and are electrophilic. One of the products of the reaction of 1'-acetoxysafrole with guanylic acid is 6-(isosafrol-3'-yl)guanylic acid; the other products of the latter reaction and the products of the reactions of the epoxides with nucleic acids have not been identified. The parent compounds show very little or no mutagenic activity either with or without the addition of NADPH-fortified hepatic microsomes. The 1'-hydroxy metabolites are not directly mutagenic, but they have some mutagenicity (much less than that of the epoxides) on addition of NADPH-fortified microsomes. Of the epoxides that have been tested only 1'-hydroxysafrole-2',3'-oxide has shown carcinogenic activity (initiation of papillomas of the skin in mice) (23).

Ethyl Carbamate

Ethyl carbamate, $\text{CH}_3\text{CH}_2\text{-O-CO-NH}_2$, is a synthetic carcinogen of weak to moderate activity and induces tumors in a wide variety of tissues of the rat and mouse (24). Early studies showed that ethyl carbamate was relatively unique, since carbamates with other alkyl groups or with substitution of the nitrogen with methyl groups showed no more than 1% of the activity of ethyl carbamate in the induction of lung adenomas and in the initiation of skin tumors in mice. A metabolite, ethyl N-hydroxycarbamate, has carcinogenic activity nearly as great as that of the parent compound, but it apparently is not a proximate carcinogenic metabolite of ethyl carbamate (24).

We recently showed that vinyl carbamate, $\text{CH}_2=\text{CH-O-CO-NH}_2$, is 10-50 times more active than ethyl carbamate in the induction of lung adenomas and in the initiation of skin tumors in mice (25). Furthermore, vinyl carbamate is mutagenic for *S. typhimurium* TA1535 and TA100 in assays that contain NADPH-fortified hepatic microsomes. Vinyl carbamate is not mutagenic in the absence of the fortified microsomes, and ethyl carbamate is not active either with or without the addition of microsomes.

^3H and ^{14}C from ethyl carbamate containing these isotopes in the ethyl moiety become bound to the liver nucleic acids of mice (26,27). ^{14}C from [carbonyl- ^{14}C]ethyl carbamate and ^{18}O from [ethoxy- ^{18}O]ethyl carbamate were not incorporated into the hepatic nucleic acids at detectable levels. Ethylation of the phosphate backbone was suggested to account for all of the incorporation of ^{14}C from [ethyl- ^{14}C]ethyl carbamate into mouse liver DNA (28). This report is inconsistent with our finding of a much lower level of binding of ^3H than of ^{14}C to the hepatic DNA of mice after administration of [ethyl-1- ^{14}C ;1,2- ^3H]ethyl carbamate. The latter observation is consistent with the formation of vinyl carbamate as a proximate carcinogenic metabolite of ethyl carbamate, but attempts to detect metabolically formed vinyl carbamate have not succeeded. These metabolic data and the qualitatively similar, but much stronger, carcinogenic activity of vinyl carbamate as compared to that of ethyl carbamate suggest that these two carbamates may form similar or identical electrophilic reactants that initiate the carcinogenic process.

CONCLUSIONS

Great progress has been made in the past decade in the elucidation of the electrophilic natures of ultimate carcinogenic metabolites, in the establishment of their abilities to induce mutations, and in the characterization of some of the products formed on interaction with cellular macromolecules. Much more difficult challenges lie ahead in the elucidation of the molecular mechanisms by which the alterations of macromolecules initiate and promote carcinogenesis.

ACKNOWLEDGEMENTS

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DNA Binding and Polycyclic Hydrocarbon Carcinogenesis

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INTRODUCTION

The covalent reactions of chemical carcinogens with nucleic acids are primarily of interest because of strong and continuing suspicions that the modification of genetic material in somatic cells may be involved, perhaps indirectly, in the initiation of malignancy. The earliest report of an in vivo reaction of a polycyclic hydrocarbon with a tissue constituent concerned protein (Miller, 1951), but this observation was then extended by others showing that covalent reactions with nucleic acids also occurred in tissues treated with radioactively-labelled hydrocarbons (Heidelberger and Davenport, 1961; Brookes and Lawley, 1964; Goshman and Heidelberger, 1967). Demonstrations that the metabolism of the polycyclic hydrocarbon was a necessary prerequisite for such interactions soon followed (Grover and Sims, 1968; Gelboin, 1969) and in the succeeding ten years, much attention has been directed towards the identification of the reactive metabolites that are formed when carcinogenic hydrocarbons are activated by metabolism, latterly with some success.

Hydrocarbon Metabolism

A detailed understanding of the metabolic processes that are undergone by the polycyclic hydrocarbons and that convert them into more polar, hydroxylated derivatives has been of great assistance in the identification of the reactive species responsible for the in vivo reactions of hydrocarbons with nucleic acids. The first step in the metabolism of an unsubstituted polycyclic hydrocarbon like benzo[a]pyrene (Fig. 1) is the formation of a simple epoxide, a reaction catalysed by the NADPH-dependent microsomal mono-oxygenases and these simple epoxides can then either (a) rearrange non-enzymically to phenols, (b) be hydrated to give dihydrodiols, a reaction catalysed by microsomal epoxide hydratase or (c) be conjugated with glutathione, a reaction that is catalysed by soluble glutathione transferases and that is the first step in the formation of mercapturic acids. The formation and the involvement of simple epoxides as intermediates in these metabolic conversions was first predicted by Boyland (1950), but it was many years before unequivocal evidence for their existence as metabolites of the polycyclic hydrocarbons was obtained (Sims *et al.*, 1971; Grover *et al.*, 1972).

Simple Epoxides

The first epoxides to be detected as metabolites were the K-region epoxides: epoxides of this type had been the first to be prepared by synthesis (Newman and Blum, 1964)

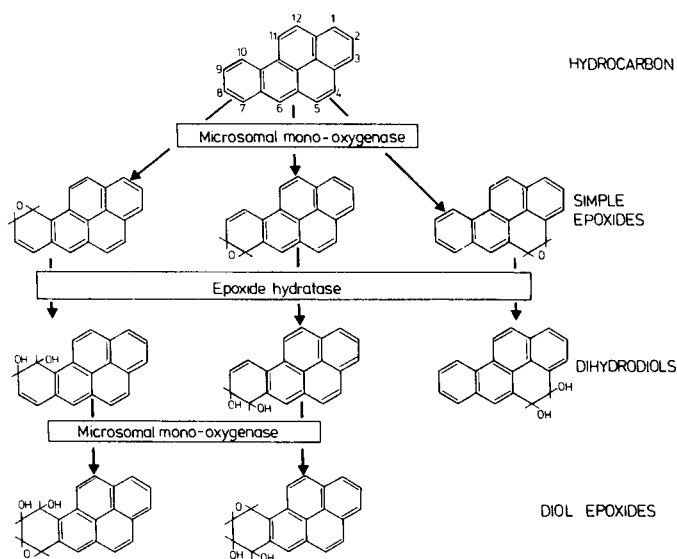


Fig. 1. Some of the pathways involved in the metabolism of benzo[a]pyrene

and a considerable amount of work, which has been reviewed (Jerina and Daly, 1974; Sims and Grover, 1974) and will not be discussed here, was carried out on those properties of K-region epoxides that might be relevant to their possible role as active intermediates in the initiation of tumours in tissues that had been treated with the parent hydrocarbons. In short, K-region epoxides were alkylating agents that (a) reacted with nucleic acid in solution and with the nucleic acids and proteins of cultured mammalian cells, (b) induced the malignant transformation of rodent fibroblasts in culture and (c) were mutagenic in a variety of test systems. K-Region epoxides were, however, less carcinogenic in whole animals than the hydrocarbons from which they were derived. Taken together, these properties placed K-region epoxides under suspicion but it was then very clearly shown that the nucleic acid products that are formed in cells treated either with 7-methylbenz[a]anthracene or with benzo[a]pyrene were quite different in their chromatographic properties on LH20 Sephadex columns from the less polar products that were formed when the corresponding K-region epoxides reacted with nucleic acids (Baird *et al.*, 1973, 1975, 1976).

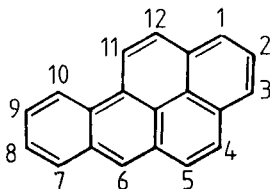
Vicinal Diol-Epoxides

Dihydrodiols were known to be formed and also to be further metabolized by rat-liver preparations (Booth *et al.*, 1973) but they were still considered at that stage to be true detoxication products of the hydrocarbons. The full significance of the further metabolism of dihydrodiols in relation to the metabolic activation of the carcinogenic polycyclic hydrocarbons only started to become apparent when it was reported that the further metabolism, by hepatic microsomal preparations, of one particular non-K-region dihydrodiol, the 7,8-dihydrodiol of benzo[a]pyrene in the presence of DNA resulted in the formation of some species that reacted extensively with nucleic acid (Borgen *et al.*, 1973). Work with the non-K-region 8,9-dihydrodiol of benz[a]anthracene led to the detection and identification of 8,9-dihydro-8,9-dihydroxybenz[a]anthracene 10,11-oxide (Booth and Sims, 1974), a new type of hydrocarbon metabolite in which the isolated olefinic double bond adjacent to the dihydrodiol grouping had been oxidized by the microsomal mono-oxygenase to an epoxide. Other studies with benz[a]anthracene then showed that the nucleic

acid products that were formed when this vicinal diol-epoxide reacted with DNA were considerably more polar than those that were formed when DNA was treated with the corresponding K-region epoxide. More interestingly perhaps, when DNA hydrolysates were examined on Sephadex LH20 columns the diol-epoxide-nucleoside products possessed chromatographic characteristics that were very similar to those of the adducts that were formed when benzo[a]anthracene itself was activated by metabolism in cells in culture (Swaisland *et al.*, 1974). When the metabolic activation of benzo[a]pyrene was re-examined in the light of this new information, it was found that the reaction of the 7,8-diol 9,10-oxide, which was prepared chemically from the 7,8-diol using *m*-chloroperoxybenzoic acid or biochemically using rat-liver microsomal preparations, with DNA led to the formation of hydrocarbon-nucleoside products that were indistinguishable in their chromatographic characteristics on Sephadex LH20 columns from the nucleic acid products that were formed when benzo[a]pyrene was activated by metabolism in cells in culture (Sims *et al.*, 1974). These findings led, not unnaturally, to an increased interest in the role that might be played by vicinal diol-epoxides in the metabolic activation of the polycyclic hydrocarbons as a class of chemical carcinogens. Some of the evidence that has recently become available concerning the metabolic activation of several polycyclic hydrocarbons through their conversion to vicinal diol-epoxides that react with nucleic acids will be discussed below.

Benzo[a]pyrene

The metabolic activation of benzo[a]pyrene (I), via a diol-epoxide mechanism has



I

been investigated in much more detail so far than that of any other hydrocarbon and it will be impossible to refer here to all the studies that have been carried out on this compound. The metabolites formed from benzo[a]pyrene have been examined by high pressure liquid chromatography (Selkirk *et al.*, 1974) and include two non-K-region dihydrodiols, the 7,8- and 9,10-diols, together with the K-region 4,5-diol (Fig. 1), which were formed predominantly as the (-)-enantiomers (Yang and Gelboin, 1976; Thakker *et al.*, 1977). Examination by photon-counting (Daudel *et al.*, 1975) or by low-temperature (Ivanovic *et al.*, 1976) spectrophotofluorimetry of DNA isolated from mouse skin or from hamster embryo cells that had been treated with benzo[a]pyrene confirmed the initial finding (Sims *et al.*, 1974) that activation of the hydrocarbon occurred in the 7,8,9,10-ring since the hydrocarbon moieties that were bound to the nucleic acid retained an intact pyrene nucleus: spectrophotofluorimetry was unable to differentiate, however, between activation involving the 7,8-diol 9,10-oxide or activation through the 9,10-diol 7,8-oxide. When ^3H -labelled dihydrodiols derived from benzo[a]pyrene were applied to mouse skin, the 7,8-diol yielded hydrocarbon-nucleoside products indistinguishable in their chromatographic properties from those arising either from the activation of benzo[a]pyrene in this tissue and in human bronchial epithelium or from the reaction of the 7,8-diol 9,10-oxide with DNA in solution (Grover *et al.*, 1976). The 9,10-diol, which is probably further metabolized by a pathway that does not involve diol-epoxide formation (Booth and Sims, 1976; Thakker *et al.*, 1978a), and the 4,5-diol, which cannot be directly converted into a vicinal diol-epoxide since it lacks an olefinic double bond adjacent to the dihydrodiol grouping, did not yield hydrocarbon-nucleoside products

in these experiments. Vicinal diol-epoxides exist in two isomeric forms (Fig.2), the syn, in which, in the case of 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene 9,10-

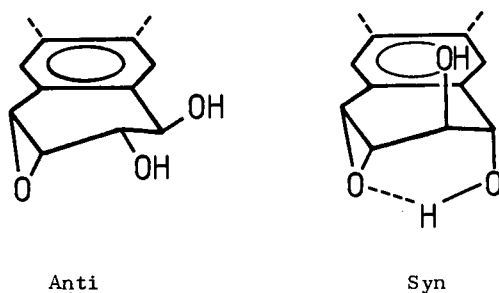


Fig. 2. Anti and syn isomers of a vicinal diol-epoxide

oxide, the epoxide grouping in the 9,10-position is on the same face of the ring as the hydroxyl group in the 7-position and the anti, in which the epoxide group is on the opposite face. The syn and anti isomers of benzo[a]pyrene 7,8-diol 9,10-oxide show, as predicted by Hulbert (1975) and by Yagi *et al.* (1975), marked differences in reactivity: when the products of the reactions of these two isomers with DNA were isolated and their chromatographic characteristics compared with those of the products that were formed when benzo[a]pyrene was metabolically-activated either by a microsomal preparation or by rodent cells in culture, the results indicated that benzo[a]pyrene was activated predominantly, in terms of reaction with nucleic acid, through the anti diol-epoxide (King *et al.*, 1976). However, it was subsequently shown that the structure of the DNA-benzo[a]pyrene adducts varied with the time of exposure to the hydrocarbon; products resulting from reactions with the syn isomer were formed first and that, with longer exposure times, products resulting from reactions of the anti isomer predominated (Baird and Diamond, 1977), a result that is in accord with others obtained using mouse skin (Moore *et al.*, 1977). Intensive research by several groups using modern physico-chemical techniques has led to the elucidation of the structures and to the absolute configuration of some of the nucleic acid adducts that are formed when benzo[a]pyrene is activated by metabolism in cells or in tissues in culture. For example, Weinstein and his group showed that one of the major RNA adducts formed in cultured bronchial mucosa results from the reaction of the anti 7,8-diol 9,10-oxide with guanine and have provided evidence showing that the adduct contains a covalent bond linking the C10 position of the 7,8,9-trihydroxybenzo[a]pyrene with the 2-amino group of guanine (Weinstein *et al.*, 1976; Jeffrey *et al.*, 1976). The absolute configuration of this product has been established (Nakanishi *et al.*, 1977) and it appears to arise from activation of the (-)-enantiomer of the trans-7,8-dihydrodiol and to have the structure shown in Fig. 3. Other less well-characterized reactions of the benzo[a]pyrene 7,8-diol 9,10-oxides with nucleic acids have also been reported and include, for example, the formation of labile phosphotriesters (Koreeda *et al.*, 1976), products in which the N₂ and N₇ positions of deoxyguanosine may be linked to the C₁₀ position of the hydrocarbon (Osborne *et al.*, 1976, 1978; Jeffrey *et al.*, 1977) and products containing deoxyadenosine and deoxycytidine (Meehan *et al.*, 1977). The biochemical and biophysical data showing that the 7,8-diol 9,10-oxides of benzo[a]pyrene are involved in the metabolic activation of this carcinogen and in its *in vivo* interactions with nucleic acids are also supported by a considerable amount of information on the biological activities of benzo[a]pyrene diols and diol-epoxides. Although it is possible that the malignant transformation of cells in culture (Marquardt *et al.*, 1976; Mager *et al.*, 1977) and the initiation of tumours in mouse skin (Chouroulinkov *et al.*, 1976; Slaga *et al.*, 1976) or in newborn mice (Kapitulnik *et al.*, 1977) following treatment with the 7,8-diol does not result from reactions

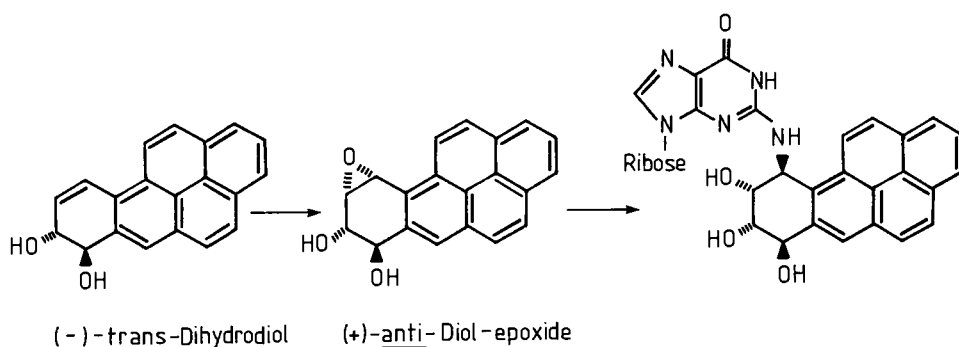
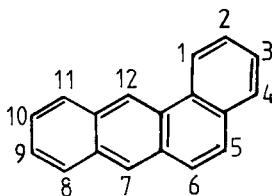


Fig. 3. Formation of an *in vivo* benzo[a]pyrene-guanine product through activation of the (-)-trans-7,8-dihydrodiol

of the related diol-epoxides with nucleic acids, it is unlikely that the mutagenicity of the diol-epoxides in *S. typhimurium* and in V79 Chinese hamster cells (Wood *et al.*, 1976a; Newbold and Brookes, 1976; Malaveille *et al.*, 1977a) is unrelated to their known reactivity towards genetic material.

Benz[a]anthracene

The metabolic activation of this hydrocarbon (II), which is only weakly carcinogenic, has not been investigated from the point of view of vicinal diol-epoxide



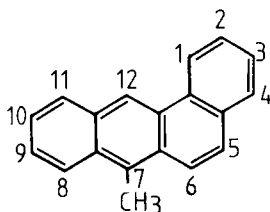
II

formation in nearly as much detail as benzo[a]pyrene but all the available data suggest that the most reactive diol-epoxides are the 3,4-diol 1,2-oxides. The metabolites formed from benz[a]anthracene by rat liver preparations appear to include all five possible trans-diols (Tierney *et al.*, 1978a) although the metabolism of the compound in a tissue in which it is carcinogenic has not been examined so far. The hydrocarbon-deoxyribonucleoside products formed in mouse skin treated with the hydrocarbon appear, from chromatographic comparisons carried out with reference adducts on Sephadex LH20 columns, to result from interaction with DNA of the 3,4-diol 1,2-oxide (P.G. Gervasi, personal communication) rather than the 8,9-diol 10,11-oxide (Swaisland *et al.*, 1974) although the chromatographic characteristics of the two sets of reference adducts are very similar. Further structural characterization of the nucleic acid adducts formed when benz[a]anthracene is activated *in vivo* is obviously required. The strongest evidence for the involvement of the 3,4-diol 1,2-oxide in the metabolic activation of benz[a]anthracene comes from the results of tests for biological activity. The 3,4-diol and the related syn and anti 3,4-diol 1,2-oxides were much more potent as mutagens in *S. typhimurium* or in V79 Chinese hamster cells (Wood *et al.*, 1976b, 1977a; Slaga *et al.*, 1978; Marquardt *et al.*, 1978) and as carcinogens on mouse skin (Wood *et al.*, 1977b; Levin *et al.*, 1978; Slaga *et al.*, 1978) or in newborn mice (Wislocki *et al.*, 1978) than other

metabolites that were tested although the 3,4-diol failed to induce malignant transformation of cultured mammalian cells (Marquardt *et al.*, 1978).

7-Methylbenz[a]anthracene

Like benz[a]anthracene itself, the 7-substituted derivative (III), which is a more potent carcinogen, can be activated by vicinal diol-epoxide formation either in the

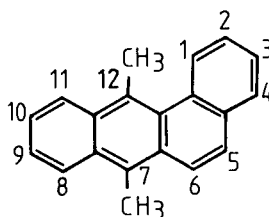


III

1,2,3,4- or in the 8,9,10,11-ring but this could also be accompanied by metabolism of the methyl group. When the metabolism of 7-methylbenz[a]anthracene by rat-liver preparations, by mouse skin (Tierney *et al.*, 1977) or by human skin maintained in short-term organ culture was examined using high pressure liquid chromatography, metabolites with chromatographic characteristics comparable to each of the five possible reference *trans*-dihydrodiols (Tierney *et al.*, 1978b) were detected although there were quantitative differences in the amounts of the individual diols that were formed. Photon-counting spectrophotofluorimetry revealed that the hydrocarbon moieties that became bound to the DNA of mouse skin or in hamster embryo cells treated with the hydrocarbon possessed an anthracene type of fluorescence spectrum consistent with activation, by vicinal diol epoxide formation, in the 1,2,3,4-ring and did not possess the phenanthrene like spectrum that would have indicated activation in the 8,9,10,11-ring (Vigny *et al.*, 1977a). The hydrocarbon-deoxyribonucleoside adducts formed in mouse skin *in vivo* possessed chromatographic characteristics on LH20 Sephadex columns that were similar to those of the deoxyribonucleoside adducts formed when the 3,4-diol 1,2-oxide reacted with DNA in solution but that were dissimilar from those of the 1,2-diol 3,4-oxide adducts (Tierney *et al.*, 1977); this indicates that the *in vivo* metabolic activation of 7-methylbenz[a]anthracene most probably involves the formation of 3,4-dihydro-3,4-dihydroxy-7-methylbenz[a]anthracene 1,2-oxide. This conclusion has been strongly supported by information obtained from experiments in which the different dihydrodiols have been tested for biological activity in systems in which they can be converted by metabolism to the related vicinal diol-epoxides *in situ*. The 3,4-diol was clearly more active than the other diols as a mutagen in *S. typhimurium* (Malaveille *et al.*, 1977b) and in V79 Chinese hamster cells, in the induction of malignant transformation in M2 mouse fibroblasts (Marquardt *et al.*, 1977) and sister chromatid exchanges in CHO cells (Pal *et al.*, 1978) and as an initiator of tumours in mouse skin (Chouroulinkov *et al.*, 1977). Absolute proof that this conclusion is correct, however, must await the synthesis of the different pairs of vicinal diol-epoxides and the full structural characteristics of the nucleic acid-hydrocarbon adducts that are formed when 7-methylbenz[a]anthracene is activated by metabolism in a target tissue *in vivo*.

7,12-Dimethylbenz[a]anthracene

Work on the metabolic activation of this potent carcinogen (IV) is still at an early stage; the *trans* 3,4-, 5,6-, 8,9- and 10,11-dihydrodiols, but not the 1,2-diol, were identified as metabolites when the hydrocarbon was metabolized by rat-liver preparations (Tierney *et al.*, 1978a) or by mouse skin in short term organ culture (A.D. MacNicol, personal communication). Fluorescence spectral studies

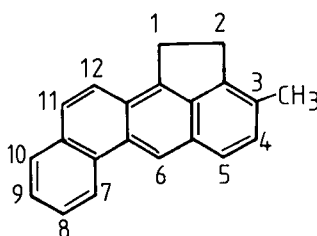


IV

have indicated that metabolic activation occurs in the 1,2,3,4-ring because the hydrocarbon moieties that become bound to DNA in mouse skin (Vigny *et al.*, 1977b) and in cultured mouse or hamster embryo cells (Moschel *et al.*, 1977; Ivanovic *et al.*, 1978) yield anthracene-like spectra. This is supported by other work on the photosensitivity of hydrocarbon-deoxyribonucleoside adducts (Baird and Dipple, 1977) but although the chromatographic characteristics of the adducts have been examined on LH20 Sephadex columns (Biggar *et al.*, 1978; Dipple and Nebzdoski, 1978) they have not been identified as resulting from the reaction of any particular vicinal diol-epoxide with DNA, partly because the authentic diol-epoxides have not been synthesized. In tests for biological activity, the 3,4-dihydrodiol, was more active as a mutagen in microsome-mediated tests with *S.typhimurium* (Malaveille *et al.*, 1978) and in V79 Chinese hamster cells (Marquardt *et al.*, 1978) than the other diols known to be metabolites; the 3,4-diol also induces malignant transformation of M2 mouse fibroblasts (Marquardt *et al.*, 1978). Tests on the ability of the diols derived from 7,12-dimethylbenz[a]anthracene to initiate tumours in mouse skin are still in progress but preliminary results show that both the 3,4-diol and, surprisingly, the K-region 5,6-diol are active.

3-Methylcholanthrene

The dihydrodiols that have been identified by comparison with authentic reference diols as metabolites of 3-methylcholanthrene (V) in rat liver preparations include



V

the *trans* 4,5-, 7,8-, 9,10- and 11,12-derivatives (Tierney *et al.*, 1978c) as well as the 1-hydroxy-9,10-diol (Thakker *et al.*, 1978b). The fluorescence spectral characteristics of the hydrocarbon residues bound to DNA in mouse skin, where the 9,10-diol is formed as a metabolite (Tierney *et al.*, 1978c), or in hamster embryo cells treated with the hydrocarbon resemble those of anthracene and indicate that activation has occurred in the 7,8,9,10-ring (Vigny *et al.*, 1977b; King *et al.*, 1977). Evidence for reaction with both purine and pyrimidine nucleosides has been presented (Jones *et al.*, 1973) but the hydrocarbon-deoxyribonucleoside adducts formed have not yet been further characterized. The 1-hydroxy-9,10-diol is active

in microsome-mediated mutagenicity tests with *S.typhimurium* (Thakker *et al.*, 1978) and in other studies with this system, the 9,10-diol is more active than any of the other dihydrodiol metabolites (C. Malaveille and H. Bartsch, personal communication). Similar mutagenicity results were obtained with V79 Chinese hamster cells and the 9,10-diol also induces malignant transformation of M2 mouse fibroblasts (H. Marquardt, personal communication). Although the available evidence indicates therefore that 3-methylcholanthrene is activated through metabolism to the 9,10-diol 7,8-oxide, it is not yet clear if oxidation of the methylene bridge also occurs prior to reactions with nucleic acid *in vivo*.

Other Hydrocarbons

Some studies have been carried out on the role that might be played by non-K-region diols and their related vicinal diol-epoxides in the metabolic activation of chrysene (Wood *et al.*, 1978) and of 5-methylchrysene (Hecht *et al.*, 1978) and preliminary mutagenicity data suggest the involvement of the 1,2-diol and of an unidentified diol in the 1,2,3,4-ring respectively. Work on the activation of dibenz[a,h]anthracene has shown the 3,4-diol to be more mutagenic in microsome mediated mutagenicity tests with *S.typhimurium* than the 1,2- and 5,6-dihydrodiols (Wood *et al.*, 1978).

Activation Mechanisms

The studies on the metabolic activation of the polycyclic hydrocarbons mentioned above, although in many cases incomplete, certainly provide support for the hypothesis that the formation of vicinal diol-epoxides is a general mechanism of activation applicable to the polycyclic hydrocarbons as a class of chemical carcinogens. In addition they also support the prediction, made from quantum mechanical considerations, that the most reactive diol-epoxides of this type will be diol-epoxides in which the epoxide grouping is adjacent to a 'bay-region' (Jerina *et al.*, 1976) as shown in Fig. 4. The possession of a 'bay-region' is not, however, of itself

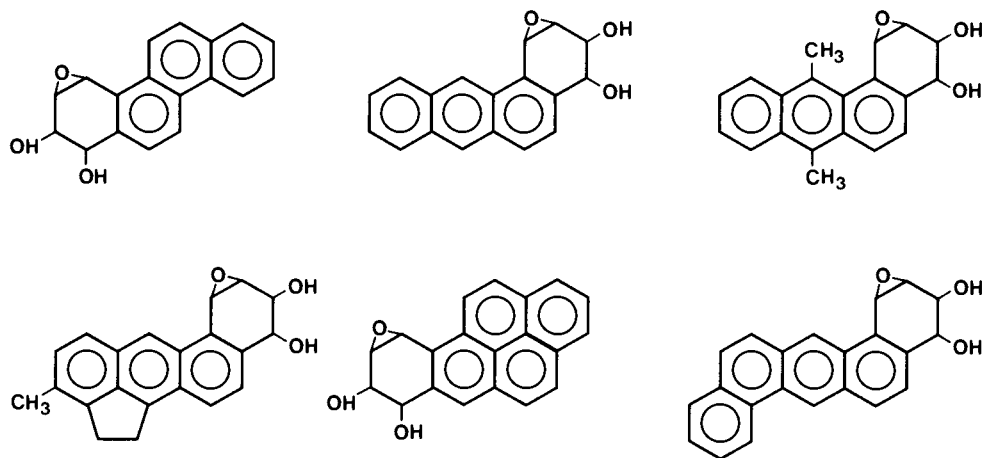


Fig. 4. Biologically active bay-region diol epoxides

sufficient to ensure that a compound is carcinogenic (Fig. 5) and, conversely, compounds such as cyclopenta[c,d]pyrene (Eisenstadt and Gold, 1978) and pyrene (Scribner, 1973), which do not possess 'bay-regions', may show varying degrees of biological activity. The chemical reactivity of 'bay-region' diol-epoxides is related to the relative ease of benzylic carbonium ion formation but the correlation

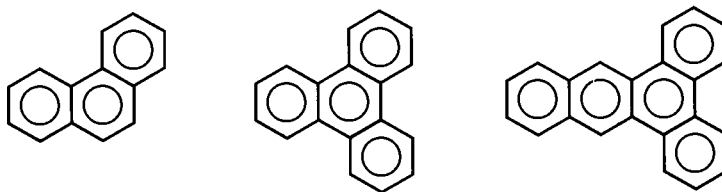


Fig. 5. Inactive hydrocarbons possessing bay-regions

between the calculated values for the ease with which such carbonium ions may be formed for a series of hydrocarbons and the carcinogenicity of these hydrocarbons is not apparently complete (Jerina *et al.*, 1976). This is not really surprising since there are likely to be a variety of other factors, which may be related, for example, to the pathways by which each hydrocarbon is metabolized and to the relative rates at which active and inactive metabolites are formed and further metabolized, involved in the metabolic activation of, and the biological activity shown by, polycyclic hydrocarbons *in vivo*.

Another correlation has recently been found between the microsome-mediated mutagenic activity of the *trans*-3,4-dihydrodiols of benz[a]anthracene, 7-methylbenz[a]anthracene and 7,12-dimethylbenz[a]anthracene and the carcinogenic potency of the present hydrocarbons expressed as Iball indices (C. Malaveille and H. Bartsch, personal communication): it may be that correlations between mutagenicity in this test system and the carcinogenicity of the hydrocarbons will be better in cases where only a single activation step has to be carried out by the microsomal preparation in order to form the diol-epoxides that appear to be involved in metabolic activation in *in vivo* situations. The precise mechanism by which diol-epoxides react with nucleic acids remains uncertain and evidence has been advanced in support of both a base-displacement model (Frenkel *et al.*, 1978) and an intercalation model (Drinkwater *et al.*, 1978).

DNA Reactions and Carcinogenesis

The information that is available concerning the metabolic activation of the polycyclic hydrocarbons is almost entirely restricted to an identification of the intermediates that react with DNA and which are themselves biologically active and we know very little about the cellular targets involved in a malignant transformation. When the metabolic activation of 7,12-dimethylbenz[a]anthracene was compared in the skin of mice of strains showing different susceptibilities to carcinogenesis by this hydrocarbon, no significant differences were found either in the rate at which the hydrocarbon became bound to or was removed from DNA (Fig. 6) or in the Sephadex LH20 column elution profiles of the hydrocarbon-deoxyribonucleoside adducts that were formed in the skin of the different strains (Phillips *et al.*, 1978). Of course the initiation of tumours is a relatively rare event and if appreciably greater extents of reaction with DNA occurred in only a few cells in the epidermis of a susceptible strain they would be masked

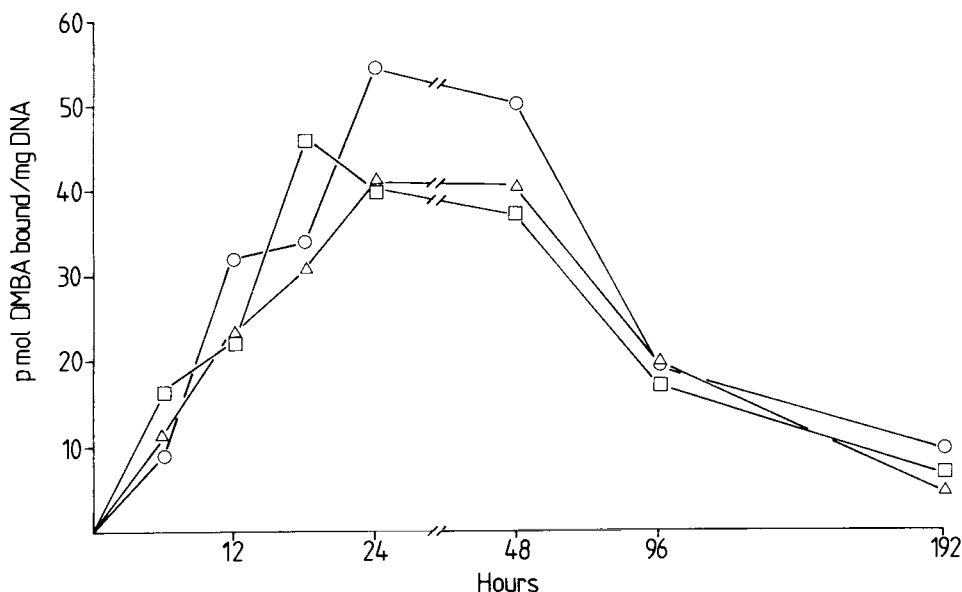


Fig. 6. The reaction of 7,12-dimethylbenz[a]anthracene (1 μ mol) with the DNA of the skin of mice of different strains (□, Swiss; Δ, C57Bl; O, DBA/2)

if the overall levels of reaction in the different strains were similar. There may also be inter-strain differences in, for example, the promotional aspects of skin carcinogenesis in these mice, in the relative rates of epidermal cell division, in the fidelity of DNA repair mechanisms or in immunological responsiveness and some of these possibilities may be worth examining experimentally.

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Environmental Chemicals Causing Cancer and Genetic Birth Defects: Mutagenicity Testing for Reactive Metabolites*

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Damage to DNA by Environmental Mutagens as a Cause of Cancer and Genetic Birth Defects

Damage to DNA appears to be the most likely cause of most cancer and genetic birth defects, and may contribute to heart disease and aging as well (1). These are the major diseases now confronting our society: currently almost one-fourth of us will develop cancer, while 5 to 10% of our children are born with birth defects.

Environmental mutagens, both natural and man made, appear to be the agents causing much of this genetic damage. Damage to the DNA of our germ cells results in genetic defects that show up in our children and in future generations. Somatic mutation could give rise to cancerous cells by changing the normal cellular mechanisms, coded for in the DNA, that control and prevent cell multiplication. Many mutagens are present among the natural chemicals in our diet; among man-made chemicals to which we are exposed, such as industrial chemicals, pesticides, hair dyes, cosmetics, and drugs; and in complex mixtures such as cigarette smoke and contaminants in the air we breathe and the water we drink.

Identifying Mutagens and Carcinogens

Identifying the mutagens and carcinogens causing cancer in people is tremendously difficult owing to a long lag period of 20 to 30 years for the appearance of most types of human cancer following exposure to a carcinogen. This is dramatically illustrated in the case of cigarette smoking (Fig. 1). Men started smoking cigarettes about 1900, but the resulting increase in lung cancer did not appear until 20 to 25 years later. Similarly, women started smoking in appreciable numbers about the time of World War II, and now the lung cancer rate for women is climbing rapidly. This same 20-year lag has been shown to apply for most types of cancer caused by the atomic bomb and for cancer in factory workers exposed to a variety of chemicals. Cigarette smoking has been much easier to identify as a cause of cancer than the usual environmental carcinogen because there is a clear control group of non-smokers and it causes a characteristic type of cancer (of the lung) that is

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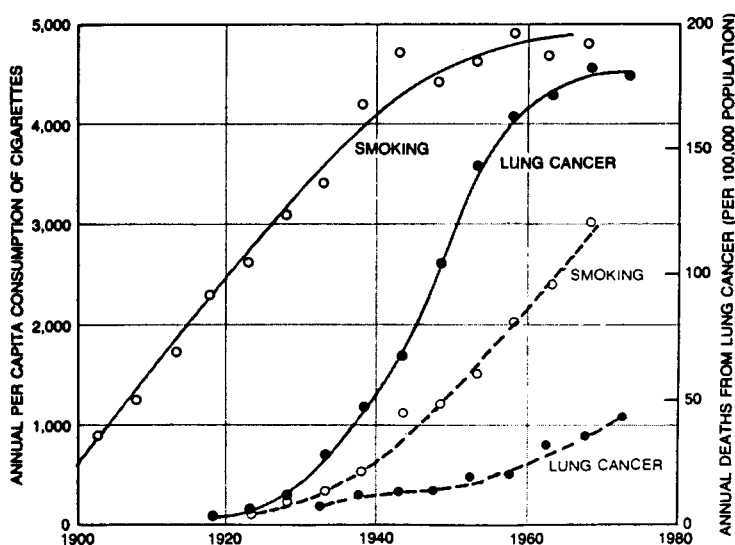


Fig. 1. Cigarette smoking and lung cancer are unmistakably related, but the nature of the relation remained obscure because of the long latent period between the increase in cigarette consumption and the increase in the incidence of lung cancer. The data are for England and Wales. In men (solid line) smoking began to increase at the beginning of the 20th Century, but the corresponding trend in deaths from lung cancer did not begin until after 1920. In women (dotted line) smoking began later, and lung cancers are only now appearing. (From J. Cairns, "The Cancer Problem." Copyright 1975 by Scientific American, Inc. All rights reserved.)

infrequent in the control group. However, with most environmental chemicals, such as the Japanese food additive AF-2 (discussed later) or vinyl chloride in spray cans, there is no clear-cut unexposed group; should certain carcinogens cause small increases in breast cancer or other common types of cancer, it will be even more difficult to show cause and effect, though the number of individuals affected might be large.

Human genetic defects are not easy to monitor or to attribute to a specific cause by epidemiology; thus a considerable increase in birth defects (exceeding the current rate of 5 to 10% among births) could easily go unnoticed.

New Chemicals in Our Environment

Clearly, many more chemicals will be added to the current list of human mutagens and carcinogens. It has been estimated that over 50,000 chemicals with significant production are currently used in commerce and close to 1,000 new ones are introduced each year (2). Only a small fraction of these chemicals--from flame retardants in our children's pajamas to pesticides accumulating in our body fat--were tested for carcinogenicity or mutagenicity before their use. In the past this problem has been largely ignored, and even very high production chemicals, with extensive human exposure, were produced for decades before adequate carcinogenicity or mutagenicity tests were done. Such chemicals include vinyl chloride (5 billion lb/yr, U.S.A.) and 1,2-dichloroethane (ethylene dichloride, 8 billion lb/yr, U.S.A.)

(Fig. 2), and a host of high-production pesticides that have only recently been shown to be carcinogenic and mutagenic.

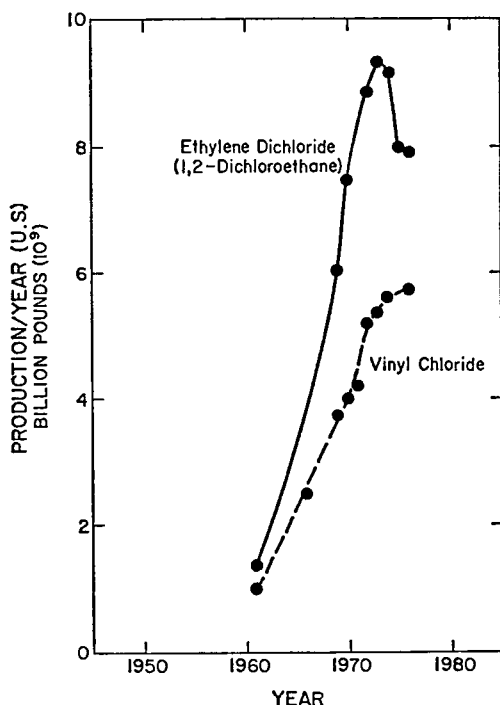


Fig. 2. Production of two mutagens/carcinogens with widespread human exposure: ethylene dichloride and vinyl chloride (production data from "Top-50 Chemicals" issues of *Chemical and Engineering News*). Approximately 100 billion lb of ethylene dichloride and over 50 billion lb of vinyl chloride have been produced since 1960. Ethylene dichloride is a volatile liquid that is the precursor of vinyl chloride and is also used extensively as a fumigant, solvent, gasoline additive (200 million lb/yr), and metal degreaser. Ethylene dichloride was first shown to be a mutagen in *Drosophila* in 1960 (3), and later in barley and *Salmonella*, but this fact was ignored. The first adequate cancer test in animals has just been completed by the N.C.I. (January 1978) and is positive in both sexes of both rats and mice. Vinyl chloride gas is used to make polyvinyl chloride (PVC; vinyl) plastic. It was shown to be a carcinogen in rats and in people in the mid-1970s, and a mutagen in *Salmonella* and other systems shortly afterwards.

Even if we could easily identify new hazardous chemicals by human epidemiology, people would already have been exposed for decades, and the discovery would be too late for those exposed. The tremendous increase in production of chemicals, such as vinyl chloride (which was used in millions of spray cans), that started in the mid 1950s (Fig. 2) may result in a steep increase in human cancer if many of these thousands of chemicals are indeed powerful mutagens and carcinogens with widespread human exposure. We will see any effect in the 1980 decade.

Animal Cancer Tests

A key method for detecting carcinogens is the animal bioassay, usually with rats and mice. The dozen or so organic chemicals known to cause cancer in humans also cause cancer in experimental animals when adequately tested. Those chemicals to which we are exposed in appreciable amounts should be screened in animal tests. One limitation of animal cancer tests, however, is their sensitivity due to the statistical problems inherent in the small sample size--usually 50 animals or less. An environmental carcinogen causing cancer in 1% of 100 million people would result in a million cases of cancer. Another problem in testing for carcinogens only by using animals is the enormity of coping with a backlog of the approximately 50,000 commercial chemicals that lack adequate testing for cancer in animals when each test costs \$150,000 and takes 2 to 3 years. In addition, chemical and drug companies need to have a method for weeding out hazardous chemicals while they are still under development and while alternatives can be chosen. Also needed is a less expensive and quicker test for identifying the carcinogens in the many complex mixtures of chemicals that surround us, such as cigarette smoke, impurities in water and air, natural carcinogens in our diet, and complex industrial products. Animal tests are usually not suitable as bioassays for identifying the active agent in a complex mixture because of the time and expense.

The *Salmonella*/Mammalian Tissue Test

Over the past 14 years we have developed a simple test for identifying chemical mutagens and have shown that almost all organic chemical carcinogens tested are mutagens (4-6). The method is being widely used to detect environmental carcinogens as mutagens. This test, and other short-term tests that have been developed, should enable society to solve some of the problems that could not be approached using human epidemiology or animal cancer tests alone.

Our test detects carcinogens and mutagens by means of their mutagenicity, and we have shown that about 90% of organic carcinogens tested can be detected as mutagens. DNA damage is measured using special strains of *Salmonella* bacteria in combination with homogenized liver tissue from rats (tissue homogenates from human autopsy material or from other mammals can also be used). The compound to be tested, about 1 billion bacteria of a particular tester strain (several different histidine-requiring mutants are used), and homogenized liver are combined on a petri dish and after incubation at body temperature (37°C) for two days, the number of bacterial colonies is recorded. Each colony (a *revertant* colony) is composed of the descendants of a bacterium that has been mutated from a defective histidine gene to a functional one. Because the Millers (reviewed in their accompanying paper) and other workers had shown that many carcinogens must be converted by enzymes in liver or other tissues to an active electrophilic form that is the true carcinogen (and mutagen), the mammalian liver tissue is added to the test to provide mammalian metabolism.

Figure 3 shows an example of the type of results obtained and the quantitative dose-response curves that are generated. These curves are almost always linear, which suggests that there is no threshold for a mutagen in bacteria. Most mutagens are detected at very low doses, in some cases in nanogram amounts.

The simplicity, sensitivity, and accuracy of this test for screening large numbers of environmental sources of potential carcinogens has resulted in its current use in over 1,000 government, industrial, and academic laboratories throughout the world. Many companies are making important economic decisions on the basis of the test.

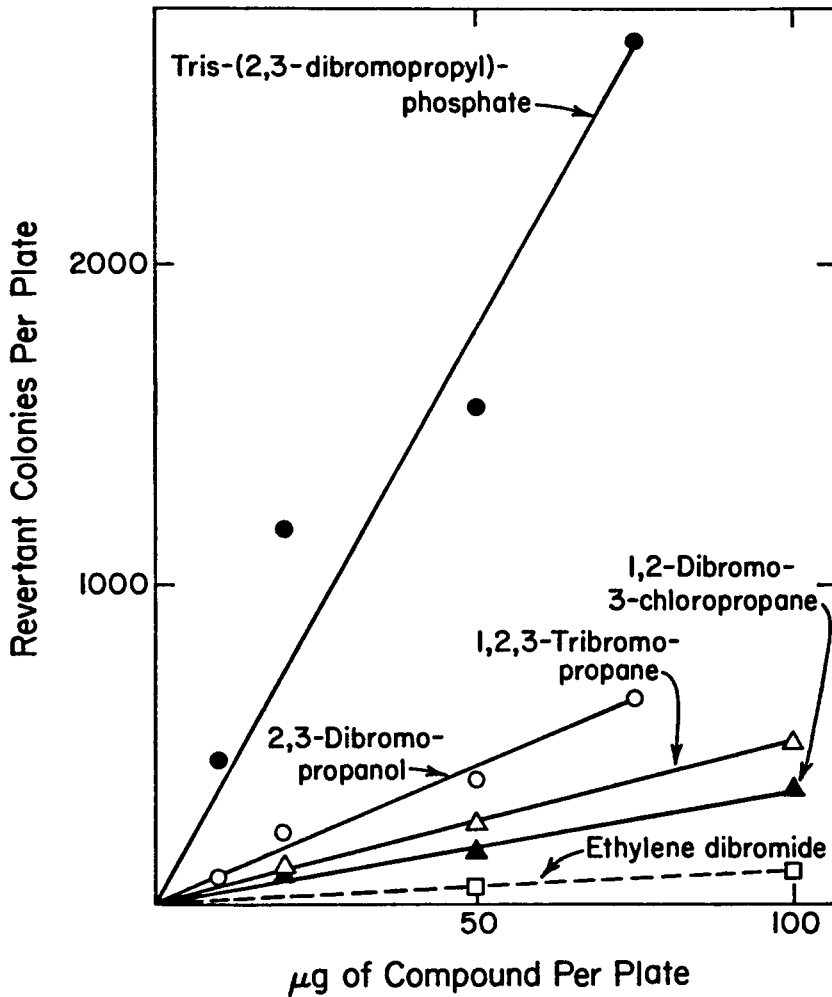


Fig. 3. The flame retardant *tris*-(2,3-dibromopropyl)phosphate and the pesticide dibromochloropropane (which has been recently shown to have caused sterility in numerous factory workers) were in the presence of rat liver homogenate. All compounds were tested on *Salmonella* strain TA100. The amount of the industrial chemical ethylene dibromide added was ten times that indicated on the scale. (Reprinted, with permission, from A. Blum and B. N. Ames, *Science*, 195 [1977], 17-23; copyright 1977 by the American Association for the Advancement of Science.)

We have validated the test for the detection of carcinogens as mutagens by examining over 300 chemicals reported as carcinogens or non-carcinogens in animal carcinogenicity experiments (5-6). The results show that almost all (90%: 158/176) of these chemical carcinogens are mutagenic in the *Salmonella* test. The percentage of carcinogens detectable would, of course, depend on how representative any particular list of carcinogens was of those existing in the real world. For this reason we also examined the organic chemicals known or suspected as human carcinogens and found that almost all (16/18) were mutagens in the test (6). Nevertheless, it is important to emphasize that some important carcinogens (6), such as many heavily chlorinated chemicals, do not show up in the test, although we are working on this problem. Even with test improvements, some carcinogens (e.g., griseofulvin) will never be detected because they are not acting through a direct interaction with DNA.

Thus, almost all carcinogens are mutagens, and the converse also appears to be true: mutagens are carcinogens with few (if any) adequately documented exceptions. We found that almost all (95/108) "non-carcinogens" tested were not mutagenic and those few that were may in fact be carcinogens but were not detected as such due to the statistical limitations of animal carcinogenicity tests (6).

Our test system has been independently validated, with similar results, in studies by Imperial Chemical Industries and by the National Cancer Institute in Tokyo.

Further validation is offered by several cases involving extensive human exposure, where chemicals initially detected as mutagens have subsequently been found to be carcinogens. One incident involved the food additive AF-2 (furylfuramide; 7), which was used extensively in Japan from 1965 until recently as an antibacterial additive in a wide variety of common food products such as soybean curd and fish sausage. It showed no carcinogenic activity in tests on rats in 1962 and on mice in 1971. In 1973, however, Japanese scientists found it to be highly mutagenic in a strain of *Escherichia coli* bacteria (it was also found to be extraordinarily potent in reverting our *Salmonella* tester strain TA100). The mutagenic activity of this chemical in food was such that one could easily demonstrate the mutagenicity of a slice of fish sausage put on a petri plate. It was subsequently examined in higher (eukaryotic) organisms and found to be mutagenic in yeast and *Neurospora*, to cause chromosome breaks in human white blood cells, and more recently to mutate embryos when even low doses were fed to pregnant Syrian hamsters (8). Animal tests for carcinogenicity, more extensive than the previous ones, were initiated, and these tests have recently shown that AF-2 is, in fact, a carcinogen in rats and mice. As a consequence, the Japanese government prohibited the use of AF-2 as a food additive, and all products containing AF-2 were removed from the market. Since AF-2 had already been tested for carcinogenicity in two animal systems and found negative, it is unlikely that further tests would have been conducted if it had not been shown to be mutagenic. Any deleterious effects of AF-2 on the Japanese population would not have been evident for decades, and it is possible that a catastrophe may have been avoided by the early detection of this carcinogen with a simple bacterial mutagenicity test. Unfortunately, for the past eight years, the Japanese people have consumed relatively large amounts of AF-2; it is still too early to predict the consequences of this exposure.

Ethylene dichloride, a 4×10^9 -kilo-a-year chemical, discussed in Fig. 2, has been shown previously to be a mutagen in *Drosophila* and in *Salmonella* and has now been found to be a carcinogen.

Another example of a carcinogen initially detected as a mutagen is 1,2-dibromoethane (ethylene dibromide), a widely used industrial chemical and gasoline additive, which was detected as a mutagen in several microbial systems (including the *Salmonella* test [see Fig. 3]) over eight years ago. It is closely related in structure to the pesticide dibromochloropropane (DBCP), also a mutagen and carcinogen.

Another related dibromo chemical, *tris*-(2,3-dibromopropyl)phosphate, commonly called "Tris," the main flame retardant in children's polyester pajamas, is a potent mutagen in our test system, as are its metabolically produced breakdown product, dibromopropanol, and its impurity, the carcinogen dibromochloropropane (Fig. 3; 9-10). Fifty million children wore sleepwear that contained this material, at about 5% of the weight of the fabric. We argued that Tris would pose a serious hazard to children because non-polar (relatively fat soluble and water insoluble) chemicals such as these are generally absorbed through human skin at appreciable rates. Since its detection as a mutagen in *Salmonella*, it has been shown to be active in a number of short-term tests: it is a potent mutagen in *Drosophila*, it interacts with human DNA, and it damages mammalian chromosomes. The compound has been tested recently at the National Cancer Institute, and the results show that Tris is a potent carcinogen in both rats and mice. It has also been shown, like dibromochloropropane and ethylene dibromide, to cause sterility in animals. It has now been banned for use in sleepwear. We have recently shown that the mutagenic Tris metabolite, dibromopropanol, is present in the urine of children wearing Tris-treated sleepwear (11).

Studies in this laboratory have shown that most common hair dyes are mutagens (12). Eighty-nine percent (150/169) of commercial oxidative-type (hydrogen peroxide) hair dye formulations were mutagenic, and of the 18 components of these hair dyes, 8 were mutagenic. Most semi-permanent hair dyes tested were also shown to be mutagenic. Hair dye components are known to be absorbed through the skin, yet very few of the hair dyes or their components had ever been tested adequately for carcinogenicity. Since the work on mutagenicity in *Salmonella*, a number of these ingredients have been shown to be mutagens in other short-term tests for mutagenicity. Several of the chemicals are being tested at the National Cancer Institute and now appear to be carcinogens. About 25 million people (mostly women) dye their hair in the United States, and the hazard could be considerable if these chemicals are mutagenic and carcinogenic in humans.

The sensitivity of the *Salmonella*/mammalian liver assay makes it useful as a tool for rapidly obtaining information about the mutagenic and potential carcinogenic activity of complex mixtures, where it can be used as an assay to identify the mutagenic components of the mixtures. A detailed study, for example, has been made of the mutagenic activity of cigarette smoke condensate and 12 standard smoke condensate fractions (13). (In the test, the condensate from less than 0.01 cigarette could easily be detected.)

We have recently developed a simple method for examining human urine in our test system and have found mutagens in the urine of cigarette smokers but not in the urine of non-smokers (14).

Other Short-Term Tests

Since our development of the *Salmonella* test and the demonstration that almost all carcinogens are mutagens, there has been a tremendous resurgence of interest in other short-term test systems for measuring mutagenicity. Many such systems have been developed and some, including the use of animal cells in tissue culture and cytogenetic damage in cells in tissue culture, have been validated with a reasonable number of chemicals. In addition, a number of the old systems, such as mutagenicity testing in *Drosophila*, have become much more sophisticated (the first mutagens known, such as X-rays and mustard gas, were first identified in *Drosophila* before they were known to be carcinogens). In addition, the development of several tissue-culture systems with animal cells, having as an end point the "transformation" of the cells to a tumor cell, are an important advance.

No one of these short-term tests, however, is completely ideal; for example, most tests using animal cells in culture require the addition of liver homogenate, just as our bacterial test does, because the animal cells useful for these tests are not capable of metabolizing all foreign chemicals to active mutagenic forms. A number of short-term systems have been validated and seem to be effective in detecting known carcinogens. Because each system detects a few carcinogens that others do not, the idea of a battery of short-term tests is now favored.

In the case of a substance like Tris, or the food additive AF-2, the combination of a widespread human exposure to the chemical and a positive result in a number of short-term tests should have been sufficient evidence to stop its use, considering that alternatives were available. Yet Tris and AF-2 were not removed from the market until the results from animal cancer tests indicated that they were carcinogens. It is becoming apparent that a positive result in many of these short-term test systems is meaningful, and that the systems may not only be a complement to animal cancer testing but may also provide much additional toxicological information as well. Mutagens should be treated with respect, even apart from their carcinogenicity.

Summary of Applications of the *Salmonella* Test and Other Short-Term Tests

1. The active metabolic forms of chemical carcinogens, and their metabolic routes, can be determined using the test as a bioassay. (This is illustrated by many papers at this Congress, and I have not tried to review the field.)
2. Chemical and drug companies can now afford to test routinely all new compounds at an early stage of development so that mutagens can be identified and this information taken into consideration before there is a large vested interest in the compound. The *Salmonella* test is now being used by about 150 major chemical and drug companies.
3. If a drug is found to be mutagenic, a variety of derivatives can be synthesized to find a nonmutagenic form.
4. The mutagenicity of a chemical may be due to a trace of impurity, and such knowledge could save a useful chemical (15).
5. Complex mixtures or natural products with carcinogenic activity can be investigated using the test as a bioassay for identifying the mutagenic ingredients.
6. Human feces and urine can be examined to see if ingested products or drugs are giving rise to mutagens.
7. The variety of substances that humans are exposed to, both pure chemicals and mixtures, are being assayed for mutagenicity by hundreds of laboratories.
8. The test system is useful in clarifying basic mechanisms of mutagenesis by chemical carcinogens, e.g., the demonstration that many aromatic carcinogens are reactive frameshift mutagens with particular base-sequence specificity and the clarification of the role of different repair systems in mutagenesis by various carcinogens.
9. The sensitivity of the *Salmonella* test may make it particularly useful for detecting chemicals that have weak carcinogenic activity and that would be difficult to identify in animal tests because of statistical limitations.

10. The tests are being used for setting priorities in selecting chemicals for carcinogenesis bioassay in animals.

Carcinogenic Potency and Human Risk Assessment

There is human exposure to a large number of environmental carcinogens, both man made and natural. Many of these chemicals are quite useful, and it is clearly impractical to ban every carcinogen. We must have some way of setting priorities for regulation of these chemicals, and this requires an assessment of human risk, a difficult and complex problem.

We believe it is important for the assessment of human risk to have knowledge of carcinogenic potency, which can be accomplished in part through a quantitative analysis of animal cancer tests. We (C. Sawyer, K. Hooper, A. Friedman, and B. Ames) have been working on the potency problem for over a year (following the lead of M. Meselson [16] and collaborating with R. Peto on the theoretical aspects), and are nearing completion of the first stage of our analysis of 1,000 published animal cancer tests in which the dose is given continuously for the lifetime of the animal by feeding.

Our results to date show that it is essential to consider carcinogens in more quantitative terms. We have shown that the potency of carcinogens (the TD_{50} , the daily dose required to produce cancer in half of the animals) can vary over a million-fold. Such a range of potency must be considered in assessing the hazard of chemicals for man. This quantitative analysis of carcinogenic potency for all of the cancer tests in the scientific literature that are suitable for calculation is almost complete. The results should be useful for the determination of:

1. Which chemicals, among the thousands of carcinogens to which people are exposed, may present the greatest human hazard and require the most immediate attention. This setting of priorities also requires an estimate of the amount of human exposure to a given chemical--information that is often available or can be obtained.
2. Allowable exposure levels for carcinogens for workers or the general population.
3. The significance of negative cancer tests. Each particular cancer test has a limit of sensitivity (because of dose level and other factors) and can detect only those carcinogens having potencies above a certain level. Because cancer tests vary enormously in sensitivity, rather than using the quantitatively meaningless term *non-carcinogen*, we must express the results of a negative cancer test by assigning the chemical a maximum potency value. For human risk assessment, it is essential to know the maximum potency values for "non-carcinogens."
4. To what extent carcinogenic potency is, or is not, species and sex specific, and which animal species and strain is the best model for humans for each particular class of carcinogens. Our analysis so far indicates that potency values do not vary much for a given chemical when comparing males and females and that values for rats and mice are often quite similar.
5. The relation of carcinogenic potency to potency as measured in many short-term tests, such as the *Salmonella* mutagenicity test. It is clear that mutagenicity tests also show a tremendous range in potency--there is about a million-fold range in *Salmonella*.

Potency in Short-Term Mutagenicity Tests

Although we can now make a start on human risk assessment based on animal cancer tests, few of the chemicals in the environment to which people are exposed have actually undergone cancer testing in animals. Furthermore, many of the tests that have been done lack the quality needed for making a quantitative analysis on the data. Thus we are faced with the question: can short-term tests provide any information about human risk? We are trying to answer this question, and our results so far suggest that these tests may be useful in giving an approximate idea of carcinogenic potency.

There is over a million-fold range in mutagenic potency in the *Salmonella* test and a similar range in carcinogenic potency. Although one would not expect a precise quantitative correlation between mutagenicity in bacteria using rat liver homogenate and carcinogenicity in rats, even a rough quantitative correlation would be useful in human risk assessment. Work done by Meselson and Russell (16) suggests there is a quantitative correlation of potencies, not only for carcinogens in the same class, but also across a broad range of classes, although some nitrosamines did not fit this general relationship. Our own work (K. Hooper, A. Friedman, C. Sawyer, and B. Ames) comparing the potency of chemicals in causing tumors in rats with potency in the *Salmonella* test (using a rat liver homogenate for activation) shows a good correlation so far, with some exceptions. Additional work will show how general this correlation is.

The theoretical basis for a correlation may be that using rat liver homogenate as a model for a rat's metabolism of foreign chemicals is a reasonable first approximation, especially because we are only analyzing the carcinogenic potency for chemicals that are ingested. The liver is the primary organ for metabolizing foreign chemicals and in general is much more active than other tissues for metabolic activation. (Most carcinogens that are ingested do not cause liver cancer, but this may be explainable by the increased DNA-repair capabilities of the liver [17].) Our analysis should, in any case, give some indication of the chemicals for which it is necessary to use tissues other than liver in mutagenicity tests and areas where the test needs improvement. We plan to examine to what extent those cases of a chemical differing in carcinogenic potency between species can be correlated with mutagenic potency assayed using the liver homogenates (or other tissues if necessary) from the different species. Other short-term tests that are currently being developed can also be calibrated against our carcinogenic potency index to see how well they correlate. The quantitative agreement between *Salmonella* and another short-term test (inhibition of DNA synthesis in human HeLa cells in tissue culture) has been recently examined and appears good (18). If several short-term tests can be shown to provide rough quantitative results consistent with those from animal cancer tests, a battery of short-term tests could then be used for establishing priorities among the many mutagens, both natural and man made, that have never been tested in animal cancer tests and to which there is significant human exposure.

Dietary Carcinogens and Mutagens

Man-made chemicals have been emphasized in the previous sections, and it has been pointed out that the major effect of these as carcinogens and mutagens will only become apparent in the next decades. Much of the cancer today, on the other hand, appears to be due to cigarette smoke, radiation (e.g., ultraviolet light induces skin cancer), and the ingestion of a wide variety of natural carcinogens present in our diet. Plants have developed a wide assortment of chemicals to discourage insects and animals from eating them and many of these are mutagens and carcinogens present in the human diet (1). In addition, powerful nitrosamine and nitrosamide carcinogens are formed from certain normal dietary biochemicals containing nitrogen,

by reaction with nitrite. Nitrite is produced by bacteria in the body from nitrates that are present in ingested plant material and water (1). A number of molds produce powerful carcinogens such as aflatoxin and sterigmatocystin that can be present in small amounts in food contaminated by molds, such as peanut butter and corn (1).

Two test cases of major importance are in progress using the *Salmonella* test as a bioassay for natural carcinogens. Dr. W. R. Bruce and his colleagues in Toronto have found a considerable amount of a powerful mutagen in human feces (1). It appears to be a nitrosamine formed from a component of dietary fat and could be a major cause of colon and breast cancer, two common cancer types associated with high fat intake. Dr. Bruce is identifying its chemical structure using *Salmonella* mutagenicity as a bioassay. He is also finding that high vitamin C or vitamin E intake lowers the amount of the mutagen. In another instance, Dr. Sugimura and his colleagues in Tokyo have discovered that when fish are broiled (a common practice in Japan), mutagenic chemicals are formed (19). Using the *Salmonella* test as a bioassay, they have found that the broiling protein produces mutagens and that broiling tryptophan (a component of protein) produces potent mutagens. They have identified one active mutagen chemically and shown that it is also very active in another short-term assay (transformation) using animal cells. They are currently doing an animal cancer test on the substance. An animal cancer test could never have been used as the bioassay for identifying the chemical because of the time involved. Thus, based on the mutagenic activity for *Salmonella*, it appears that broiling food so that burnt protein is formed may contribute a fairly substantial dose of mutagens to our diet.

Prevention of DNA Damage

We believe that the problem of cancer and genetic birth defects can be usefully attacked by prevention. Nevertheless, it seems clear that we cannot ban all the carcinogens and mutagens because too many exist and many are of great economic importance, but we must identify them and treat them with respect. Thus, setting priorities and trying to minimize human exposure are essential. The carcinogen and mutagen vinyl chloride is still used in the plastics industry to make vinyl floor tiles and PVC pipe, but vinyl chloride is no longer used in millions of cosmetic spray cans, and workers are no longer breathing in a dose that may give a high percentage of them cancer.

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Biological Activity of Polycyclic Hydrocarbon Metabolites and the Bay Region Theory

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Evidence for the metabolic activation of a polycyclic aromatic hydrocarbon to reactive intermediates was first obtained in 1951 from a key study by E.C. Miller. She applied benzo[a]pyrene (BP) to mouse skin and found covalent binding of metabolites of this polycyclic hydrocarbon to skin protein (1). This demonstration of the metabolic activation of a polycyclic aromatic hydrocarbon was followed by many other investigations indicating that application of carcinogenic polycyclic hydrocarbons to mouse skin resulted in the covalent binding of metabolites to DNA, RNA and protein. Data which suggested the importance of DNA-binding for the carcinogenicity of polycyclic hydrocarbons on mouse skin was presented by Brookes and Lawley (2,3). In 1957, the liver microsomal metabolism of BP to several hydroxylated products and quinones by an inducible monooxygenase was described (4) and several years later the microsomal-mediated metabolism of BP to DNA-bound metabolites was demonstrated (5,6). More recently, Borgen et al (7) found that metabolism of BP 7,8-dihydrodiol by liver microsomes resulted in significantly higher binding of hydrocarbon metabolite(s) to DNA than did the metabolism of BP or several other BP metabolites. Shortly thereafter, Sims et al (8) provided evidence that a BP 7,8-diol-9,10-epoxide was involved in the binding of BP 7,8-dihydrodiol to DNA, and the synthesis and high chemical reactivity of the diastereomeric 7,8-diol-9,10-epoxides of BP was described by Yagi et al (9). The biotransformation of BP to BP 7,8-oxide, BP 7,8-dihydrodiol and its diol epoxides (BP 7,8-diol-9,10-epoxides-1 and 2) is illustrated in Figure 1. It should be noted that each of the BP metabolites shown in Figure 1 can exist as optically active enantiomers and that metabolism of BP by rat liver microsomal enzymes can be highly stereoselective. The relative amounts of individual diastereomers and optical enantiomers formed depends on the source of the monooxygenase enzyme preparation. Although the data presented in this manuscript will be concerned with the metabolism and biological activities of racemic polycyclic hydrocarbon derivatives, the stereoselective metabolism of BP and the biological activities of its optically active metabolites are described elsewhere in this symposium (10). In the present manuscript, we describe the biological activities of metabolites of BP, benzo[a]-anthracene, dibenzo[a,h]anthracene and chrysene, and we discuss a generalized theory of polycyclic hydrocarbon carcinogenesis (bay region theory) that has evolved from this research.

CARCINOGENICITY OF BENZO[a]PYRENE DERIVATIVES

Although studies on the mutagenicity and covalent binding of polycyclic hydrocarbon metabolites provide important information that helps in the identification of proximate and ultimate carcinogens, high mutagenicity and covalent binding

need not be accompanied by tumor formation. Because of these considerations, the identification of proximate and ultimate carcinogenic metabolites of BP and other polycyclic hydrocarbons can only come from carcinogenicity studies. These studies have been ongoing in our laboratories for the past several years.

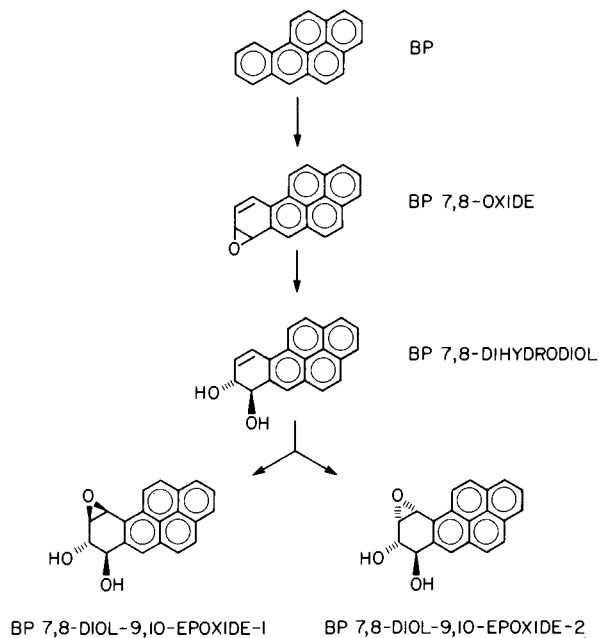


Fig. 1. Metabolism of benzo[a]pyrene to the BP 7,8-diol-9,10-epoxides.

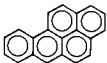
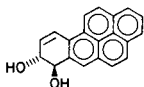
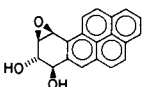
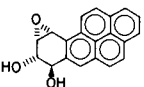
Carcinogenicity of BP 7,8-oxide and BP 7,8-dihydrodiol - When the carcinogenicities of BP and BP 4,5-, 7,8-, 9,10-, and 11,12-oxides were tested by the topical application of 0.4 μmol of each compound once every two weeks to the backs of C57BL/6J mice for 60 weeks, only BP and BP 7,8-oxide were highly active in causing skin tumors (11,12). Application of 0.15 μmol of BP 7,8-oxide once every two weeks for 60 weeks resulted in fewer tumors than similar treatment with an equimolar dose of BP. The results also indicated that BP 7,8-dihydrodiol was a considerably more potent carcinogen on mouse skin than BP 7,8-oxide (13,14). Studies on the carcinogenicity of the four arene oxides in newborn mice indicated that only BP 7,8-oxide had high tumorigenicity but that it was less active in causing lung tumors than BP. BP 7,8-dihydrodiol was more tumorigenic in this animal model than either BP 7,8-oxide or BP (15-17). Data obtained from initiation-promotion studies paralleled data from chronic studies on skin in that BP 7,8-oxide had appreciable but lower tumor initiating activity than BP while the other three arene oxides were much weaker in activity, and BP 7,8-dihydrodiol was highly active in this test system (18-20). 7,8-Epoxy-7,8,9,10-tetrahydro BP and 7,8-dihydroxy-7,8,9,10-tetrahydro BP, compounds identical to BP 7,8-oxide and BP 7,8-dihydrodiol but with the double bond removed from the 9,10-position, were completely inactive in eliciting tumors when they were applied to mouse skin once every two weeks for 60 weeks (13,14). The lack of carcinogenicity of BP 7,8-oxide and BP 7,8-dihydrodiol when the 9,10-double bond was saturated suggests that the carcinogenicity of BP 7,8-oxide and BP 7,8-dihydrodiol is due to metabolic conversion of these compounds to one or more of the BP 7,8-diol-9,10-epoxides. The high tumorigenic activities of BP 7,8-oxide and BP 7,8-dihydrodiol together with their lack of high mutagenic activity suggest that these compounds are proximate carcinogenic

metabolites of BP.

Carcinogenicity of BP 7,8-diol-9,10-epoxide-2 - The high chemical reactivity (9) and high mutagenicity (21,22,23) of BP 7,8-diol-9,10-epoxides 1 and 2 (see fig 1 for structures) and the finding of adducts of both of these diol epoxides bound to cellular DNA, RNA and protein after metabolism of BP (24,25,26,27) suggested that these diol-epoxides were prime candidates for ultimate carcinogenic metabolites of BP. In carcinogenicity tests on mouse skin, we found that BP 7,8-diol-9,10-epoxide-1 was inactive as a complete carcinogen while BP 7,8-diol-9,10-epoxide-2 had weak activity compared to BP (14). Additional studies revealed that BP 7,8-diol-9,10-epoxide-2 was considerably less active than BP and BP 7,8-dihydrodiol as a tumor initiator on mouse skin (19). In newborn mouse studies, we found that 1400 nmol of racemic BP 7,8-dihydrodiol administered i.p. to newborn mice during a two-week interval was more than 10 times as active as an equimolar dose of BP in causing malignant lymphomas and pulmonary adenomas (table 1, ref. 15). Racemic BP 7,8-diol-9,10-epoxides-1 and 2 were highly toxic, and survival of the animals could be achieved only when 2% of the above dose of the diol-epoxides was adminis-

TABLE 1

CARCINOGENICITY OF BP, BP 7,8-DIHYDRODIOL
AND THE BP 7,8-DIOL-9,10-EPOXIDES IN
NEWBORN MICE

COMPOUND	TOTAL DOSE (nmol)	% MICE WITH MALIGNANT LYMPHOMA	LUNG TUMORS PER MOUSE
CONTROL	—	0	0.13
 BENZO[a]PYRENE	1400 28	0 0	6.3 0.24
 BP 7,8-DIHYDRODIOL	1400 28	70 0	75 1.77
 BP 7,8-DIOL-9,10-EPOXIDE-1	28	0	0
 BP 7,8-DIOL-9,10-EPOXIDE-2	28	2	4.42

SWISS-WEBSTER MICE WERE INJECTED I.P. WITHIN 24 HRS. OF BIRTH AND AT 8 AND 15 DAYS OF AGE WITH 1/7, 2/7, AND 4/7 OF THE TOTAL DOSE OF COMPOUND RESPECTIVELY. THE MICE WERE SACRIFICED WHEN THEY WERE 6-7 MONTHS OLD.

tered. Animals treated with the very low dose of 28 nmol of BP during their first two weeks of life had about twice as many pulmonary adenomas per mouse as animals treated with the DMSO vehicle (table 1). BP 7,8-diol-9,10-epoxide-1 did not cause lung tumors, whereas BP 7,8-diol-9,10-epoxide-2 and BP 7,8-dihydrodiol were highly tumorigenic (15,16). After correcting for the small number of lung

adenomas in control mice, the data in table 1 indicate that BP 7,8-dihydrodiol and BP 7,8-diol-9,10-epoxide-2 were, respectively, 15- and 39-fold more active than BP in causing pulmonary adenomas in newborn mice (16). The data described here indicate that BP 7,8-dihydrodiol is a proximate carcinogenic metabolite and that BP 7,8-diol-9,10-epoxide-2 is an ultimate carcinogenic metabolite of BP in the newborn mouse. *These results are the first demonstration of proximate and ultimate carcinogenic metabolites for the polycyclic hydrocarbon class of compounds.* The carcinogenic activities of the optically active (+)- and (-)-enantiomers of BP 7,8-dihydrodiol and BP 7,8-diol-9,10-epoxide-2 are markedly different and these results are presented elsewhere in this symposium (10).

THE BAY REGION THEORY OF POLYCYCLIC HYDROCARBON CARCINOGENESIS

In an attempt to correlate the structures of polycyclic hydrocarbons with their carcinogenic activities, the literature was surveyed to determine the effects of halogen or alkyl substituents on carcinogenic activity, and it was found that substituents on angular benzo rings of polycyclic hydrocarbons reduce carcinogenic activity, presumably by blocking metabolism at that portion of the molecule (28). Furthermore, perturbational molecular orbital calculations for a number of polycyclic hydrocarbons predicted that benzylic carbonium ions derived from epoxides on an angular tetrahydrobenzo ring of carcinogenic hydrocarbons would have an unusual ease of formation when the epoxide is located in the bay region of the hydrocarbon (29). The prototype of a bay region in a polycyclic hydrocarbon is the sterically hindered region between C-4 and C-5 of phenanthrene. Examples of bay regions and bay region diol-epoxides of polycyclic hydrocarbons are given in Fig. 2. Examination of the properties of the BP 7,8-diol-9,10-epoxides that account for their high biological activity revealed that 9,10-epoxy-7,8,9,10-tetrahydro BP (H₄-BP 9,10-epoxide) is an extremely potent mutagen whereas 7,8-epoxy-7,8,9,10-tetrahydro BP (H₄-BP 7,8-epoxide) is less potent (table 2, ref. 21). In addition, the BP 7,8-diol-9,10-epoxides are much more

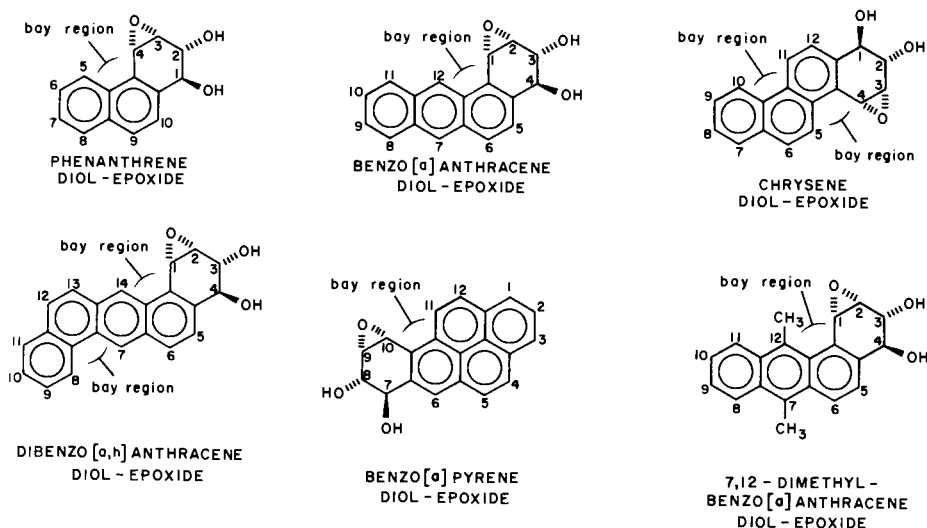


Fig. 2. Structures of bay region diol epoxides.

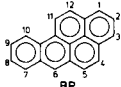









mutagenic than BP 7,10-diol-8,9-epoxide, the BP 9,10-diol-7,8-epoxides, or the BP 7,8- and 9,10-arene oxides (table 2, ref. 21,30,31). These results indicate that a saturated benzo ring and a bay region 9,10-epoxide group in BP are required for high biological activity.

Benzo[a]anthracene - Studies with benzo[a]anthracene (BA) were initiated as a test

of the bay region theory. Even though BA is a weak carcinogen, perturbational molecular orbital calculations indicated an unusual ease of carbonium ion formation at the bay region 1-position for the BA 3,4-diol-1,2-epoxides, when compared with other BA diol epoxides (29,32). The relative ease of carbonium ion formation is indicated by values for $\Delta E_{\text{deloc}}/\beta$ which estimate the change in pi-electron energy which occurs when a diol epoxide on a saturated benzo-ring is converted to a benzylic triol carbonium ion. The value of $\Delta E_{\text{deloc}}/\beta$ at C-1, C-4, C-8 and C-11 of BA are 0.766, 0.628, 0.526 and 0.572, respectively, suggesting that BA 3,4-diol-1,2-epoxides, which possess an epoxide group in the bay region, would be more reactive than diol epoxides elsewhere on the BA molecule. Our prediction that BA 3,4-dihydrodiol, the expected metabolic precursor of BA 3,4-diol-1,2-epoxide, would undergo metabolic activation to mutagens to a greater extent than BA or the other metabolically possible BA dihydrodiols was confirmed. Metabolic activation of BA and of the 1,2-, 3,4-, 5,6-, 8,9- and 10,11-dihydrodiols of BA revealed that BA 3,4-dihydrodiol was metabolized by a highly purified liver microsomal monooxygenase system to products which are at least ten times more mutagenic to *Salmonella typhimurium* strain TA 100 than are the metabolites of BA or the other four metabolically possible *trans* dihydrodiols of BA (33). As expected from the perturbational molecular orbital calculations, the diastereomeric BA 3,4-diol-1,2-epoxides were much more mutagenic than the BA 8,9-diol-10,11-epoxides or

TABLE 2

MUTAGENICITY OF BENZO[a]PYRENE BENZO RING EPOXIDES

 BP	RELATIVE ACTIVITY		
	S. TYPHIMURIUM		V-79 CELLS
	TA 98	TA 100	
COMPOUND			
 7,8-DIOL-9,10-EPOXIDE-1	100	100	40
 7,8-DIOL-9,10-EPOXIDE-2	35	65	100
 7,10-DIOL-8,9-EPOXIDE	<0.1	0.4	<0.1
 9,10-DIOL-7,8-EPOXIDE-1	2	0.4	<0.1
 9,10-DIOL-7,8-EPOXIDE-2	11	1	0.2
 H ₄ -9,10-EPOXIDE	95	90	40
 H ₄ -7,8-EPOXIDE	10	2	0.2
 9,10-ARENE OXIDE	1	0.6	<0.1
 7,8-ARENE OXIDE	1	0.6	<0.1

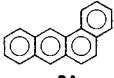
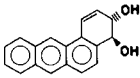
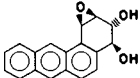
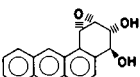
the BA 10,11-diol-8,9-epoxides (34). In analogy to the studies with BP derivatives, H₄-BA 1,2-epoxide (epoxide group in the bay region of the saturated benzo

ring) was significantly more mutagenic than H₄-BA 3,4-epoxide (epoxide group remote from the bay region) (34).

In accord with observations on the mutagenic activity of the BA derivatives, we found that BA 3,4-dihydrodiol was at least 10 times more tumorigenic than BA or the 1,2-, 5,6-, 8,9- or 10,11-dihydrodiols of BA on mouse skin (35). In addition, BA 3,4-dihydrodiol (2800 nmol, i.p.) was at least 30-fold more active than an equimolar dose of the other metabolically possible BA dihydrodiols in causing pulmonary adenomas in newborn mice and this compound also had high activity in causing malignant lymphomas (36). Examination of the tumorigenicity of very low doses of the bay region BA 3,4-diol-1,2-epoxides as initiators of skin tumors in mice revealed that BA 3,4-diol-1,2-epoxides-1 and 2 were at least 10-fold more tumorigenic than BA, and BA 3,4-diol-1,2 epoxide-2 was more tumorigenic than BA 3,4-dihydrodiol (table 3, ref. 37). Injection of 280 nmol of these compounds into newborn mice during their first 15 days of life revealed that BA 3,4-diol-1,2-epoxide-2 was at least 30-fold more active than BA 3,4-dihydrodiol and at least 60-fold more active than BA in causing lung tumors (Table 3, ref. 38). High tumor-initiating activity on mouse skin was also reported by Slaga *et al.* for BA 3,4-dihydrodiol and for BA 3,4-diol-1,2-epoxide-2, but other diol epoxides of BA had little or no tumorigenicity (39). These results, coupled with our recent observation that small amounts of BA 3,4-dihydrodiol are metabolically formed from BA by liver microsomes (40), indicate that BA 3,4-dihydrodiol is a proximate carcinogenic metabolite of BA and that BA 3,4-diol-1,2-epoxide-2 is an ultimate carcinogenic metabolite of BA. Our data suggest that the low carcinogenic activity of BA is caused in part from a low rate of metabolism of BA to BA 3,4-dihydrodiol.

TABLE 3

TUMORIGENICITY OF THE BAY REGION DIOL EPOXIDES OF BENZO[*a*]ANTHRACENE ON MOUSE SKIN AND IN THE NEWBORN MOUSE

HYDROCARBON	SKIN STUDIES ^A	NEWBORN STUDIES ^B
	PAPILLOMAS/MOUSE	LUNG TUMORS/MOUSE
CONTROL	0.03	0.15
 BA	0.07	0.22
 BA 3,4-DIHYDRODIOL	1.30	0.37
 BA 3,4-DIOL-1,2 EPOXIDE-1	0.60	0.56
 BA 3,4-DIOL-1,2-EPOXIDE-2	1.90	13.3

A. CD-1 MICE WERE TREATED ONCE WITH 0.4 μ MOL OF HYDROCARBON, FOLLOWED 7 DAYS LATER WITH BIWEEKLY APPLICATIONS OF 12-O-TETRADECANOYLPHORBOL-13-ACETATE FOR 20 WEEKS.

B. SWISS WEBSTER MICE WERE INJECTED WITHIN 24 HR OF BIRTH AND AT 8 AND 15 DAYS OF AGE WITH 40, 80 AND 160 μ MOL OF HYDROCARBON, RESPECTIVELY. MICE WERE KILLED AT 26 WEEKS OF AGE.

Dibenzo[a,h]anthracene - Dibenzo[a,h]anthracene (DBA) is a strong carcinogen with two equivalent bay regions (Fig. 2). This hydrocarbon is metabolized to significant amounts of the 1,2-, 3,4- and 5,6-dihydrodiols. The bay region theory predicts that one or more of the DBA 3,4-diol-1,2-epoxides are ultimate carcinogenic metabolites of DBA. Metabolic activation of DBA and the 1,2-, 3,4- and 5,6-dihydrodiols of DBA by liver microsomes or by a highly purified monooxygenase system revealed that DBA 3,4-dihydrodiol (precursor of bay region diol epoxides) was activated to products that were more mutagenic to Salmonella typhimurium TA 100 than were the metabolites of DBA or the metabolites of the 1,2- or 5,6-dihydrodiols of DBA (41). With microsomes, but not with the purified monooxygenase system, DBA 5,6-dihydrodiol was also significantly activated to mutagenic products but this only occurred in the presence of a relatively high substrate concentration (41). Saturation of the double bond at the 1,2-position of DBA 3,4-dihydrodiol produced a tetrahydrodiol (H₄-DBA 3,4-diol) which was poorly activated by microsomes or the purified monooxygenase system (41). The high activity of DBA 3,4-dihydrodiol in both metabolic activation systems and the importance of the double bond in the 1,2-position of the dihydrodiol strongly suggests that a bay region 3,4-diol-1,2-epoxide is a biologically important metabolite of DBA. Examination of the tumorigenicity of the 1,2-, 3,4- and 5,6-dihydrodiols of DBA revealed that only the 3,4-dihydrodiol of DBA had significant tumorigenic activity on mouse skin or in the newborn mouse (table 4, ref. 42). Saturation of the 1,2-

TABLE 4

TUMORIGENICITY OF DIBENZO(A,H)ANTHRACENE DIHYDRODIOLS ON MOUSE SKIN AND IN NEWBORN MICE

HYDROCARBON	SKIN STUDIES ^A		NEWBORN MOUSE STUDIES ^B	
	DOSE (NMOL)	PAPILLOMAS/MOUSE	DOSE (NMOL)	LUNG TUMORS/MOUSE
NONE	-	0.10	-	0.2
DBA	10 40 160	0.36 1.40 2.14	70 420	2.9 41.2
DBA 1,2-DIHYDRODIOL	10 40 160	0.14 0.07 0.18	70 420	0.1 0.2
DBA 3,4-DIHYDRODIOL	10 40 160	0.69 1.17 1.52	70 420	1.9 23.3
DBA 5,6-DIHYDRODIOL	10 40 160	0.17 0.17 0.03	70 420	0.1 0.1
H ₄ -DBA 3,4-DIOL	160	0.68	-	-

A. CD-1 MICE RECEIVED A SINGLE TOPICAL DOSE OF COMPOUND FOLLOWED 7 DAYS LATER BY BIWEEKLY APPLICATIONS OF 12-O-TETRADECANOYLPHORBOL-13-ACETATE FOR 25 WEEKS.

B. SWISS WEBSTER MICE WERE INJECTED WITHIN 24 HOURS OF BIRTH AND AT 8 AND 15 DAYS OF AGE WITH 1/7, 2/7, AND 4/7 OF THE TOTAL DOSE OF COMPOUND RESPECTIVELY. MICE WERE SACRIFICED AT 26 WEEKS OF AGE.

double bond of DBA 3,4-dihydrodiol produced a compound (H₄-DBA 3,4-diol) that was considerably less tumorigenic than DBA 3,4-dihydrodiol. Application of 160 nmol of H₄-DBA 3,4-diol to mouse skin resulted in the same number of tumors as 10 nmol of DBA 3,4-dihydrodiol.

Chrysene - Chrysene is a weak carcinogen with two equivalent bay regions (Fig. 2). The bay region theory predicts that chrysene 1,2-diol-3,4-epoxide is an ultimate carcinogenic metabolite of chrysene and that chrysene 1,2-dihydrodiol is a proximate carcinogenic metabolite. Metabolic activation of chrysene 1,2-dihydrodiol by liver microsomes or a purified monooxygenase system resulted in the formation of metabolites that were 20 times more mutagenic to strain TA 100 of Salmonella

typhimurium than were the metabolites formed from chrysene or chrysene 3,4- or 5,6-dihydrodiol (43). When the double bond in the 3,4-position of chrysene 1,2-dihydrodiol was saturated, the resulting tetrahydrodiol (H_4 -chrysene 1,2-diol) could not be metabolically activated to mutagenic metabolites, suggesting that a bay region chrysene 1,2-diol-3,4-epoxide was the active mutagenic metabolite formed from chrysene 1,2-dihydrodiol (43). Studies on the tumorigenicity of chrysene and its metabolically possible dihydrodiols on mouse skin revealed that chrysene 1,2-dihydrodiol had about twice the tumor initiating activity of the parent hydrocarbon chrysene (Fig. 3, ref. 44). The 3,4- and 5,6-dihydrodiols of chrysene had no appreciable tumorigenic activity and H_4 -chrysene 1,2-dihydrodiol had less than 25% of the tumorigenic activity of chrysene 1,2-dihydrodiol. These results suggest that chrysene 1,2-dihydrodiol is a proximate carcinogenic metabolite of chrysene and that a chrysene 1,2-diol-3,4-epoxide, in which the epoxide group is in the bay region of the molecule, is a likely candidate as an ultimate carcinogenic metabolite of chrysene.

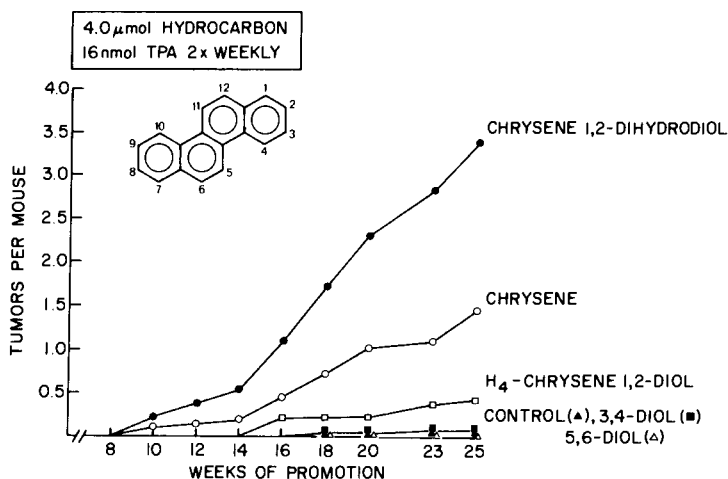


Fig. 3. Tumorigenic activity of chrysene and chrysene dihydrodiols.

Other polycyclic hydrocarbons - Evidence was recently obtained indicating that 7-methyl BA (45,46), 7,12-dimethyl BA (47-49), 5-methylchrysene (50) and 3-methylcholanthrene (49,51-53) undergo metabolism to reactive intermediates at the bay region. The data with 3-methylcholanthrene suggests that there are several biologically active metabolites, and one of these is an immediate metabolic precursor of a bay region diol epoxide (53). Data presented in this manuscript and elsewhere have demonstrated the importance of metabolic activation at the bay region for the carcinogenicity of several polycyclic aromatic hydrocarbons. Indeed, every hydrocarbon which has been examined to date has been shown to meet the predictions of the bay region theory. The rates of formation of biologically active bay-region diol epoxides relative to the rates of formation of other biologically inactive metabolites should play an important role in explaining differences in the carcinogenic activities of different polycyclic hydrocarbons.

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Stereoselective Metabolic Activation of Polycyclic Aromatic Hydrocarbons

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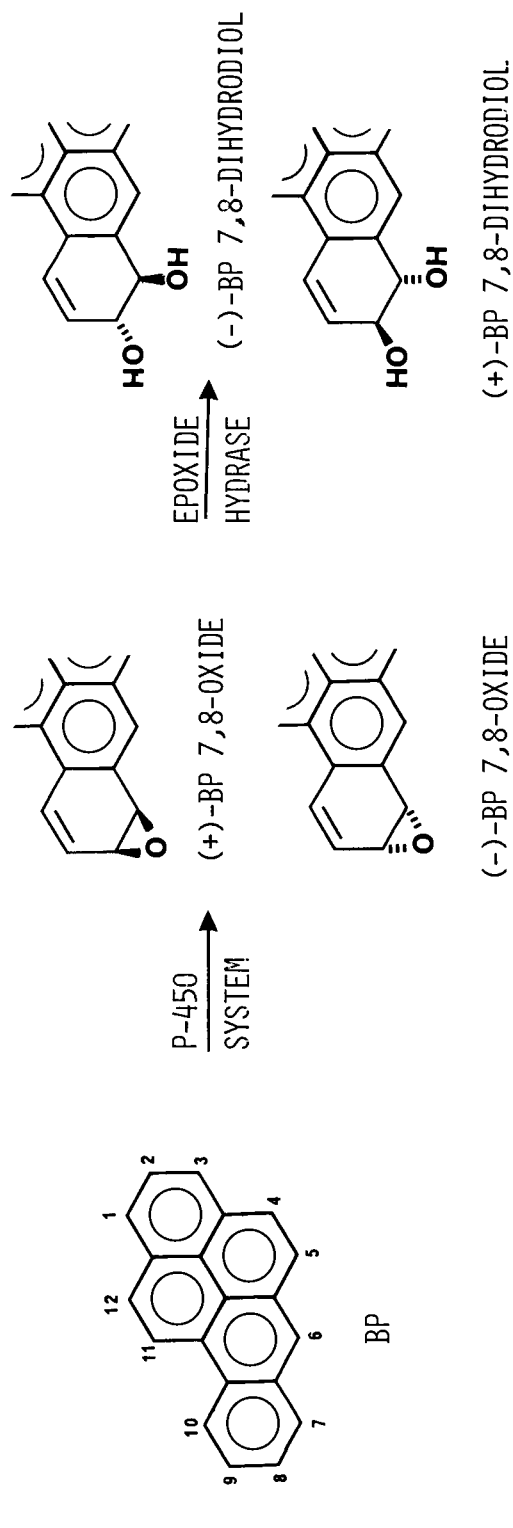
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Although changes in relative and absolute stereochemistry have long been known to result in marked differences in the biological activity of isomeric molecules, little effort has been expended to determine what role such factors play in the expression of carcinogenicity by metabolites of the polycyclic aromatic hydrocarbons. Since drug metabolism in mammals is well recognized to occur with varying degrees of stereoselectivity (1-3), the metabolic formation and disposition of the various metabolites along the pathways which lead to ultimate carcinogenic species might well be expected to occur with diastereomeric and enantiomeric preferences. Questions as to which of such stereoisomers are most extensively formed from the polycyclic hydrocarbons and which of these isomers have the highest biological activity are the subjects of the present chapter.

As discussed elsewhere in this symposium (4), tumor studies have established that "bay region" epoxides of angular benzo-ring dihydrodiols are important ultimate carcinogenic metabolites of the polycyclic hydrocarbons as predicted by the "bay region" theory (5,6). For the hydrocarbon benzo[a]pyrene (BP, Fig. 1.), this pathway consists of initial oxidation to BP 7,8-oxide by the cytochrome P-450 system, hydration of BP 7,8-oxide to the *trans* isomer of BP 7,8-dihydrodiol by epoxide hydrazide, and subsequent oxidation to BP 7,8-diol-9,10-epoxides by the cytochrome P-450 system. Each of the metabolically possible stereoisomers on this pathway has been prepared and has been assigned absolute stereochemistry as indicated in Fig. 1.

RESOLUTION AND ASSIGNMENT OF ABSOLUTE STEREOCHEMISTRY TO BP METABOLITES

BP 7,8-dihydrodiol has proved to be a pivotal compound in the assignment of absolute stereochemistry to the several BP derivatives shown in Fig. 1. Resolution of the 7,8-dihydrodiol into its (+)- and (-)-enantiomers has been achieved by chromatographic separation of diastereomeric diesters; both (-)-menthoxyacetic acid (7) and (-)- α -methoxy- α -trifluoromethylphenylacetic acid (8) have been used as resolving agents. The 7- and 8-mono-esters of (\pm)-*trans*-7,8-dihydroxy-7,8,9,10-tetrahydro BP with (-)- α -methoxy- α -trifluoromethylphenylacetic acid have also been resolved with the advantage that these derivatives are not expected to be carcinogenic (9). Exciton chirality circular dichroism spectra of the bis-N,N-dimethylaminobenzoates of BP 7,8-dihydrodiol (7) and *trans*-7,8-dihydroxy-4,5,7,8,9,10,11,12-octahydro BP (9) have allowed the assignments of absolute stereochemistry shown in Fig. 1. The octahydro derivative has the advantage that the spectral basis for configurational assignment is completely unambiguous.



STEREOMERS OF BP 7,8-DIOL-9,10-EPOXIDES

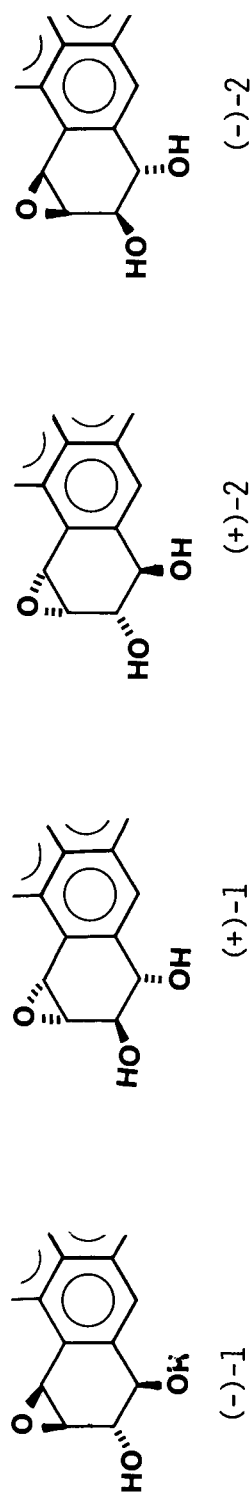


Fig. 1. Absolute stereochemistry of BP metabolites responsible for the carcinogenicity of the hydrocarbon.

Enantiomerically pure BP 7,8-oxide has been prepared by resolution of *trans*-7-hydroxy-8-bromo-7,8,9,10-tetrahydro BP as its esters with (-)-menthoxyacetic acid (10) and subsequent conversion to the desired arene oxides. Assignment of absolute configuration to the arene oxides was achieved by chemical correlation of intermediates in the synthesis with the (+)- and (-)-enantiomers of *trans*-7,8-dihydroxy-7,8,9,10-tetrahydro BP.

The BP 7,8-diol-9,10-epoxides derived from *trans* BP 7,8-dihydrodiol exist as two possible diastereomers in which the epoxide oxygen is either *cis* (isomer-1 series) or *trans* (isomer-2 series) to the benzylic 7-hydroxyl group. Nuclear magnetic resonance studies have allowed unequivocal assignment of relative stereochemistry to these diastereomers (11,12). Synthesis of the optically pure diastereomers of the BP 7,8-diol-9,10-epoxides from the optically pure 7,8-dihydrodiols has allowed the assignments shown in Fig. 1 (9).

TUMORIGENICITY OF (+)- AND (-)-BP 7,8-DIHYDRODIOL

Metabolism of BP by rat liver microsomes is highly stereoselective in the formation of (-)-BP 7,8-dihydrodiol. As shown in Table 1, liver microsomes from control, phenobarbital-treated, or 3-methylcholanthrene-treated Long-Evans rats form 92-96% of the (-)-enantiomer with only 4-8% of the (+)-enantiomer (8,13). Although other workers have found that liver microsomes from 3-methylcholanthrene-treated Sprague-Dawley rats form only the (-)-enantiomer (14), this result has not been confirmed.

TABLE 1 Metabolism of BP to (+)- and (-)-BP 7,8-dihydrodiol by liver microsomes from Long-Evans rats

pretreatment	percent of each enantiomer	
	(-)-[7R,8R]	(+)-[7S,8S]
control	93	7
phenobarbital	92	8
3-methylcholanthrene	96	4

In direct analogy to the enzymatic hydration of naphthalene 1,2-oxide (15), oxygen-18 experiments have indicated that epoxide hydase attacks (±)-BP 7,8-oxide at the 8-position (14,16). These results are consistent with the observation that epoxide hydase is completely selective in the conversion of (+)-BP 7,8-oxide to (-)-BP 7,8-dihydrodiol (13, see Fig. 1.). Since the rates of hydration of (+)- and (-)-BP 7,8-oxide are similar (13), the data suggest that cytochrome P-450 forms mainly (+)-BP 7,8-oxide. Previously, studies on the metabolism of naphthalene and naphthalene 1,2-oxide to the *trans* 1,2-dihydrodiol had indicated that the cytochrome P-450 system forms (+)-[1R,2S]-1,2-naphthalene oxide of less than 10% optical purity (17). This enantiomer is superimposable on (+)-BP 7,8-oxide which has [7R,8S] absolute stereochemistry. Preliminary results of skin tumor studies with CD-1 mice indicate that (+)-BP 7,8-oxide is 3 to 5 times more active as a tumor initiator than is the (-)-enantiomer at 22 weeks of promotion (13).

There are also marked differences in the tumorigenic activity of the (+)- and (-)-enantiomers of BP 7,8-dihydrodiol. Initiation-promotion experiments on the backs of CD-1 mice have shown that the (-)-enantiomer is 5- to 10-fold more potent than the (+)-enantiomer as a tumor initiator (18). The (-)-enantiomer was somewhat

more active than BP when tested at equimolar doses. Even larger differences in the tumorigenicity of these enantiomers were observed when they were injected intra-peritoneally into newborn, Swiss-Webster mice (19, Table 2). The results indicate that the (-)-enantiomer of BP 7,8-dihydrodiol is at least 10 to 20-fold more active than BP or the (+)-enantiomer of the dihydrodiol in causing pulmonary adenomas and lymphomas in these animals.

TABLE 2 Tumorigenicity of the optically pure (+)- and (-)-enantiomers of BP 7,8-dihydrodiol in newborn, Swiss-Webster mice

treatment (dose,nmol)	percent of mice with		lung adenomas per animal
	lymphoma	lung adenoma	
control	0	8	0.10
BP (1400)	0	74	4.1
(+)-BP 7,8-dihydrodiol (140)	0	16	0.16
(700)	3	54	2.3
(-)-BP 7,8-dihydrodiol (140)	4	98	9.3
(700)	55	100	32.2

Newborn, Swiss-Webster mice (60-80 animals) were injected i.p. on days 1,8, and 15 of life with 1/7, 2/7, and 4/7 of the total dose of compound and were sacrificed at 17 weeks of age.

At the doses tested, (+)-BP 7,8-dihydrodiol and BP showed little or no ability to produce malignant lymphomas. The (-)-dihydrodiol was much more toxic than the (+)-enantiomer.

The (+)- and (-)-enantiomers of BP 7,8-dihydrodiol have also been compared as complete carcinogens on mouse skin (13, Table 3). The (-)-enantiomer was at least 10-fold more active than the (+)-enantiomer and had a decreased latency period compared to BP. Thus, in three different tumor models, (-)-BP 7,8-dihydrodiol has been found to be more active than its (+)-enantiomer. The (-)-enantiomer is the predominant stereoisomer produced from BP by rat liver.

TABLE 3 Carcinogenicity of the optically pure (+)- and (-)-enantiomers of BP 7,8-dihydrodiol on the skin of C57/B6 female mice

treatment	percent of mice with tumors at			
	30 weeks	37 weeks	43 weeks	60 weeks
control	0	0	0	0
BP	0	17	61	100
(+)-BP 7,8-dihydrodiol	0	0	0	8
(-)-BP 7,8-dihydrodiol	17	66	79	100

Each treatment group consisted of 30 animals. The minimum survival rate (80%) was found for the group treated with (+)-BP 7,8-dihydrodiol.

TUMORIGENICITY OF THE (+)- AND (-)-ENANTIOMERS
OF THE DIASTEREOMERIC BP 7,8-DIOL-9,10-EPOXIDES

The two diastereomeric BP 7,8-diol-9,10-epoxides are highly reactive molecules which undergo rapid, spontaneous hydrolysis to mixtures of tetraols in water (20). Analysis of the ratio of these tetraols produced on metabolism of BP 7,8-dihydrodiol provides a measure of the extent to which each of the diastereomeric diol epoxides was formed (8,21-23). Liver microsomes metabolize BP and BP 7,8-dihydrodiol at similar rates. The rate of metabolism (nmol product/nmol hemeprotein/min) of both substrates is enhanced by pretreatment of animals with 3-methylcholanthrene but not with phenobarbital (8-21). The regiospecificity of metabolism by liver microsomes varies with pretreatment of the animals (8-21). Microsomes from control and phenobarbital-treated rats form as much as 40% of a phenolic metabolite, whereas 85-95% of the metabolites formed by microsomes from 3-methylcholanthrene-treated animals are derived from the 7,8-diol-9,10-epoxides. Interestingly, liver microsomes from 3-methylcholanthrene-treated rats as well as a highly purified and reconstituted system containing cytochrome P-448 also show the highest stereospecificity on conversion of the enantiomeric BP 7,8-dihydrodiols into the diastereomeric BP 7,8-diol-9,10-epoxides (8, Table 4). Metabolism of the (-)-enantiomer produces mainly diol epoxide-2 (4 to 1) while the (+)-enantiomer produces predominantly diol epoxide-1 (19 to 1) relative to the other diastereomer. The (-)-enantiomer is 1.8-fold more active as a substrate than the (+)-enantiomer, and the average of the rates for the two enantiomers equals that of the racemic substrate. A particularly interesting stereochemical aspect of these metabolism studies is the fact that the major diol epoxide from either enantiomer of the dihydrodiol (see Fig. 1., (+)-diol epoxide-1 and (+)-diol epoxide-2) is produced by epoxidation on the same face of the molecule; i.e., both diol epoxides have [9R,10R] absolute stereochemistry at the epoxide group. The BP diol epoxides covalently bind to nucleic acid in the cell with the exocyclic ²N-amino group of guanine as a principle target (24-26). When cultured bronchial explants were exposed to BP and when BP was applied to mouse skin, adducts of (+)-diol epoxide-2 and, to a lesser extent, adducts of (+)-diol epoxide-1 were found bound to the nucleic acid in these tissues (27,28). Of the four possible BP 7,8-diol-9,10-epoxides (Fig. 1.), the two which are formed most extensively from BP by liver are the two which are found bound to the nucleic acid of target tissue.

TABLE 4 Product stereoselectivity in the metabolism of (±)-, (+)-, and (-)-BP 7,8-dihydrodiol to diol epoxides-1 and -2 by a purified and reconstituted system containing cytochrome P-448

substrate	relative amounts of		rate
	diol epoxide-1	diol epoxide-2	
(-)-BP 7,8-dihydrodiol	19	81	4.64
(+)-BP 7,8-dihydrodiol	95	5	2.57
(±)-BP 7,8-dihydrodiol	41	59	3.70

Rates are expressed as nmol of all products per nmol hemeprotein per min.

The four optically pure isomers of the BP 7,8-diol-9,10-epoxides have been evaluated individually for their mutagenic and tumorigenic activity (Table 5). In Chinese hamster V79 cells, (+)-diol epoxide-2 was from 6 to 18 times as mutagenic as the other three isomers (29). The importance of the stereochemical control of

this mutagenicity is emphasized by the fact that the enantiomeric (-)-diol epoxide-2 was the least active compound in the study. On intraperitoneal injection into newborn, Swiss-Webster mice, (+)-diol epoxide-2 showed exceptional tumorigenicity, whereas BP and the other three optically pure isomers of the BP 7,8-diol-9,10-epoxides had little or no activity at the low dose tested (30). Initiation-promotion experiments on mouse skin have also indicated that (+)-diol epoxide-2 is highly tumorigenic, whereas the other three isomers lacked significant activity (31). Such a high degree of stereochemical control over the biological activity of these enantiomers could not have been anticipated.

TABLE 5 Mutagenicity and tumorigenicity of the optically pure BP 7,8-diol-9,10-epoxides

isomer	mutagenicity in V79 cells (mutant colonies/nmol/10 ⁵ survivors)	lung adenomas/mouse (14 nmol dose)
(-)-diol epoxide-1	60	0.25
(+)-diol epoxide-1	34	0.33
(-)-diol epoxide-2	22	0.12
(+)-diol epoxide-2	400	7.67

MUTAGENICITY OF THE (+)- AND (-)-ENANTIOMERS OF BP 4,5-OXIDE

Although BP 4,5-oxide is very weak as a carcinogen when compared to BP on mouse skin (32), it is the next most mutagenic metabolite of BP after the 7,8-diol-9,10-epoxides (33). Thus, the (+)- and (-)-enantiomers of BP 4,5-oxide provide an additional opportunity to explore the effects of absolute stereochemistry on biological activity (13, Table 6).

TABLE 6 Mutagenic and cytotoxic activity of mixtures of (+)- and (-)-BP 4,5-oxide in Chinese hamster V79 cells

percent total dose		percent cell death	mutation frequency
(+)-BP 4,5-oxide	(-)-BP 4,5-oxide		
100	0	5	2
90	10	15	4
75	25	22	11
50	50	38	18
25	75	46	24
10	90	17	16
0	100	18	12

Cells were treated with a total of 30 nmol of arene oxide in 5 ml of culture medium. Mutation frequency is expressed as 8-azaguanine-resistant colonies/10⁵ surviving cells.

The (-)-enantiomer of BP 4,5-oxide is 6-fold more mutagenic toward Chinese hamster V79 cells than is the (+)-enantiomer with the toxic agent 8-azaguanine as a mutagenic marker. An even more interesting feature of this study was found when mixtures of the two enantiomers were tested for cytotoxic and mutagenic activity. At each of the ratios of the two enantiomers tested (from 1:9 to 9:1), synergistic effects on both the cytotoxic and mutagenic response were observed. Maximum synergism was observed at a ratio 25:75 (-)- to (+)-enantiomer where the mutagenic and cytotoxic effects were at least twice that of the more active (-)-enantiomer. The results suggest that more than one site of reaction is involved in producing the biological activity observed. No such synergism was observed when the (+)- and (-)-enantiomers of the BP 7,8-diol-9,10-epoxides were tested for mutagenic activity in V79 cells (29).

TUMORIGENICITY OF THE (+)- AND (-)-ENANTIOMERS
OF BENZO[a]ANTHRACENE 3,4-DIHYDRODIOL

As predicted by the bay region theory (4-6), the 3,4-dihydrodiol of benzo[a]-anthracene (BA) has been found to be the most tumorigenic of the five metabolically possible dihydrodiols of this hydrocarbon (34). For this reason, we have examined the tumorigenic activity of the (+)- and (-)-enantiomers of BA 3,4-dihydrodiol in initiation-promotion experiments on the backs of CD-1 mice. The (-)-[3R,4R]-enantiomer was found to be at least 5-fold more active as a tumor initiator than the (+)-[3S,4S]-enantiomer (35). Metabolism studies with rat liver microsomes have indeed shown that the 3,4-dihydrodiol is a metabolite of BA (36). Although this dihydrodiol is a minor metabolite (2-4%), it is formed in a sufficient amount to account for the tumorigenicity of BA. More recent studies (13, Table 7) have shown that the more tumorigenic (-)-[3R,4R]-enantiomer is the major enantiomer formed by rat liver microsomes from BA.

TABLE 7 Enantiomeric composition of the dihydrodiols formed from BA by rat liver microsomes

isomer	% (-)-enantiomer	% (+)-enantiomer
3,4-dihydrodiol	67% [R,R]	33% [S,S]
5,6-dihydrodiol	38%	62%
8,9-dihydrodiol	90% [R,R]	10% [S,S]
10,11-dihydrodiol	82% [R,R]	18% [S,S]

Although the 1,2-dihydrodiol is also a metabolite, an insufficient amount was formed to permit accurate determination of its enantiomeric composition.

CONCLUSIONS

Chemically induced cancer is one of the unfortunate aspects of man's existence in an environment of naturally occurring and xenobiotic carcinogens. The present report represents the first attempt to summarize our present knowledge of the biological consequences of the stereoselective metabolism of polycyclic hydrocarbons to ultimate carcinogens. Although the metabolism of foreign chemicals by the non-specific cytochrome P-450 system and epoxide hydase of liver is a necessary aspect of man's existence in his environment, one cannot fail to be impressed by the fact that these enzymes invariably made all of the wrong stereochemical choices in their conversion of BP to an ultimate carcinogen. At each step in the formation of the

BP 7,8-diol-9,10-epoxides, a predominant amount of the more tumorigenic isomer was produced. The reason for this unfortunate series of stereochemical selections is presently unclear. A similar pattern may also exist for BA since the more tumorigenic (-)-[3R,4R]-dihydrodiol of BA is directly superimposable on the more tumorigenic (-)-[7R,8R]-dihydrodiol of BP when the bay regions of the hydrocarbons are aligned. Stereochemical considerations such as those described herein will provide important probes of the nature of the carcinogen "receptor site" as well as the active sites of drug metabolizing enzymes.

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Metabolic Inactivation of Reactive Metabolites

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ABSTRACT

Many compounds which are not electrophilically reactive as such are transformed by mammalian enzymes to reactive metabolites which are, in many cases, responsible for cytotoxic, mutagenic and/or carcinogenic effects of the compounds in question. The essential role of activating systems in this situation has become common knowledge during the last decade. However, many reactive metabolites are also subject to inactivation by mammalian enzymes. This important parameter is frequently not taken into account.

Compounds possessing aromatic or olefinic moieties are very widely occurring and activation of these often proceeds via an electrophilically reactive epoxide. This may be transformed to electrophilically non-reactive diols by epoxide hydratase or to glutathione conjugates by glutathione S-transferases. The former system is limited by the fact that in some special cases (e.g. location of the epoxide at an angular benzoring of a polycyclic hydrocarbon opposite a bay region) the inactivation of the epoxide yields a dihydrodiol which is the precursor of an (even more reactive) dihydrodiol-epoxide. The glutathione S-transferase system is limited by its location in the cytosol fraction, which diminishes its efficiency in inactivating epoxides derived from large lipophilic compounds. Even with compounds where the efficiency of these inactivating systems is limited as discussed above (e.g. benzo(a)pyrene), they still are of decisive importance. Depending on the quantity and quality of monooxygenase forms present in a situation in question, epoxide hydratase can play the role of an inactivating or of a predominantly coactivating system. However, with the majority of epoxides, both the epoxide hydratase and glutathione S-transferase enzymes represent simple and effective inactivating mechanisms.

Activities of activating and inactivating enzymes are often decisive for species differences in toxic effects. As enzyme activities become more accurately known in different species, strains, sexes, organs and developmental stages, they will become of predictive value. However, other mechanisms such as differences in repair or other host defense mechanisms will also contribute substantially to differences in toxic manifestations in these different situations.

INTRODUCTION

The enigma of the way in which chemically inert compounds can exert potent toxic effects has at least partially been solved during the last decade. Inert precursor compounds are transformed into ultimate carcinogens, mutagens and frequently also cytotoxins and allergens by enzymatic conversion to electrophilic metabolites (1). Although the nature of the reactive metabolite(s) produced varies with the structural components of the compound in question they have in

common an electrophilic reactivity.

The discussion in this paper is restricted to aromatic and olefinic moieties. They represent one of the best studied structural element in respect of metabolic activation and inactivation. Moreover, they are especially important since their structural elements occur very widely in foreign compounds. Aromatic and olefinic compounds can be transformed to electrophilically reactive epoxides by microsomal monooxygenases. Due to their electrophilic reactivity such epoxides can covalently bind to nucleophilic centres in DNA, RNA and proteins. Such alterations of critical cellular macromolecules can lead to fundamental disturbances of the normal biochemistry of a cell. Cytotoxic, mutagenic and/or carcinogenic effects can be manifestations of such disturbances (for reviews see ref. 2-9).

PATHWAYS OF BIOTRANSFORMATION OF EPOXIDES

Several pathways of biotransformation of epoxides compete with their reaction with nucleophilic moieties in tissue macromolecules: (a) molecular rearrangement (to phenols in the case of arene oxides, and to aldehydes or ketones in the case of alkene oxides); (b) addition of the elements of water by the action of epoxide hydratase; (c) addition of glutathione by the action of glutathione S-transferases (for reviews see ref. 2-9).

RELATIVE IMPORTANCE OF ENZYMES IN THE CONTROL OF MUTAGENICALLY REACTIVE EPOXIDES

To investigate the relative importance of the individual enzymes contributing to the control of mutagenically reactive metabolites by their formation or further biotransformation, we used an *in vitro* system where the various factors can be individually manipulated (10). Since many epoxides are too unstable for chemical quantitative determination the *Salmonella typhimurium* mutants TA 1535, TA 1537, TA 1538, TA 98 and TA 100 developed by Ames (11) were used as analytical tools. Tissue preparations (typically liver microsomal suspensions) were used for the *in vitro* generation of metabolites (10). Addition or removal of cofactors or of homogeneous enzymes was used for evaluation of the relative roles of the various enzymes (9, 10, 12-15).

This system may give results weighted toward the relatively most stable reactive metabolites, which best survive passage from the microsomal membranes to the bacteria, after generation of short-lived reactive metabolites of lipophilic compounds in the microsomal membranes of such preparations. We therefore investigated whether the physiochemical properties of the surface of the microsomes and bacteria would lead to binding of the two. By electron microscopic studies it could be demonstrated that a tight binding actually occurs (13). In the eucaryotic cell lipophilic reactive metabolites which are generated in those parts of the endoplasmic reticulum which are in continuous contact with the nuclear membrane can reach the latter by simple lateral diffusion within the lipid matrix of the membrane. The system used is analogous to the situation in eucaryotic cells in that lipophilic metabolites may reach the bacterial membrane by simple diffusion within the lipid matrix of the microsomes.

If the chemically inert premutagen benzo(a)pyrene is introduced into the above described system in the absence of microsomes or of the cofactor NADPH required for monooxygenase activity no increase above the spontaneous reversion rate is observed. However, in the presence of microsomes and NADPH transformation to mutagenically reactive metabolites occurs, manifested by a marked increase in the reversion rate (16). Compounds which are composed exclusively of aromatic rings such as benzo(a)pyrene may be considered as models for aromatic and olefinic

moieties in more complex molecules.

If pure epoxide hydratase (17,18) is added to the system in order to titrate out reactive epoxides which are substrates of the enzyme, the results depend on the quantity and pattern of monooxygenase forms present in the microsomes which are used for activation (14). If liver microsomes from untreated mice are used epoxide hydratase can reduce the mutagenic effect by 75 - 98 %. After pretreatment of the animals with phenobarbital the situation is similar. However, 3-methylcholanthrene pretreatment fundamentally changes the situation. Epoxide hydratase now has a weak and multiphasic effect (14).

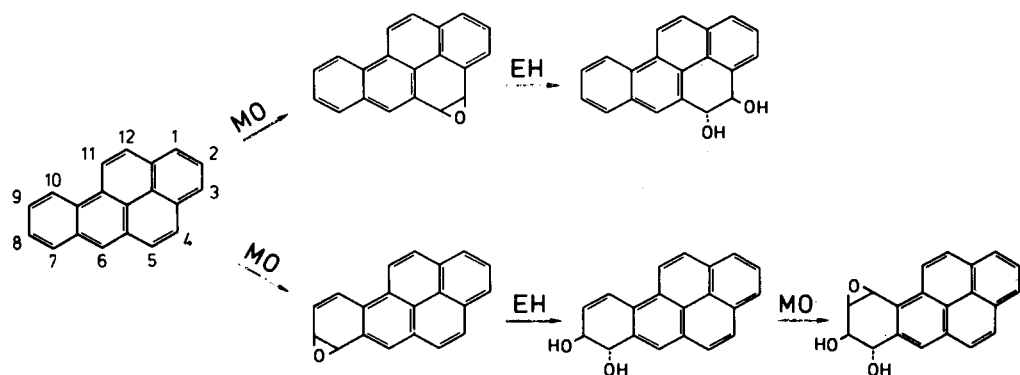


Fig. 1: Two major pathways of benzo(a)pyrene metabolism. Upper pathway: formation of 4,5-oxide followed by inactivation to 4,5-dihydrodiol; lower pathway: formation of 7,8-oxide followed by inactivation to 7,8-dihydrodiol representing at the same time biosynthesis of the precursor of the even more reactive 7,8-dihydrodiol 9,10-oxide. MO, monooxygenase; EH, epoxide hydratase.

The difference in the relative accumulation of different mutagenically reactive metabolites in these situations is caused by a combination of two factors: (1) Control and phenobarbital-induced cytochrome P-450-dependent-monooxygenase forms preferentially attack the benzo(a)-pyrene molecule at the 3,4 and 5-positions (Fig. 1, upper pathway) whilst 3-methylcholanthrene induced cytochrome P-448 (P1-450)-dependent monooxygenase forms preferentially attack the benzo(a)pyrene molecule at the 7,8,9 and 10-positions (Fig. 1, lower pathway) (19-21). The greatly increased monooxygenase activity after 3-methylcholanthrene-induction additio-

nally favors the pathway indicated in the lower part of Fig. 4: The extra activity allows the second monooxygenation more effectively to compete with other pathways. In the pathway depicted in the upper part of Fig. 1, epoxide hydratase plays a simple inactivating role; in the pathway indicated in the lower part a dual role: inactivating the precursor epoxide in the 7,8-position but simultaneously yielding the precursor dihydrodiol for the biosynthesis of the extraordinarily reactive 7,8-dihydrodiol-9,10-epoxide which is not inactivated by epoxide hydratase (14, 22-24). This shows that the relative quantities of the different monooxygenase forms represent one important contributing factor to the pattern of mutagenically reactive metabolites derived from aromatic hydrocarbons, to their relative accumulation and to the roles of inactivating enzymes. In the first two metabolic situations (control and phenobarbital-induced liver microsomes) epoxide hydratase acts efficiently as an inactivator. In the last situation (3-methylcholanthrene-induced liver microsomes) epoxide hydratase has only a weak and multiphasic effect which includes activating phases.

DIHYDRODIOL DEHYDROGENASE, A PROTECTIVE ENZYME COMPLEMENTARY TO EPOXIDE HYDRATASE

Since we could not find any enzyme inactivating the 7,8-dihydrodiol-9,10-epoxide, we tried to sequester the metabolic precursor 7,8-dihydrodiol. Benzene dihydrodiol can be converted to catechol by a dihydrodiol dehydrogenase (25). Dehydrogenation of benzo(a)pyrene 7,8-dihydrodiol might compete with dihydrodiol-epoxide formation. However, the enzyme uses pyrimidine nucleotide cofactors as does the monooxygenase system required for bioactivation. The role of this enzyme could therefore not be studied by simple cofactor removal or addition. Thus it was necessary for the enzyme to be isolated in pure form.

In the metabolic situation where the action of epoxide hydratase was weak and complex (3-methylcholanthrene-induced liver microsomes) pure dihydrodiol dehydrogenase could effectively reduce the mutagenic effect of metabolically activated benzo(a)pyrene (Fig. 2).

Thus it depends on the monooxygenase forms and activity present which types of mutagenically reactive metabolites are formed. If amongst these metabolites monofunctional epoxides predominate, epoxide hydratase represents an efficient inactivating enzyme, but if dihydrodiol epoxides predominate it does not. In the latter situation it could be demonstrated that another enzyme, dihydrodiol dehydrogenase does provide protection. These two enzymes therefore complement each other in their protective action.

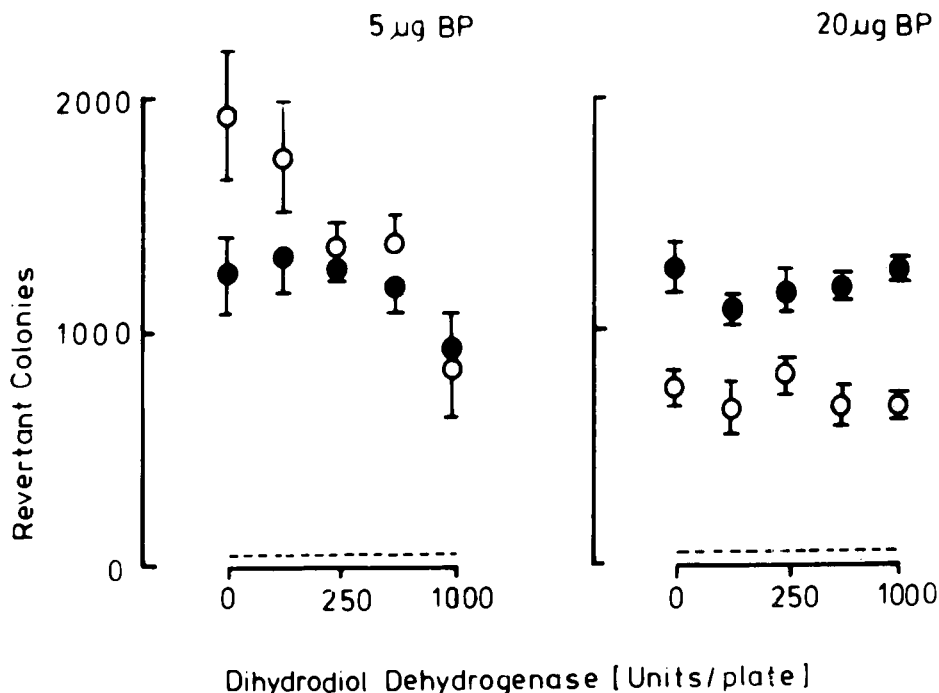


Fig. 2: Dihydrodiol dehydrogenase-mediated reduction of the mutagenicity of benzo(a)pyrene activated by microsomes from 3-methylcholanthrene-treated mice. *Salmonella typhimurium* TA 98 were incubated with a low (5 µg BP) or high (20 µg BP) concentration of benzo(a)pyrene in the presence (●) or absence (○) of the epoxide hydratase inhibitor 1, 1, 1-trichloropropene oxide (1 mM). His⁺ revertant colonies were counted after two days. Values represent means ± S.D. Horizontal dashed lines represent the mean of spontaneous mutations in the absence of benzo(a)pyrene.

CONCLUSION

As a model for aromatic and olefinic moieties of more complex molecules a compound exclusively composed of aromatic rings was studied. Thus links between the metabolism of such moieties and mutagenic effects were investigated whilst interaction with other structural elements was not taken into consideration.

Chemicals possessing several fused aromatic rings and an angular structure are metabolized to many derivatives. Several of them are mutagenic but contribute very little to the total mutagenicity, whilst the two major groups of mutagenically reactive metabolites are: monofunctional arene oxides and dihydrodiol-epoxides.

Various monooxygenase forms exist which differ in their preferred site of oxidative attack on

large molecules. The monooxygenase forms which are present will in part dictate whether benzo(a)pyrene is predominantly epoxidized at the 4,5-position (monofunctional epoxide) or at the 7,8,9,10-position (dihydrodiol-epoxide). A further contributing factor is the relative monooxygenase activity. Higher activity favors the second monooxygenation necessary for dihydrodiol-epoxide formation over other competing pathways.

In metabolic situations where monofunctional arene oxides represent the majority of the mutagenically reactive metabolites, epoxide hydratase efficiently inactivates; if dihydrodiol-epoxides predominate, it does not. In the latter situation another enzyme was found which exerts protective effects complementary to those of epoxide hydratase dihydrodiol dehydrogenase.

The enzyme activities investigated play an important role, yet they only partially determine the final toxic effects. Other metabolizing enzymes also contribute. After occurrence of the primary lesion differences in repair capacity and other host defence mechanisms will also contribute to differences in toxic manifestations.

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Factors Influencing DNA Damage and its Repair with Cellular Implications for Toxicology*

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DNA is recognized as an important target molecule for several types of toxic agents including carcinogens, mutagens and many necrogenic agents useful in cancer chemotherapy. Although the role of altered DNA in the genesis of cancer remains to be established, there is increasing circumstantial evidence implicating it in the complex multifactorial pathologic process.

However, it is becoming evident that the interactions of carcinogens with DNA are subject to major modulations that may well determine the subsequent biological consequences of such alterations in DNA. In this presentation, we would like to discuss some of these aspects, particularly the complexity of the structure and organization of DNA in chromatin in relation to (a) interaction of carcinogens with the DNA in chromatin resulting in 'damaged' DNA, (b) the repair of such potential 'damage', and (c) some of the factors which influence the conversion of chemical damage to DNA into cellular damage. In this context, it is also important to distinguish between the chemical damage to DNA *per se* as a consequence of carcinogen interaction and cellular damage resulting from its biological effects. For example, with methylating agents such as N-methyl-N-nitrosourea (MNU) or with low doses of ethylating agents such as diethylnitrosamine (DEN), one can induce extensive chemical damage without obvious cellular damage. However, if these same alkylating agents are given in an environment of proliferating hepatocytes, serious toxicological consequences occur.

Interactions with DNA

Carcinogens, either directly or after suitable metabolic activation, can interact with both the purines and pyrimidines in the DNA and in some cases with the phosphate as well (1-3). One or more carcinogens have been shown to alkylate, arylate or otherwise interact with one or more available sites in the bases, e.g., N⁷, N³, O⁶, C⁸ and 2-NH₂ of guanine; N⁷, N³, N¹ and 6-NH₂ of adenine; O⁴ of thymine and N³, N¹ and C⁴-NH₂ of cytosine (1-3). However, no specific chemical interaction has been found common to all the different mutagens and carcinogens studied so far.

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It is, therefore, premature to generalize and single out any one chemical event per se and correlate it with cellular damage. This difficulty is further highlighted by the recent data with the potent carcinogen aflatoxin B₁, in which the major interaction is with the N⁷ position of guanine (4), a position less favored for consideration as an important target site in mutagenesis and carcinogenesis with methylating and ethylating agents (5).

Preferential Interaction of Carcinogens with Different Regions of Chromatin-DNA

In addition to the pure chemical factors that influence the sites of interaction of carcinogens with DNA, the availability of these sites in chromatin-DNA is determined by the several hierarchies of organization of the DNA in chromatin. One level of organization resulting from the association of DNA with histones gives the repeating unit structures, the nucleosomes (6-8). Further coiling and supercoiling of these unit structures, together with the association of nonhistone proteins, results in the highly complex chromosome structure in which the DNA is ultimately contracted by a factor of 10,000 (9) and its availability for transcriptional and other enzymes is strictly regulated (10,11). It is, therefore, not unreasonable to visualize that this same organizational complexity would impose constraints on the availability of DNA for carcinogen interaction as well as repair enzymes. This is evident from our recent work on the nonrandom nature of carcinogen interaction with chromatin-DNA (12-14) and the selective effects of spermine and distamycin A on the methylation of chromatin-DNA by MNU and MMS (15-17). A brief discussion of some of these results on the preferential interaction of carcinogens with different regions of chromatin-DNA will be presented here.

Several methods have been developed to determine whether there are preferred sites in DNA for carcinogen interaction (see Table 1).

TABLE 1 Probes Useful in Determining the Preferred Sites of Interaction of Carcinogens with Chromatin-DNA

1. Nuclease accessibility of DNA in Chromatin:

- | | |
|--------------------------|--|
| (a) Micrococcal nuclease | :Digests the linker regions of chromatin-DNA (11) |
| (b) DNase I | :Digests transcribable genes (18) |
| (c) DNase II | :Fractionates transcribable from nontranscribable genes (19) |

2. Fractionation of eu- from hetero-chromatin

:Gradient centrifugation (20)

3. Compounds that interact with specific regions of DNA (minor groove of DNA):

- | | |
|--------------------------------|--|
| (a) Spermine | (interacts with PO ₄ ⁻⁻⁻)(21) |
| (b) Distamycin A and Netropsin | (interact with A-T base pairs)(22) |
| (c) Actinomycin D | (interacts preferentially with GC base pairs)(23) |
| (d) Phleomycin (24) | |

These studies revealed that carcinogens do exhibit preferential interaction with some regions of the DNA in chromatin (Table 2)(12,13,25-30); e.g., dimethylnitrosamine (DMN) methylates predominantly DNase I digestible regions of chromatin-DNA (12,13) while N-hydroxy-2-acetylaminofluorene (14) and bromomethylbenz(a)anthracenes (29) interact preferentially with the DNase I nondigestible regions. N-acetoxy-2-acetylaminofluorene (27) and benz(a)pyrene metabolites (28) in vitro exhibit preferential interaction with the micrococcal nuclease digestible regions of chromatin-DNA, presumably representing linker regions. Using yet another tool, gradient centrifugation technique, it was observed that N-hydroxy-2-acetylaminofluorene (31-33), 3-methylcholanthrene and 1,2,4,6-dibenzanthracene (34) interact predominantly with euchromatin.

TABLE 2 Preferential Interaction of Chemical Carcinogens in Regions of Chromatin-DNA Detectable by Nuclease Digestion

<u>Carcinogen</u>	<u>Predominant Interaction In</u>	
	<u>DNase I</u>	<u>Micrococcal Nuclease</u>
Dimethylnitrosamine (in vivo)	Accessible (12,13)	-
N-hydroxy-2-acetylaminofluorene (in vivo)	Inaccessible (14,26)	Accessible (26)
N-acetoxy-2-acetylaminofluorene (in vitro)	-	Accessible (27)
Benz(a)pyrene metabolites (in vitro)	-	Accessible (28)
7-Bromo-methylbenz(a)anthracene (in vitro)	Inaccessible (29)	-
7-Bromo-methyl-12-methylbenz(a)anthracene (in vitro)	Inaccessible (29)	-

A further specificity apparently exists in the distribution of the individual alkylated bases. Although DMN methylates guanine in DNA, yielding N⁷-methylguanine (N⁷meG) and O⁶-methylguanine (O⁶meG), O⁶meG in rat liver chromatin is preferentially located in the micrococcal nuclease digestible regions, possibly representing linker regions. Such preferential distribution of N⁷meG, however, is not obvious (Table 3). These results suggest that the guanines susceptible for methylation by DMN at O⁶ position and at N⁷ position are distributed differently along the DNA.

Another approach we have recently initiated to determine whether chemical carcinogens exhibit preferential interaction with certain regions of chromatin-DNA is to study the effect of some agents that specifically interact with the minor groove of DNA and stabilize its structure on the interaction of chemical carcinogens with chromatin-DNA. The agents selected were spermine and distamycin A. Both these agents inhibited the formation of N⁷meG in the DNA by MNU but not that by methylmethane sulfonate (MMS)(Fig. 1). Based on the following facts, viz., (i) spermine interacts with phosphate groups and distamycin A with A-T base pairs, but both compounds interact at the minor groove (21,22); (ii) both agents increase thermal melting of DNA (35,36); and (iii) the helical structure is stabilized in A-T rich region (21,37), it was concluded that MNU methylates regions of DNA that are readily denaturable, probably rich in A-T base pairs located at or close to the binding site of spermine and distamycin A, possibly in the minor groove (15,16). On the contrary, MMS may methylate randomly throughout the DNA (17).

Even though both MNU and MMS yield N⁷meG, its distribution along the DNA is differ-

ent, depending on whether it is derived from MNU or from MMS. If this proves to be a general phenomenon, then, although several carcinogens yield the same chemical lesion in DNA, the biological potential of the lesion may vary depending on its intragenomic distribution.

TABLE 3 Preferential Distribution of O^6 -methylguanine in the Micrococcal Nuclease Digestible Regions of *in vivo* DMN-Methylated Rat Liver Chromatin-DNA

DNA digested ($A_{260\text{nm}}$) (percent of total chromatin-DNA)	N ⁷ meG and O ⁶ meG Released (% of Total in Chromatin-DNA) [*]	
	N ⁷ meG	O ⁶ meG
1 ± 0.3	4 ± 0.6	15 ± 0.5
9 ± 2.3	11 ± 0.8	26 ± 2.6
17 ± 1.8	19 ± 1.4	41 ± 10.9
30 ± 4.5	27 ± 4.2	47 ± 3.8
48 ± 4.3	50 ± 7.5	65 ± 5.9

*Four hours after the administration of ^{14}C -DMN (55 $\mu\text{Ci}/0.5\text{ mg}/100\text{ g}$ body weight; I.P.) rats were killed and the liver chromatin was prepared (16). Digestion of rat liver chromatin-DNA with micrococcal nuclease and determination of $A_{260\text{nm}}$, O⁶meG, N⁷meG in the material that was rendered acid soluble by the nuclease, in the acid precipitated material and in the total chromatin-DNA have been described earlier (13,16). Values are corrected for acid blanks and are the average of 3 experiments ± S.D.

Factors which Influence the Expression of Chemical Damage in DNA in Terms of Cellular Damage

Chemical damage to DNA in itself is not sufficient to account for cellular damage. Other factors such as repair and replication play a significant role in this process. Mammalian cells, either *in vivo* or *in vitro*, have the capacity to remove the DNA-bound carcinogen or its metabolite and repair the carcinogen-induced lesions in DNA (38-40). A persistence of unrepaired damage in DNA represents a potential mutagenic or carcinogenic hazard and therefore repair of such lesions becomes an important factor in determining the biological consequences of chemical damage. However, very few studies have been carried out to determine the kinetics of removal of DNA-bound carcinogen or its metabolite (13,14,26,32,33) and the subsequent repair of DNA damage (41,42) from different regions of chromatin-DNA. These studies demonstrate that the DNA-bound carcinogen or its metabolite is removed faster from some regions of chromatin-DNA than others, thereby indicating that the availability of the DNA in chromatin to repair enzymes is also subject to restrictions imposed by the hierarchy of folding of DNA in the nucleus.

Another significant factor involved in the conversion of chemical damage into cellular damage is the replication of DNA with unrepaired damage through cell proliferation. We shall briefly discuss the results of our experiments which implicate the replication of damaged DNA in subsequent cellular damage. Recently, Solt and Farber (43) have developed an experimental model where putative preneoplastic lesions can be induced in rat liver following the administration of necrogenic doses of DEN. It may be postulated that under these conditions of compensatory liver cell proliferation following necrosis, the DEN-induced unrepaired damage is fixed in the daughter strand, a process that may be important in the initiation phase of the development of preneoplastic lesions and ultimate liver cell cancer. Supportive evidence for this concept comes from the recent observations that admin-

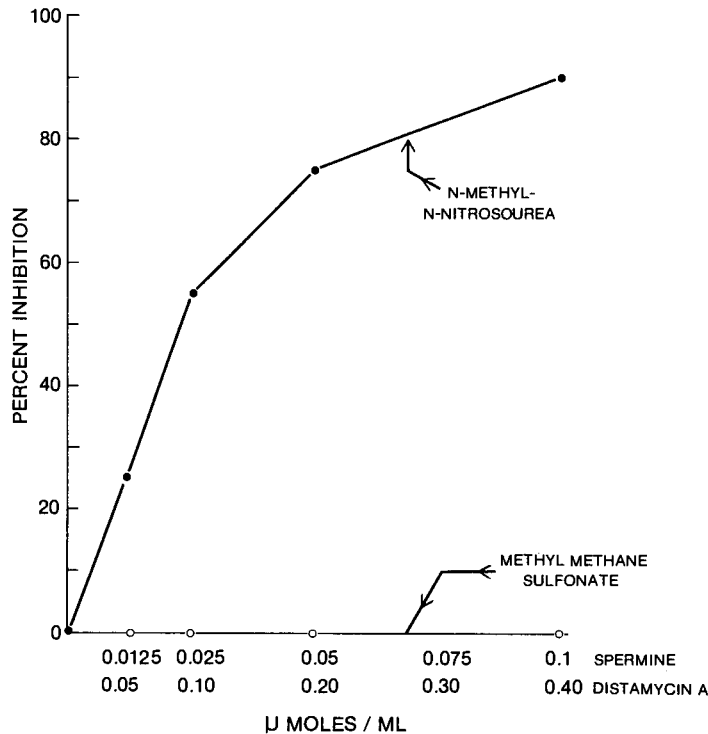


Fig. 1. Selective inhibition by spermine and distamycin A of the formation of N⁷-methylguanine in chromatin-DNA by MNU and not by MMS.

Chromatin (150-200 μg DNA/ml) was incubated in a total volume of 4 ml containing 0.01M Tris-HCl pH 7.8, either with ¹⁴C-MNU (2 μmoles/ml; sp.act. 1.7 x 10⁶ DPM/μmole) for 2 or 18 hr at 37°. The pH of the incubation mixture was maintained constant throughout the reaction at 7.8 using dilute sodium hydroxide. Freshly prepared aqueous solutions of spermine hydrochloride (pH 7.8) or distamycin A were used. Isolation of methylated DNA and the determination of N⁷-methylguanine in the methylated DNA were detailed earlier (16).

istration of diethyldithiocarbamate 4 or 8 hr after DEN administration inhibited not only the DEN-induced necrosis (44), but also the incidence of the putative preneoplastic lesions in rat liver (unpublished observations, T.S. Ying). Equally convincing evidence was obtained using nonnecrogenic doses of another carcinogen, MNU. Putative preneoplastic lesions were seen only in the livers of those rats that were partial hepatectomized 3 hr following the administration of MNU and not in the livers of rats that were given either MNU alone or given MNU and sham operated (45).

Although the precise role of cell proliferation in this process of cellular damage is not fully understood, the results are compatible with the hypothesis that rep-

lication of DNA with unrepaired damage helps fix the damage in the daughter strand, a step that may be obligatory in the toxicologic, mutagenic and carcinogenic process in the cell.

The specific significance of these observations concerning the distribution of carcinogen interaction in chromatin-DNA and the differential rates of subsequent removal of DNA-bound carcinogen from different regions as influenced by the organization of the chromatin is not evident at this time. However, the findings are compatible with the concept that carcinogens and mutagens may have a selective effect on some genes and not on others when the genetic material is in its more natural state as chromatin. A knowledge of specific regions of DNA with which carcinogens or their metabolites predominantly interact may also help to devise chemotherapeutic agents that specifically compete for these specific regions. The observations that distamycin A and spermine inhibit the *in vitro* methylation of DNA by MNU and not by MMS offer hope for such an approach.

It is equally important to devise more sensitive probes to determine the structural and conformational requirements and the organizational restrictions imposed on the DNA in chromatin which regulate its accessibility to carcinogen interaction and DNA repair enzymes.

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New Aspects of Nitrosamine-Induced Carcinogenesis

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Since the carcinogenic action of dimethylnitrosamine for the rat liver was reported by Magee and Barnes in 1956 (1) and for the rat kidney by the same authors in 1962 (2), more than one hundred different N-nitroso compounds have been shown to be carcinogenic (3,4). The earlier work was done by Druckrey, Schmahl, Preussmann, Ivankovic and their colleagues (5) and more recently Lijinsky and his coworkers (6,7,8), as well as several other groups, have added to the list. Many N-nitroso compounds are powerfully mutagenic (9,10) and several have been shown to be teratogenic (11,12). The compounds are cytotoxic when administered in relatively higher doses, the nitrosamines being primarily hepatotoxic (13) while the nitrosamides attack tissues with rapid cell turnover and several 2-chloroethyl derivatives are extensively used in cancer chemotherapy (14). The chemical structures of some carcinogenic N-nitroso compounds are shown in Fig. 1. Relatively few nitrosamines have been reported to be non-carcinogenic. These include diphenylnitrosamine, tert butylethylnitrosamine (5) and N-nitroso proline (15, 16).

Of the compounds shown in Fig. 1 the nitrosamines are thought to require metabolic activation by enzymes for their various biological actions, while N-methylnitrosourea and N-methyl-N-nitro-N-nitrosoguanidine do not have this requirement. Several nitrosamines and other N-nitroso compounds have been shown to decompose in the body to yield alkylating intermediates which react with cellular components, including nucleic acids and proteins, and there is increasing evidence, which will be discussed further, that the biological actions of the nitroso compounds are mediated by these alkylating products.

A wide range of species, including mammals, birds, amphibians and fish are susceptible to carcinogenesis by one or more nitroso compounds (17). Some of these are listed in Table 1. Recent studies on the induction of embryonal neoplasms in the opossum (18) are of great interest. Single or repeated doses of ethylnitrosourea, administered to the animals when in the pouch, resulted in the induction of a variety of embryonal neoplasms of the eye, liver, brain, kidney, muscle and jaw which closely resembled their human counterparts, e.g., retinoblastoma and nephroblastoma (Wilms tumor).

Organs in which tumors have been induced by one or more N-nitroso compound are listed in Table 2. The high degree of organ specificity of some of the compounds is well shown by the esophagus, the bladder, and the exocrine pancreas, as well as the kidney, the islets of Langerhans and the nervous system. The latter three organ specificities will be discussed in greater detail. Organotropy for the esophagus

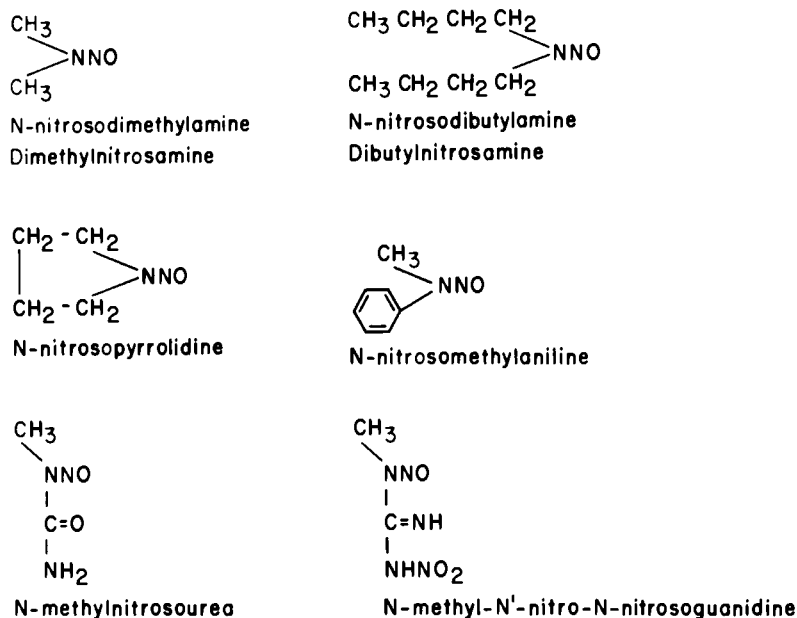


Fig. 1 Some carcinogenic N-nitroso compounds

is shown by a variety of unsymmetrical nitrosamines (5), the specificity for this organ being virtually complete for N-phenylmethylnitrosamine (N-nitrosomethylaniline) and N-benzylmethylnitrosamine, even after administration by routes other than the oral. Dibutylnitrosamine induces bladder tumors in rats (5) and other rodents (4) with some liver and esophageal tumors. However, butyl(4-hydroxybutyl)nitrosamine is completely specific for the bladder. Dibutylnitrosamine and its metabolites have been extensively studied by Hashimoto, Okada and their colleagues who have demonstrated rapid and selective induction of bladder cancer in rats with ethyl(4-hydroxybutyl)nitrosamine (19) and by the intravesicular instillation of butyl(4-hydroxybutyl)nitrosamine and its principal urinary metabolite, butyl(3-carboxypropyl)nitrosamine (20). It was further shown that epithelial cells of rat urinary bladder could be transformed *in vitro* by exposure to butyl(3-carboxypropyl)nitrosamine (21). Tumors of the exocrine pancreas are induced in high yield by 2, 2'-dihydroxy-di-n-propyl-nitrosamine, (N-nitroso-bis (2-hydroxypropyl)amine) (22) and by N-nitroso-bis (2-oxopropylamine) (23). The specificity is not complete as tumors are also induced at other sites. It is of interest that exocrine pancreatic tumors could also be induced by N-nitroso-bis(2-acetoxypopyl)amine after single as well as repeated subcutaneous injections (24). The significance of acetoxy derivatives of nitrosamines will be further discussed.

Several N-nitroso compounds, including dimethyl- and diethylnitrosamine, are known to induce tumors of the kidney in rats after single large doses. N-nitrosomorpholine causes renal tumors in this species after limited exposure (4). If the rats are given a protein deficient diet for a short period before and after the single dose of the nitrosamine the incidence of the mesenchymal tumors can be increased to 100%, with epithelial tumors in about 30% of the surviving animals (25,26). In the rat, the renal neoplasms are of two types, adenomas and adenocarcinomas, clearly of epithelial origin, and a distinct type of highly invasive non-epithelial tumor (2). Under these conditions a smaller number of tumors may appear in the lung but usually none elsewhere. The development of these neoplasms from the first hours after

TABLE 1 Species Susceptible To Carcinogenesis by
N-Nitroso Compounds

<u>Mammals</u>		<u>Birds</u>
Monkey	Syrian Hamster	Fowl Grass Parakeet
Rat	Chinese Hamster	<u>Fish</u>
Mouse	European Hamster	Rainbow Trout
Guinea Pig	Opossum	Various Aquarium Fish
Rabbit	Dog	<u>Amphibia</u>
	Pig	Newt Frog

TABLE 2 Organs in which Tumors Have Been Induced by
One or More N-Nitroso Compounds

Liver	Nervous System	Brain Cord Peripheral
Kidney	Mammary Gland	
Bladder	Pancreas	Exocrine Islets of Langerhans
Esophagus	Skin	
Fore Stomach	Subcutaneous	
Glandular Stomach	Heart	
Small Intestine	Bone	
Large Intestine	Ovary	

carcinogenic treatment up to the appearance of the established tumors has been intensively studied by Gordon Hard and his colleagues (27,28,29). Using an in vivo/in vitro model, it has been shown that cells with altered properties can be cultivated from the kidneys of exposed animals during the first day after treatment and that these cells show the properties of malignant transformation in vitro after five subcultures (30). The transformed cells give rise to malignant neoplasms after implantation into syngeneic hosts. The growth pattern of the transformed cells after injection into the kidneys of recipients appears closely similar to that of the primary tumors (31). These findings are interesting because they clearly indicate that some change has occurred in the cells of the target organ within hours of the administration of the single dose of the carcinogen which results in an irreversible process leading to cancer.

Another remarkable example of organotropism is the production of tumors of the islets of Langerhans by the glucosyl derivative of N-methylnitrosourea, streptozotocin (N-D-glucosyl-(O)-N'-nitrosomethylurea). This agent was isolated from mold cultures and investigated as a possible antibiotic. It was early found to cause

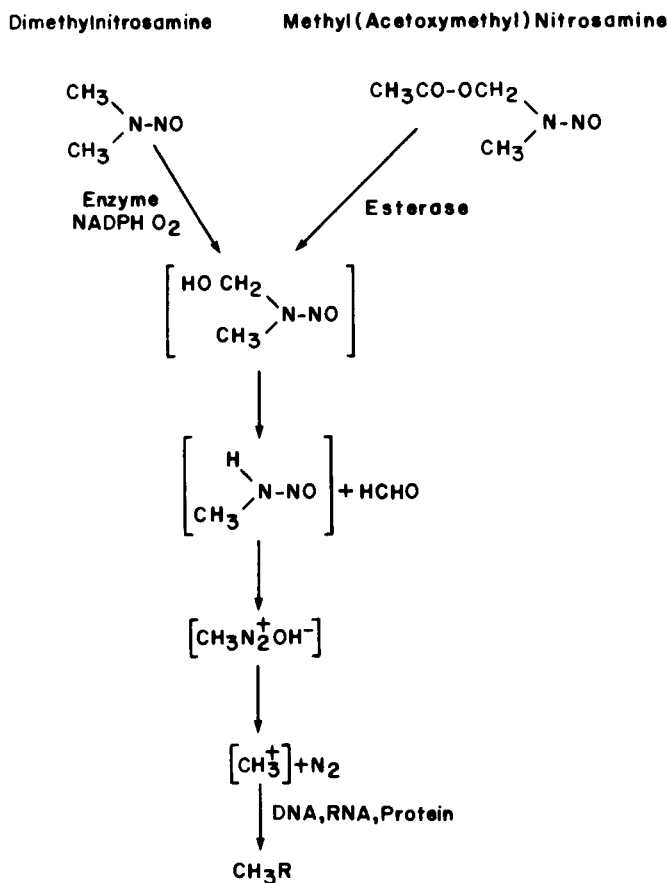


Fig. 2 Suggested metabolic pathways of dimethylnitrosamine and methyl(acetoxyethyl)nitrosamine

diabetes in rats and other rodents and to induce renal tumors in rats. The diabetogenic effects of streptozotocin in rats (32) and in monkeys and dogs (33) can be prevented by pre- and post treatment of the animals with nicotinamide. The work in rats led to the very interesting findings that tumors of the pancreatic islets of Langerhans could be induced in this species by the combined action of streptozotocin and nicotinamide as above (34). Recent work by Tjälve and his colleagues (35), using whole body autoradiographic techniques, has shown that [^{14}C] streptozotocin, is taken up and retained by islets of Langerhans to a greater extent than by any other organ in mice, following intravenous injection.

METABOLISM OF N-NITROSO COMPOUNDS

A suggested metabolic pathway of dimethylnitrosamine is shown in Fig. 2, together with that for N-acetoxyethyl-methylnitrosamine. Dimethylnitrosamine, in doses sufficient to induce hepatic necrosis and kidney tumors in some of the surviving animals (30 mg/kg body wt.) is quite rapidly metabolized in the rat such that most, if not all, of the compound is degraded within 24 hr. following its injection (36). Post-mitochondrial and microsomal fractions of rat liver have been shown to metabolize demithylnitrosamine *in vitro*, with requirements for oxygen and reduced pyridine nucleotides and the enzymes involved have been widely assumed to be mixed function oxidases dependent on cytochrome P-450. It has been clear, however, for

some years, that the activity of these liver preparations was considerably less with dimethylnitrosamine as substrate than with compounds such as aminopyrine (37,38) and there is some disagreement in the results reported from different laboratories (39), which may reflect the lack of uniformity in the concentrations of dimethylnitrosamine that have been used (40). In recent years, however, Lake and his colleagues have questioned whether the initial activation step is mediated by a P-450 enzyme system at all or whether another type of enzyme system is involved, at least in part. These workers have demonstrated that methanol, as well as formaldehyde, is produced by the *in vitro* metabolism of dimethylnitrosamine and that several inhibitors of alcohol metabolism, including pyrazole and 3-amino-1,2,4-triazole profoundly inhibited the formation of these products (41). Further work showed that the stability in the frozen state of the hepatic preparations catalysing the decomposition of dimethylnitrosamine was considerably greater than that for typically P-450-dependent mixed function oxidases and thus resembling non-cytochrome P-450-dependent N-oxidase enzymes. Aminoacetonitrile and other known monoamine oxidase inhibitors were shown to be more effective inhibitors of dimethylnitrosamine demethylation *in vitro* than typical P-450 enzyme inhibitors and the monoamine oxidase substrate B-phenethylamine also inhibited demethylation of dimethylnitrosamine (42,43), as did benzylamine. The authors recognize that most of the known monoamine oxidase activity of rat liver is located in the mitochondrial fraction but they point out that some activity has been reported in the microsomal fraction and conclude that dimethylnitrosamine may be metabolized in part by enzymes unrelated to the mixed function oxidase complex. Clearly further work is required to resolve these questions but the marked effects of aminoacetonitrile, not only on the metabolism of dimethylnitrosamine, but also on its toxic and carcinogenic effects (44,45) certainly give support to the views of Lake and his colleagues.

In common with other N-demethylation reactions, the formation of hydroxymethylmethylnitrosamine as an early step in the metabolism of dimethylnitrosamine has been postulated. This expected product has never been isolated, however, presumably because of its great chemical instability. As a model for the hydroxymethyl derivative acetoxymethylmethylnitrosamine has been prepared in two laboratories (46,47). The acetoxo derivative is hydrolysed by unspecific esterases and has proved to be a locally acting carcinogen as well as inducing tumors at distant sites. After oral administration to rats, acetoxymethylmethylnitrosamine selectively induced squamous cell carcinomas of the fore stomach (48) and intraperitoneal injection resulted in the induction of intestinal tumors (49,50). Intrarectal administration induced rectal carcinomas, as well as myosarcomas and neurosarcomas, subcutaneous injection resulted in local sarcomas and intravenous injection induced tumors of the heart, lung, kidney and ear duct (51). These findings are clearly different from the carcinogenic actions of dimethylnitrosamine, which appears only to be carcinogenic for the liver, kidney and lung in the rat and to have no local effect after subcutaneous or intrarectal treatment (4). Consistent with the above findings with acetoxymethylmethylnitrosamine is the observation that N-nitro-bis (2-acetoxypropyl) amine is a pancreatic carcinogen in the Syrian golden hamster (24). Several α -acetoxo derivatives of nitrosamines have been shown to be mutagenic in microorganisms (52,53) and in *Drosophila* (54,55,56). The synthesis of α -acetoxynitrosopyrrolidine has been reported and it has been shown to be mutagenic against two tester strains of *Salmonella typhimurium* without metabolic activation (57) and direct evidence has recently been obtained for the metabolic α -hydroxylation of N-nitrosopyrrolidine after incubation with rat liver microsomes *in vitro*, and after administration to rats *in vivo*, by isolation of 2-hydroxytetrahydrofuran, the major decomposition product of α -hydroxynitrosopyrrolidine (58). Alkylation of α -aminopyridine and 2,4-dinitrophenol by α -acetoxo-N-nitrosamines in the presence of hog liver esterase has been recently demonstrated (59).

The metabolic pathway of dimethylnitrosamine, as shown in Fig. 2, shows generation of a methyl carbonium ion from the methyl diazonium cation, with release of molecular nitrogen. Some of the evidence favoring this scheme has been outlined

above and there is no doubt that a methylating species is generated metabolically since proteins (60) and nucleic acids (61) are methylated in vivo as shown by the presence of labeled methyl groups derived from the nitrosamine in defined positions of these macromolecules. Studies with fully deuterated dimethylnitrosamine by Lijinsky and his colleagues (62) have clearly shown that the methyl group is transferred intact, under the conditions in the cell, without the formation of diazomethane. This evidence does not however give any indication of what proportion of the dimethylnitrosamine is metabolized via the pathway leading to the methylating species and other metabolic pathways are likely to occur. The scheme shown in Fig. 2 requires that all of the nitrogen in the nitrosamine is released as N_2 . Since there are, apparently, no known mechanisms of nitrogen fixation in mammalian cells, no incorporation of nitrogen into body components of animals exposed to dimethylnitrosamine would be expected if this were the only pathway. However, as long ago as 1958, Heath and Dutton (63) showed that there was incorporation of ^{15}N into urinary urea and other molecules in rats that had been treated with dimethylnitrosamine labeled in both the amino and the nitroso nitrogens, i.e. $[^{15}N^{15}N]$ dimethylnitrosamine. Much more recently Grilli and Prodi (64) have identified formaldehyde, formic acid, methylamine and N-methylhydrazine after incubation with rat liver microsomes in vitro. The author, with Dr. D. Halliday and Mr. J. Holsman, has found that about 90% of the ^{15}N in an administered dose of $[^{15}N^{15}N]$ dimethylnitrosamine (20 mg/kg body wt.) is expired by rats, indicating that the production of the alkylating intermediate is the major metabolic pathway. The metabolic formation of methanol from dimethylnitrosamine after incubation with rat liver microsomal preparations in vitro has been reported (41). Recent work of the Fels Research Institute in Philadelphia indicates that some of this metabolically formed methanol is derived from methylated protein carboxyl groups (65).

ORGAN SPECIFICITY

The organ specificity of nitrosamides has been discussed in relation to the carcinogenicity of streptozotocin. Another remarkable specificity is the ability of N-methyl- and N-ethylnitrosourea, under certain experimental conditions, to induce tumors of the nervous system, sometimes virtually confined to the brain. Since both of these nitrosamides alkylate various organs to about the same extent initially, as measured by reaction on the 7-position of guanine (66,67), the organotropism was difficult to explain. Alkylation on the 7-position of guanine in DNA was studied by the earlier investigators because this is the position of maximal reaction. However alkylation occurs at a number of other sites in the DNA molecule and the possible biological importance of O^6 -alkylation was pointed out by Loveless in 1969 (68). Following the pioneering work of Goth and Rajewsky (69) there have been many reports indicating that the persistence of this alkylated base can vary very greatly in different organs. This variation is particularly well illustrated by the studies of Margison and Kleihues (70) who compared the levels of 7-methylguanine and O^6 -methylguanine in the DNAs of brain, kidney and liver of rats given weekly intravenous injections of $[^{14}C]$ methylnitrosourea. This treatment regime is known to induce a high incidence of brain tumors, a few tumors of the kidney and none in the liver of this species. O^6 -methylguanine was found to accumulate progressively in the brain, to a considerably smaller extent in the kidney and virtually not at all in the liver. Since this alkylated base is stable in DNA under physiological conditions it is evident that an enzyme system must be involved in its removal. Methyl methane-sulfonate, only methylates on the O^6 -position of guanine to a very small extent, while reacting at the same level or more as the nitroso compounds on the N-7 and other positions of the DNA molecule (71,72). When given in single doses to rats in doses sufficient to methylate kidney nucleic-acids at levels, in terms of 7-methylguanine, that would be expected to be carcinogenic to the kidney, no tumors of this organ were induced. Interestingly, however, some brain tumors did appear in these animals after longer latency periods (73) and the induction of nervous system tumors

by the transplacental administration of methylmethane sulfonate was subsequently reported (74). Since the persistence of O⁶-methylguanine in rat brain is so much greater than in the kidney or liver (7) the capacity of methylmethane sulfonate to induce brain tumors gives strong support for an important role of O⁶-alkylation in carcinogenesis.

CONCLUSIONS

Nitrosamines and other N-nitroso compounds are effective carcinogens, inducing tumors in most organs in a wide range of species. The compounds are also powerfully mutagenic and several have been shown to alkylate DNA and other cellular macromolecules in different organs. In the case of dimethylnitrosamine, the metabolic pathway leading to the alkylating intermediate is quantitatively the major one. The organ specificity or organotropism of the N-nitroso compounds may be dependent on the capacity of the organ to activate them, when necessary, and on the varying persistence of certain alkylated components of the DNA. Other factors are probably also involved.

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Tissue Specificity in Metabolic Activation

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ABSTRACT

Organ specificity of certain carcinogenic chemicals can be determined by the half-lives of ultimate carcinogens which may act to prevent their distribution in the body by covalent reactions, in organs (cells) in which they are generated. Evidence is summarized for several systemically acting carcinogenic *N*-nitrosamines, *N*-(α -acyloxy)alkyl-*N*-alkylnitrosamines and 3,3-dimethyl-1-phenyltriazene; an alternative model of organ specificity involving the formation of a transportable alkylating species is discussed.

In human liver specimens, large inter-individual differences in the activity of carcinogen-activating enzymes were noted. Benzo[*a*]pyrene-hydroxylase (AHH)-activity and microsome-mediated mutagenicity was measured using the hepato-carcinogens, *N*-nitrosomorpholine, *N*-nitroso-*N'*-methylpiperazine and vinyl chloride as substrates. When AHH-activity in liver specimens from human subjects was plotted against the respective microsome-mediated mutagenicity in *S. typhimurium*, a positive correlation was obtained for the rate of oxidative benzo[*a*]pyrene metabolism and mutagenicity in the presence of *N*-nitrosomorpholine, *N*-nitroso-*N'*-methylpiperazine or vinyl chloride. Thus, differences in tissue-specific activation processes of chemical carcinogens a) appear to be contributing factors in the production of tumours in certain organs only and b) may also condition the carcinogenic response in human individuals when exposed to the same level of environmental carcinogens.

KEY WORDS

Organotropic carcinogenesis, carcinogen metabolism in human tissues, benzo[*a*]pyrene-hydroxylase, mutagenicity, *N*-nitrosamines, vinyl chloride

INTRODUCTION

The site of tumorigenic action for many carcinogens that require metabolic activation appears to be primarily determined by the final concentration of ultimate metabolites available for reaction with cellular macromolecules. This

may result from a critical balance between activation and detoxification of the chemical *in vivo*. The physico-chemical properties of these ultimate metabolites such as their electrophilicity, their half-lives and the cell compartment where they are generated, are parameters that can be expected to influence their reactions with cellular constituents and thus alter biological responses. In this paper, experimental evidence is given to support this model for organspecific carcinogenicity of chemicals, and also to show its limitations using three distinct examples of systemically acting carcinogens: *N*-nitrosamines, *N*-(α -acyloxy)alkyl-*N*-alkyl-nitrosamines and 3,3-dimethyl-1-phenyltriazene.

Species differences in the rates of carcinogen metabolism and the magnitude of inter-individual variations in human liver samples were also investigated, using cyclic *N*-nitrosamines and vinyl chloride. The enzymic capacity of animal or human target tissues to convert these carcinogens into electrophiles was measured *in vitro* using a) mutagenicity assays with *S. typhimurium* strains and b) determination of benzo[*a*]pyrene-hydroxylase. Comparative metabolic studies, using animal and human tissues with carcinogens to which man is exposed, but for which no epidemiological studies or case reports exist, may aid in the extrapolation of animal data to man. These results may facilitate the selection of non-toxic drugs (1) which are metabolized by the same enzyme system(s) that act on environmental carcinogens to assess indirectly their *in vivo* rates of metabolism in man.

METHODS

Animal and Human Tissue Preparations

Pooled rat livers were homogenized (3 ml of 0.15% KCl/5mM Sørensen-buffer pH 7.4 per g of wet liver) at 0-4°C and centrifuged at 9000 x g for 30 min. The supernatant was used in subsequent incubations. Human diagnostic specimens with no pathological lesions were obtained from adult female and male human subjects and provided by Drs M. Boicchi, G. Della Porta and U. Veronesi, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy. Human liver samples were prepared within 3 hrs after surgery under conditions identical to those described for rat tissues. After freezing in liquid nitrogen, samples were stored at below -70°C and rapidly thawed immediately before use.

Mutagenicity Assays with *S. typhimurium*

Plate incorporation assay (Method A). Each plate contained 1-2 x 10⁸ cells of *S. typhimurium* TA1530, 9000 x g supernatant from rat or human liver, an NADPH-generating system and the *N*-nitroso compound dissolved in 100 μ l acetone or DMSO per plate (2). The number of revertant colonies was determined in triplicate after 48 hours of incubation.

Assays for volatile compounds (Method B). Mutagenicity assays for vinyl chloride were carried out in assays, adapted to test volatile compounds (3). Plates containing 1-2 x 10⁸ bacteria of TA1530 strain and other ingredients mentioned in method A, were exposed to a gaseous mixture of 20% vinyl chloride in air (v/v) 37°C in the dark. The concentration of vinyl chloride in the aqueous phase was 4 mM. After 4 hrs of exposure, vinyl chloride was removed and replaced by air. Incubation of the plates was continued for up to 48 hrs and the number of revertants scored.

Incubation in liquid suspension (Method C). The incubation medium (final vol.

220 μ l) contained 60 μ l of a purified microsomal suspension of rat liver or other tissues, an NADPH-generating system, Sørensen phosphate buffer pH 7.4, 4×10^7 cells (*S. typhimurium* TA1530 strain) and the *N*-nitrosamine dissolved in 20 μ l acetone. In assays in which rat tissue cytosol (100,000 x g supernatant) was used, the NADPH-generating system was omitted. Incubation was carried out at 37°C for 30 minutes and was stopped by dilution with ice-cold saline. The number of revertants and survivors was determined by plating on selective medium after appropriate dilution (4).

Determination of Benzo[a]pyrene-Hydroxylase

A liver fraction equivalent to 4-16 mg of wet weight tissue, an NADPH-generating system and 50 μ mol of *Tris*-HCl buffer pH 7.4, was incubated at 37°C for 10 minutes (rat liver) or 20 minutes (human liver); benzo[a]pyrene (80 μ mol) dissolved in 50 μ l acetone was added after 1 minute of preincubation (final vol. 1 ml). The reaction was stopped and the amount of fluorescent metabolites were determined, using 3-hydroxy-benzo[a]pyrene as standard (5). Activity was expressed as pmoles of phenolic metabolites formed per g of wet tissue per minute. The results were calculated from assays performed under conditions of linearity, in respect to time and liver protein concentration.

RESULTS AND DISCUSSION

Sites of Metabolic Activation of *N*-nitrosamines as related to the Target Organs in Rats

N-nitroso compounds display a remarkable organspecific carcinogenic activity. The initiation of tumours is currently associated with the formation of alkylating agents in the body, either from *N*-nitrosamides spontaneously or by SH-group catalysed hydrolysis or from *N*-nitrosamines by enzymic activation. The role of organspecific enzymic activation in determining which tissue develops the tumour in response to a given *N*-nitrosamine, becomes apparent when published carcinogenicity data (6-8) are plotted (Fig. 1) to show the percentages of compounds (of a total of 62 *N*-nitrosamines and 21 *N*-nitrosamides) which induce tumours in rats at specific sites as shown at the bottom of the figure (9). It is evident that *N*-nitrosamides and *N*-nitrosamines produce completely different patterns of organ distribution of tumours. The most frequent target organs for *N*-nitrosamines are in descending order, liver, oesophagus, nasal cavity, respiratory tract, kidney and bladder and there seems to be a positive correlation between the metabolic capacity for nitrosamine activation in the organ in which tumours are induced (10). With *N*-nitrosamides (Fig. 1), the principle target organs are different. Since there is a homogeneous distribution of the administered *N*-nitrosamines throughout the organism, Fig. 1 can be explained by the formation of short-lived alkylating metabolites by tissue-specific enzymes which have different substrate specificities that determine tumour development in response to *N*-nitrosamines. In the cases of *N*-nitrosamides, other factors such as organ specific removal of miscoding bases or cell- and pharmaco-kinetic parameters may decide in which tissue a tumour develops (11-12).

Model Compounds for Activated *N*-nitrosamines: *N*-(α -acyloxy)alkyl-*N*-alkylnitrosamines

The half-life of alkylating intermediates released from the parent *N*-nitrosamines in biological fluids and localization of the enzymes that generate them in the body, appear therefore to be of prime importance in the processes that initiate

tumour development. Due to their high reactivity, none of these intermediates has so far been isolated, however, model compounds for activated *N*-nitrosamines, which are esters of *N*-(α -hydroxy)alkyl-*N*-alkylnitrosamines have become available (13-14); these α -acyloxy derivatives are thought to yield the same intermediates after enzymic or non-enzymic release as the parent *N*-nitrosamines with microsomal mono-oxygenases (Fig. 2)

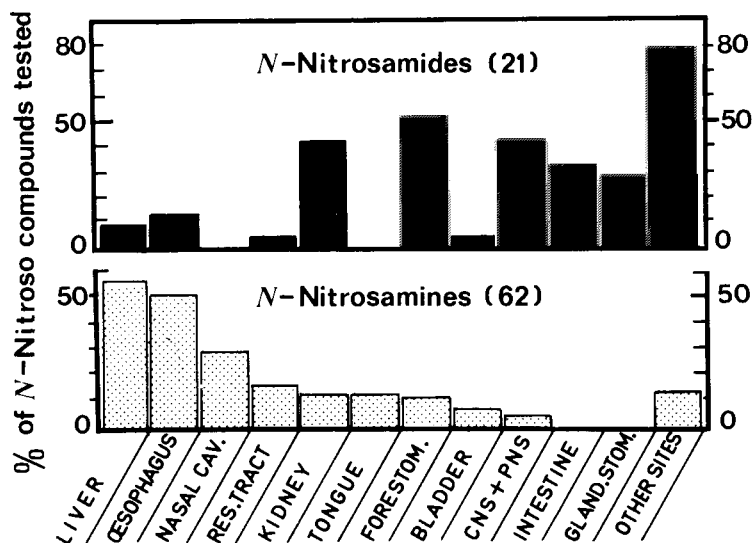


Fig. 1. Localization of tumours induced by *N*-nitroso compounds in rats. Data were compiled from ref. 6-8 on 62 *N*-nitrosamines and 21 *N*-nitrosamides found to be carcinogenic in rats (9).

The hypothesis that *N,N*-dialkylnitrosamines are activated by α -C-hydroxylation (15) is strongly supported by the finding that both enzymic cleavage and non-enzymic hydrolysis release mutagenic agents (16-17) and by our studies indicating that alkylation of bacterial DNA of *S. typhimurium* TA1530 strain by methyl-, ethyl-, *n*-propyl- or *n*-butyl-groups released from the corresponding α -acyloxy derivatives, were highly mutagenic (18). The enzymic release of alkylating reactants from *N*-(acetoxymethyl)-*N*-methylnitrosamine is mediated by two distinct enzyme systems: one is localized in the cytosol of rat tissues and probably consists of non-specific esterases, and the other is a microsomal enzyme system requiring an NADPH-generating system (Table 1). The soluble esterase(s) appear(s) to be ubiquitous and have the same activity in the different tissues studied, while the microsome-associated activity was highest in the liver and lowest in the mucosa of the small intestine.

In carcinogenicity studies (19-21), *N*-(acetoxymethyl)-*N*-methylnitrosamine caused tumours at various sites including the small intestine and colon after i.p. injection, and tumour of the forestomach after oral dosing, even though the parent compound, dimethylnitrosamine, rarely affects the gastro-intestinal tract of rats. This apparent shift in target organs may be plausibly explained by the ubiquitous distribution of non-specific esterases, which release alkylating agents from *N*-(acetoxymethyl)-*N*-methylnitrosamine at tissues where the concentration of the carcinogen is highest e.g. at the site of injection or, after oral dosing, in the

gastro-intestinal tract, which is a target organ.

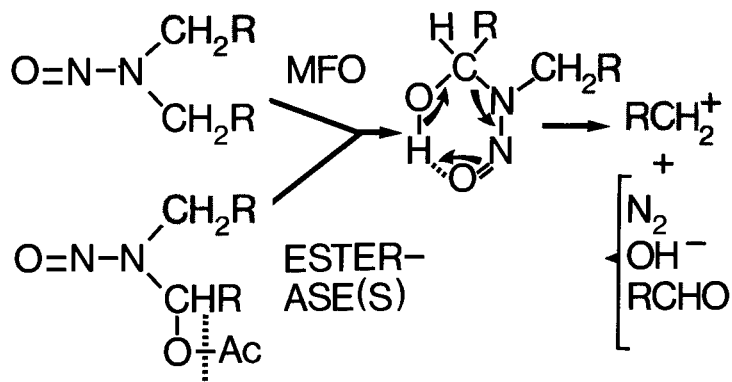


Fig. 2. Proposed scheme for the release of a common alkylating intermediate from *N,N*-dialkylnitrosamines by microsomal mixed-function oxygenases (MFO) or from *N*-(α -acyloxy)alkyl-*N*-alkyl nitrosamines by esterases. Heterolysis of the intermediate α -hydroxy compound yields an alkyl carbonium ion (RCH₂⁺)

TABLE 1 Mutagenicity induced in *Salmonella typhimurium* strain TA1530 by *N*-(acetoxy)methyl-*N*-methylnitrosamine in the presence of various rat tissue fractions

Fraction Tissue	Cytosol	Microsomes + NADPH-generating system
	Specific mutagenicity a)	
Liver	37	32
Kidney		10
Spleen		8
Small intestine		1.6

a) Number of *his*⁺-revertant colonies formed per mg of wet tissue per μ mol of the *N*-nitroso compound per ml of incubation medium per minute; assays were carried out by liquid incubation (Method C).

Half-life and methylating properties of 3-methyl-1-phenyltriazene, a mutagenic and carcinogenic metabolite of 3,3-dimethyl-1-phenyltriazene

The neurotropic carcinogen, 3,3-dimethyl-1-phenyltriazene (22-24) predominantly undergoes an oxidative *N*-demethylation in the rodent liver by microsomal enzymes to yield a carcinogenic metabolite, 3-methyl-1-phenyltriazene (25-26) which is a direct-acting mutagen for *S. typhimurium* TA1530 (27). However, the parent compound produces extra-hepatic tumours exclusively. To explain this alternative model of organ-specificity, the half-life of 3-methyl-1-phenyltriazene was measured *in vitro* by using different preincubation times and by measuring the

concentration of the residual 3-methyl-1-phenyltriazenes from its reaction with 4(4-nitrobenzyl)pyridine or by mutagenicity assays in liquid suspension (Method C). At pH 7.4 at 37°C it was found to be 1.13 minutes, which was enough to permit its distribution throughout the body. This was confirmed by s.c. injection of 3-(¹⁴C-methyl)-1-phenyltriazenes (synthesized by Dr G.F. Kolar, German Cancer Research Centre, Heidelberg, FRG) into rats, which yielded methylated bases in nucleic acids of hepatic and extra-hepatic tissues including the brain, the major target organ of the parent compound, 3,3-dimethyl-1-phenyltriazenes (G.P. Margison, in preparation). Thus, for the latter carcinogen, tissue specific removal of mis-coding bases i.e. the excision of the promutagenic base, O⁶-methylguanine, may apparently decide in which extra-hepatic tissue a tumour will develop. In contrast, the reactive alkylating agents released from *N*-nitrosamines or *N*-(α-acyloxy)-alkyl-*N*-alkylnitrosamines appear to have a much shorter half-life and consequently alkylation is limited to those tissues containing the appropriate activating enzyme systems.

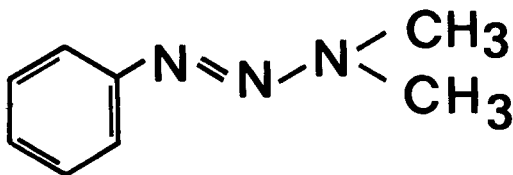


Fig. 3. Formula of 3,3-dimethyl-1-phenyltriazenes

Carcinogen Metabolism in Human Liver Specimens

The human carcinogen, vinyl chloride (28) and the animal hepatocarcinogens, *N*-nitrosomorpholine, *N*-nitrosopyrrolidine, *N*-nitrosopiperidine and *N*-nitroso-*N'*-methylpiperazine (6) are converted by rodent and human liver microsomal monooxygenases into mutagenic intermediates that bind covalently to DNA (4, 9). Binding reactions of ultimate carcinogenic metabolites to cellular macromolecules are controlled by the host's metabolism and appear to be a prerequisite for these compounds to exert their carcinogenic effects (29). We have examined the nature of the carcinogen-activating enzyme(s) in human liver and the magnitude of inter-individual variations in activities by measuring in parallel benzo[*a*]pyrene-hydroxylase and microsome-mediated mutagenicity in liver fractions in the presence of vinyl chloride and those cyclic nitrosamines as substrates. Benzo[*a*]pyrene-hydroxylase activity is considered as a marker enzyme for carcinogen activation which conditions the tumour response to polycyclic aromatic hydrocarbons in mice (30) and possibly to cigarette smoke in humans (31).

Figure 4 summarizes the relative capacity of human samples to convert some cyclic nitrosamines into mutagenic intermediates. The results are expressed as percentage of control; although large inter-individual differences were observed, the average enzymic capacity to activate 3 nitrosamines (\bar{m}) was close to that of rat liver in which for example *N*-nitrosomorpholine is a potent carcinogen. However, with *N*-nitroso-*N'*-methylpiperazine, human liver samples were up to 36 times more active than rat liver. If such differences do have relevance *in vivo*, species differences have to be taken into account when extrapolating carcinogenicity data for certain compounds from rats to man.

In the present study, we have also compared the rates of benzo[*a*]pyrene-hydroxylation with the metabolic activation of some carcinogens into mutagens. Vinyl chloride, an animal and human carcinogen, is converted by microsomal enzymes into chloroethylene oxide, a highly electrophilic mutagenic and carcinogenic agent (32) and cyclic nitrosamines are thought to yield alkylating intermediates following

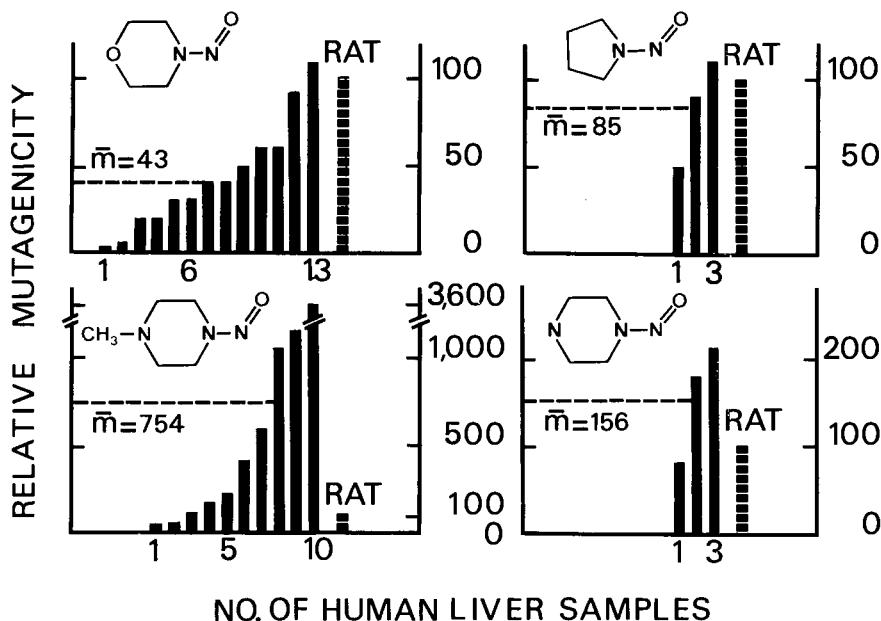


Fig. 4. Enzymic capacities of individual human liver specimens (solid bars) to convert *N*-nitrosopyrrolidine, *N*-nitrosomorpholine, *N*-nitroso-*N'*-methylnpiperazine and *N*-nitrosopiperidine into electrophiles, mutagenic to *S. typhimurium* TA1530. Mutagenic activity (Method A) is expressed relative to that obtained in assays using liver fraction from untreated rats (dashed bars) which is given as 100. Mean activity (\bar{m}) of all human samples measured is listed.

oxidative metabolism (8, 9). Benzo[*a*]pyrene hydroxylase activity in human liver was measured and the values plotted against the respective microsome-mediated mutagenicity in the presence of *N*-nitrosomorpholine, *N*-nitroso-*N'*-methylnpiperazine (Fig. 5a) or vinyl chloride (Fig. 5b). A statistically significant positive correlation was obtained between the rates of benzo[*a*]pyrene hydroxylation and the mutagenicity in the presence of *N*-nitrosomorpholine ($r = 0.84$; $p < 0.002$), *N*-nitroso-*N'*-methylnpiperazine ($r = 0.92$; $p < 0.002$) or vinyl chloride ($r = 0.88$; $p < 0.05$). Although limited by the small number of specimens, the observed positive correlation between the two enzymic activities would suggest that the substrate pairs are metabolized by a single enzyme or by enzyme systems which are under a similar regulatory control. Such a correlation may have significance for developing methods to evaluate the drug- and carcinogen-metabolizing capacity of different human individuals. Since statistical significant correlations were obtained between the rates of hydroxylation of benzo[*a*]pyrene and those of antipyrine, hexobarbital and zoxazolamine (1), such predictor drugs should be explored to see whether they are useful for assessing the *in vivo* rates of metabolism of environmental carcinogens in humans.

These results exhibited large inter-individual differences in liver activation of chemical carcinogens (Fig. 4, 5a, b) and the capacity of some samples to efficiently convert diverse chemical structures such as *N*-nitrosamines and vinyl chloride to reactive metabolites (Fig. 5c). If predictor drugs exist, they may help to identify individuals in the general population at high risk; their increased capacity to activate chemical carcinogens may be environmentally caused or genetically determined (33).

In summary, differences in tissue specific activation processes of chemical carcinogens appear to be contributing factors in the production of tumours in certain organs only (34) and may also condition the carcinogenic response in human individuals when exposed to the same level of environmental carcinogens.

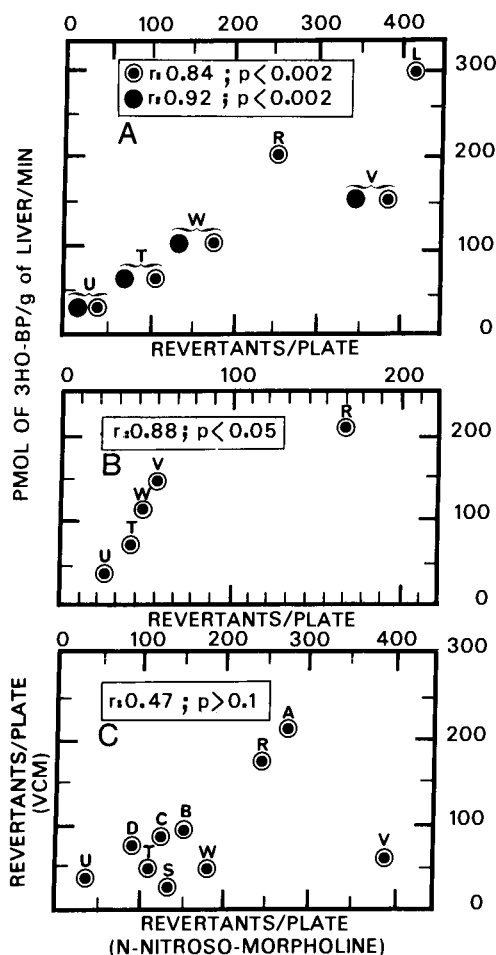


Fig. 5. - a, b, c. Relationship between benzo[a]pyrene hydroxylase activity and microsomal mutagenicity in human liver specimens in the presence of *N*-nitrosomorpholine (○), *N*-nitroso-*N'*-methylpiperazine (●) (Chart A) or vinyl chloride (Chart B). Relationship between liver microsomal mutagenicity in the presence of vinyl chloride (ordinate) and *N*-nitrosomorpholine (abscissa) (Chart C). Tissue samples from different subjects are represented by different letters. Benzo[a]pyrene hydroxylase hydroxylation was measured as described in Methods. Mutagenicity assays were carried out with *S. typhimurium* TA1530 in the presence of an *N*-nitrosamine and 150 μ l of human liver fraction/plate (Method A); revertants per 10 μ l of *N*-nitroso compound/plate are plotted. Mutagenicity assays in the presence of vinyl chloride were performed by exposing the plates containing *S. typhimurium* TA1530 and 150 μ l of human liver fraction to the gaseous mixture (Method B).

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Formation of Chemically Reactive Metabolites from Drugs

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INTRODUCTION

Over the last few years we have been involved in a systematic examination of the pathogenetic role of chemically reactive metabolites in several drug-induced tissue lesions. This work has been published in detail elsewhere by us and our colleagues and is summarized briefly here in order to provide an overview. New evidence that N-hydroxyacetaminophen is the possible toxic metabolite of acetaminophen and phenacetin is presented in greater detail.

FORMATION OF CHEMICALLY REACTIVE METABOLITES FROM DRUGS: SYNOPSIS OF RECENT WORK

A systematic examination of the role of chemically reactive metabolites in drug-induced extrahepatic tissue lesions has been undertaken. Doses of ³H-acetaminophen (750-900 mg/kg) causing renal and hepatic necrosis in Fischer rats markedly depleted target organ glutathione and resulted in large amounts of radiolabeled metabolite being covalently bound to renal and hepatic protein (1,2). Autoradiographic examination of the intraorgan localization of the bound metabolite in the kidney showed a striking correlation with the target cells for toxicity, namely, the cells of the terminal proximal tubules (pars rectus). Pretreatment with cobalt chloride decreased both the covalent binding of metabolite and the glutathione depletion in target organ tissues while concomitantly protecting against tissue damage. Pretreatment with 3-methylcholanthrene enhanced hepatic necrosis and covalent binding of metabolite to hepatic protein in vivo and to microsomal protein in vitro but had little effect on the corresponding renal parameters. Thus, acetaminophen-induced renal and hepatic necrosis apparently result from in situ activation of acetaminophen to a chemically reactive species.

Animal models were also developed for the hepatic and renal necrosis produced by the minor analgesic phenacetin (1), the ethoxy analog of acetaminophen, and for

the methemoglobinemia and hemolytic anemia caused by phenacetin (3). The effects of substituting deuterium for hydrogen in the ethyl group were then compared on both the metabolism and the toxicity of phenacetin *in vivo* (4), because the rate of deethylation of phenacetin to acetaminophen by hamster liver microsomes *in vitro* was decreased by the substitution of deuterium for hydrogen in the α -methylene position of the p-ethoxy group ($kd_0/kd_2 = 1.86$) but not by substitution in the β -methyl group ($kd_0/kd_3 = 1.12$) (5). The hepatotoxicity of phenacetin- d_0 was the same as phenacetin- d_3 and both were more hepatotoxic than phenacetin- d_2 , as measured by the extent of hepatic necrosis, glutathione depletion and covalent binding of metabolites. In contrast, the methemoglobinemia after phenacetin- d_0 and phenacetin- d_3 was less than after phenacetin- d_2 administration. As expected, measurement of acetaminophen blood concentrations showed that acetaminophen formation from deethylation of phenacetin- d_0 was similar to that from phenacetin- d_3 but was much greater than from phenacetin- d_2 ($AUCd_0/AUCd_2 = 2.71$).

Single intraperitoneal or intravenous injections of other classes of drugs, such as several substituted furans and thiophenes, including the diuretic furosemide and the antibiotic cephaloridine, produced renal tubular necrosis, hepatic necrosis or bronchiolar necrosis in mice and rats (6,7). Pretreatment of animals with substances that altered drug-metabolizing enzymes usually altered the extent of alkylation and the severity of the renal, hepatic and pulmonary necrosis produced by the furans and thiophenes. Occasionally the target organ susceptibility to injury was even switched from one tissue to the other or the intraorgan zone of the lesion was changed by the pretreatments. Similar results were obtained with another naturally occurring furanotoxin, the air pollutant 3-methylfuran which is selectively lethal to the Clara cells of pulmonary bronchioles (8) probably because these cells contain activating P-450 monooxygenases (7). Extension of these studies to the drug-induced pulmonary disease caused by the urinary antiseptic nitrofurantoin has provided evidence that this lesion might also be initiated by a drug metabolite, the nitro anion radical, which in turn generates a reactive oxygen species, thereby leading to a form of pulmonary oxygen toxicity (9).

Animal models have also been developed for the renal tubular necrosis produced by other drugs, and with techniques similar to those described above, preliminary evidence has been obtained in support of the hypothesis that reactive metabolites are responsible for the renal necrosis. The nephrotoxic drugs include

α -methyldopa, salicylate, iproniazid and its isopropylhydrazine derivative, and isoniazid and its acetylhydrazine derivative (Table 1).

TABLE 1 Effect of High Fat Diet on Susceptibility to Injury by Nephrotoxic Drugs (Data from 12)

Drug	Stock Diet	High Fat Diet Normal Vit. E	Extent of Necrosis
Acetaminophen	1000 mg/kg	500 mg/kg	+++
Phenacetin	Not observed	750	+
Furosemide	Not observed	200-400	++
α -Methyldopa	Not observed	500-1000	+
Acetylhydrazine	Not observed	30-60	+
Isopropylhydrazine	Rarely observed	25-50	+
Iproniazid	Rarely observed	100-150	+

In examining the different types and locations of the tissue lesions produced by these reactive drug metabolites, it appeared (Table 2) that at least three types of reactive species causing tissue lesions could be postulated: (a) electrophilic cations showing significant glutathione conjugation in vivo (e.g., acetaminophen, 2-furamide); (b) electrophilic cations not showing glutathione conjugation in vivo (e.g., furosemide); (c) radicals whose toxicity are potentiated by Vitamin E-deficient diets (Table 3) (e.g., nitrofurantoin, α -methyldopa, the hydrazine drugs and possibly salicylates). Accordingly, we have examined the chemical properties of some of the reactive metabolites by trapping them as adducts of sulfhydryl nucleophiles, such as cysteine, N-acetylcysteine, cysteamine, glutathione, and α -mercaptopropionylglycine, with isolation and identification by HPLC and mass spectral analysis (10). Considerable specificity for conjugation was seen among the nucleophiles, and some compounds (e.g., methionine) that are advocated as antidotes for the treatment of acetaminophen overdosage were such poor nucleophiles that adducts with the electrophilic acetaminophen metabolite could not be measured. Thus, the inherent chemical reactivity toward nucleophiles may in part determine the preferential macromolecular binding of acetaminophen and furosemide metabolites to protein (1800 pmol/ mg) rather than to DNA (20 pmol/mg) (11).

TABLE 2 Nature of Chemically Reactive Metabolites

Cations - GSH Threshold	Cations - No GSH Threshold	Radicals - Vitamin E
Acetaminophen	Furosemide	Carbon Tetrachloride
Phenacetin	Dimethylnitrosamine	Hydrazines
Acetanilide		α -Methyldopa
Halobenzenes		? Salicylate
Halonaphthalenes		Nitrofurantoin
Simple Furans		Paraquat
Simple Thiophenes		

TABLE 3 Effect of Vitamin E on the Susceptibility to Injury by Nephrotoxic Drugs (Data from 12).

Drug (Dose, mg/kg, i.p.)	n	Mean Extent of Renal Necrosis*	
		E-Repleted	E-Deficient
α -Methyldopa (500 X 3)	44	0.50	1.13
Acetylhydrazine (60)	8	0.75	2.00
Isopropylhydrazine (50)	16	0.76	1.76

*Tissues were evaluated individually and given a score between 0 - 4⁺ according to the percentage of cortical and subcortical cells exhibiting necrosis. Means = sum of individual scores divided by n.

To gain further insight into the mechanism of activation of acetaminophen, the reactive metabolite was trapped by including glutathione in the microsomal incubation mixtures (10,24). The formation of the acetaminophen adduct with glutathione was then examined during incubation under an ^{18}O atmosphere (24). If N-hydroxylation were the mechanism of the activation of acetaminophen, no ^{18}O would be incorporated into the metabolite. Conversely, if acetaminophen were activated to an arylating metabolite by 3,4-epoxidation, with a subsequent rearrangement to acetimidquinone,

some ^{18}O would be incorporated into the para- position of the metabolite.

The acetaminophen formed after Raney nickel cleavage of the glutathione conjugate from the acetaminophen incubation mixture showed a parent peak at m/e 151 corresponding to the molecular weight of ^{16}O -containing acetaminophen. The absence of a significant peak at m/e 153 (^{18}O -acetaminophen) showed that little ^{18}O was incorporated during metabolic activation of acetaminophen to a chemically reactive arylating agent. The presence of the base peak at m/e 109, corresponding to the loss of ketene ($^{16}\text{M}-42$), and the absence of a significant peak at m/e 111 ($^{18}\text{M}-42$) confirm this observation.

Recently, the converse experiments have been completed (25,26). Incubations of hamster liver microsomes, an NADPH generating system, glutathione and acetaminophen- ^{18}O yielded 3-S-glutathionyl-acetaminophen which was isolated either by TLC or HPLC as previously described (10,24). The aryl sulfur ether conjugate was reduced with Ra-Ni and the ^{18}O -content of the product acetaminophen was calculated from peak height ratios of the molecular ions at m/e 153 and 151, and the base peak at m/e 111 and 109 which correspond to a loss of the elements of ketene. Calculations from several spectra showed that the metabolite from acetaminophen contained $45 \pm 3\%$ ^{18}O compared to 48% in the acetaminophen- ^{18}O substrate, indicating an insignificant loss of the phenolic oxygen. These data are consistent with the previous observation that there is no incorporation of $^{18}\text{O}_2$ into the metabolite from acetaminophen (24).

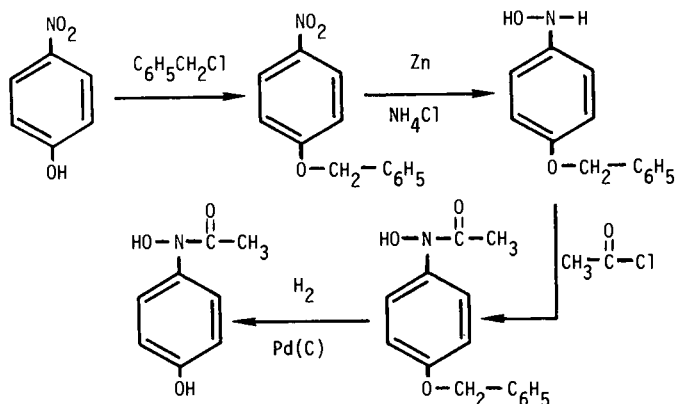
Hamsters were also injected i.p. with 50 mg/kg of acetaminophen- ^{18}O (25,26). Examination of the 24-hour urinary metabolites after acetaminophen- ^{18}O revealed that acetaminophen and its glucuronide and sulfate conjugates (all analyzed as acetaminophen after hydrolysis with Glusulase) lost no ^{18}O from the phenolic position. The same result was found for the mercapturic acid metabolite which was analyzed as acetaminophen after Ra-Ni reduction. This result provides additional evidence for the N-hydroxylation pathway shown in Fig. 1.

Preparation of N-Hydroxyacetaminophen (V)

The synthesis of this reactive acetaminophen metabolite was accomplished using a method similar in principle to that used for the preparation of N-hydroxy-4-chloroacetanilide and of N-hydroxyphenacetin (22-24). The key modification involved protection of the phenolic group by benzylation (Figure 2).

SYNTHESIS OF N-HYDROXY-ACETAMINOPHEN (N-OH-PHAA)

Figure 2
(Data from 12)



The structure of V was confirmed using numerous criteria. After its separation by TLC, V reacted positively with ferric chloride spray reagent, characteristic of other N-aryl-hydroxamic acids. Titanium trichloride, a reagent shown to reduce N-O bonds, has been found to reduce hydroxamic acids like N-hydroxylidocaine and N-hydroxy-monoethylglycine xylidine to their respective amides, lidocaine and monoethylglycine xylidine. Treatment of N-hydroxyacetaminophen with TiCl_3 resulted in its conversion to acetaminophen, as determined by TLC analysis. The physical characterization of V by IR and NMR spectrometry, and by gas liquid chromatography - chemical ionization mass spectrometry consistently supported the assignment of N-hydroxyacetaminophen as the structure of this compound.

Half-life of N-Hydroxyacetaminophen in Solution.

The stability of N-hydroxyacetaminophen in aqueous environments was determined using GLC analysis (Table 4). The disappearance of this acetaminophen metabolite from aqueous solutions in vitro was characterized by two distinct phases, both of which appeared to be first order processes dependent on N-hydroxyacetaminophen concentration. At a concentration of 3 mM, the apparent half-lives for N-hydroxyacetaminophen disappearance were markedly dependent upon the pH of the medium, seen by the progressive lengthening of half-lives as the pH of the medium was decreased. These observations are consistent with a possible base catalyzed decomposition of N-hydroxyacetaminophen through the 1,6-elimination of the elements of water to give acetimidiquinone.

TABLE 4 Apparent Half-lives for the Disappearance of N-hydroxy-Acetaminophen from Aqueous Solutions In Vitro at 37° (Data from 12)

<u>Conditions</u>	<u>pH</u>	<u>Apparent Half-life</u>	
		<u>Rapid</u>	<u>Slow</u>
Buffer	7.4	4 min	14 min
Microsomes and Buffer	7.4	6 min	22 min
Microsomes and Buffer	6.4	24 min	102 min
Microsomes and Buffer	5.4	160 min	---
Distilled Water	4.8	15 hr	79 hr

Reaction of N-Hydroxyacetaminophen with L-Cysteine.

Figure 3 shows the disappearance of N-hydroxyacetaminophen from buffered solution (pH 7.4) at 37° in the presence of an equimolar concentration of L-cysteine. As previously observed for N-hydroxyacetaminophen in buffered solutions alone, the rate of its loss in the presence of cysteine was again biphasic ($t_{1/2} = 8.7$ and 75 min). Exclusion of oxygen (N_2 atmosphere) and/or the presence of 1 mM EDTA prevented the rapid phase of disappearance, thereby suggesting a role for both metal ions and molecular oxygen in this process. Under these conditions, the rate of N-hydroxyacetaminophen loss was the same as that observed for the slow phase disappearance in the absence of these modifications (Figure 3 - "control").

The stoichiometry of these reactions was also investigated, with N-hydroxyacetaminophen loss and acetaminophen formation monitored by GLC analysis, and acetaminophen-cysteine conjugate formation by HPLC analysis. During reaction of N-hydroxyacetaminophen with cysteine under aerobic conditions and without EDTA, the loss of N-

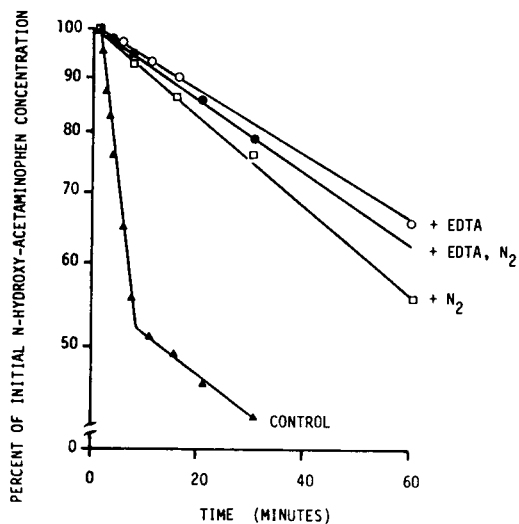


Figure 3
(Data from 12)

STOICHIOMETRY OF THE REACTION BETWEEN N-HYDROXY-
ACETAMINOPHEN (N-OH-PHAA) AND L-CYSTEINE (CYS)¹

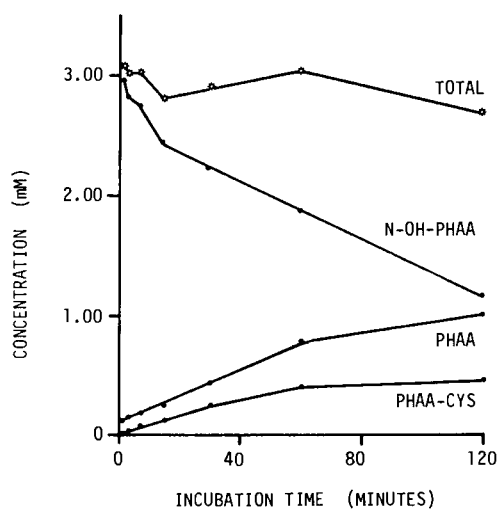


Figure 4
(Data from 12)

¹Incubated at 37° in 0.1 M phosphate buffer, in presence of 1.0 mM EDTA and under N₂ atmosphere.

hydroxyacetaminophen throughout the slow phase of disappearance could be accounted for by steadily increasing concentrations of acetaminophen and its cysteine conjugate. However, the loss of N-hydroxyacetaminophen during the rapid phase of disappearance could not be accounted for by formation of these products. Addition of EDTA and exclusion of O_2 each increased the proportion of lost N-hydroxyacetaminophen accounted for by these products, and with these modifications in combination, the accounting was nearly quantitative (Figure 4).

Reaction of N-Hydroxyacetaminophen with N-Acetylcysteine and Glutathione.

N-Hydroxyacetaminophen was shown to react chemically with other sulfhydryl nucleophiles to produce adducts *in vitro*. Incubation with N-acetylcysteine or glutathione at 37° in 0.1 M phosphate buffer also resulted in the time-dependent formation of acetaminophen-sulfhydryl conjugates, as determined by HPLC analysis. Retention times for the N-acetylcysteine and glutathione conjugates were identical to values previously reported for these adducts (10).

Production of Hepatic Necrosis by N-Hydroxyacetaminophen

Administration of N-hydroxyacetaminophen (100–300 mg/kg i.p. or i.v.) produced centrilobular hepatic necrosis in male adult Swiss mice similar to that seen after acetaminophen. Administering N-hydroxyacetaminophen in 0.1 M phosphate buffer, pH 5.6 moderately increased its hepatotoxicity, presumably by increasing the biologic availability of N-hydroxyacetaminophen. The compound was more potent than acetaminophen in producing hepatic necrosis.

Discussion.

The synthesis of N-hydroxyacetaminophen (Figure 2) has permitted investigation of the stability of this compound under conditions resembling the environment of its formation *in vivo*. Although halflives of 4 and 14 min. were observed for N-hydroxyacetaminophen in phosphate buffer (pH 7.4) at 37° *in vitro*, experiments performed under anaerobic conditions or in the presence of EDTA have suggested that these values may represent considerable underestimates of the reactivity of this acetaminophen metabolite *in vivo*.

Abolition of the rapid phase of N-hydroxyacetaminophen disappearance during its reaction with cysteine *in vitro* by the presence of EDTA and by the exclusion of oxygen suggests that both metal ions and molecular oxygen may be important determinants of *in vivo* reactivity. Such a rapid phase of loss may involve a metal ion-catalyzed oxidation of N-hydroxyacetaminophen that depends upon a dissolved molecular oxygen species to act as an electron acceptor. Limitation of this reaction by the availability of dissolved oxygen or loss of metal ion catalyst would explain the biphasic disappearance kinetics for N-hydroxyacetaminophen. Trace metal ions, especially Cu^{2+} , also accelerate the autooxidation of aliphatic hydroxylamines under neutral and basic conditions. If these factors play as important roles *in vivo* as they appear to *in vitro*, then the high metal ion concentrations and oxygen tensions encountered *in vivo* would contribute to an extremely high reactivity of this metabolite as it is formed in man and animals.

A proposed reactive metabolite of a compound should chemically give rise to the same products that are thought to arise from metabolism of the parent compound via the suspected toxic pathway *in vivo*. Direct chemical reaction of N-hydroxyacetaminophen with cysteine and N-acetylcysteine resulted in the formation of sulfhydryl adducts identical to those found after acetaminophen metabolism *in vivo*. Furthermore, reaction with glutathione produced an acetaminophen-glutathione conjugate that is the biological precursor of the acetaminophen-cysteine and acetaminophen-N-acetylcysteine conjugates which are found in urine.

Ideally, a proposed reactive metabolite of a drug should also be able to produce the same tissue lesion. The metabolite, N-hydroxyacetaminophen, produces centrilobular hepatic necrosis similar to acetaminophen but at lower doses.

Collectively, we believe these data provide strong evidence that the ultimate hepatotoxin formed from acetaminophen is N-hydroxyacetaminophen in its dehydrated acetimidoquinone form.

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Metabolic Activation of Aminostilbene Derivatives and Diethylstilbestrol

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Introduction

It is generally accepted that carcinogenic aromatic amines are metabolically activated and that the reaction of reactive intermediates with cellular nucleophiles is causally related to the carcinogenic effect. The reaction of N-arylnitrenium ions with nucleic acid bases is considered a possible ultimate step in the reaction sequence (1,2). Such ions may originate from arylhydroxylamines, their esters and the esters of arylhydroxamic acids (Fig.1). Arylhydroxamic acids are thought to play a key role in the metabolic activation of aromatic amines. Although they do not react directly with proteins and nucleic acids under physiological conditions, they give rise to the formation of several reactive intermediates: I Sulfates, catalysed by sulfo-transferase, a soluble liver enzyme, whose activity has been linked to the carcinogenic effect in this tissue (3), II Hydroxylamine acetate, due to the activity of an N,O-acyltransferase (4,5), III Nitroxide free radical, which may further disproportionate to yield N-acetoxy-N-acetyl-amino- and nitroso-derivatives (6).

With the three carcinogenic aromatic amines studied most extensively a rather simple reaction pattern was found. The hydroxamic acid esters of 2-fluorenyl-, 4-biphenyl- and 2-phenanthrylamine all react with guanine which is substituted in the 8-position by nitrogen. In addition the fluorenyl and biphenyl derivatives react with the amino-group of guanine (N²) in the position ortho to the nitrogen, and the phenanthrene derivative with adenine at N⁶ to give a 1-phenanthryl-acetamide adduct (7-9). None of the respective reaction products was found with the hydroxamic acid ester of trans-4-aminostilbene (10). The trans-stilbene nucleus thus appears to exhibit some special features.

Metabolic activation of trans-4-dimethylaminostilbene

We have studied the metabolism of trans-4-dimethylaminostilbene (trans-DAS) in more detail (Fig.1) and identified α,β -dihydroxy-acetylaminobibenzyl as a major metabolite (11). We therefore proposed that an epoxide is formed at the stilbene double bond. Such a trans-

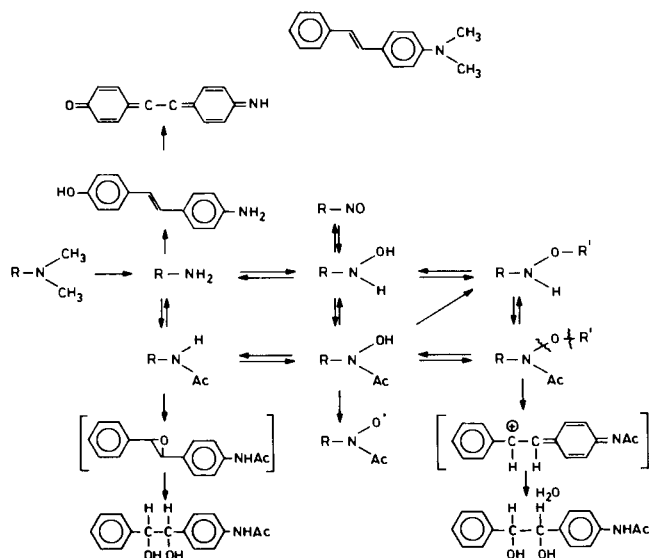


Fig.1. Metabolic activation of trans-4-di-methylaminostilbene. R = trans-stilbene, R' = different acid residues, Ac = -CO-CH₃.

disubstituted epoxide is not expected to be a substrate for epoxide hydrase (12). From rat urine the threo-diol was isolated which is also found as the solvolysis product of the synthetic epoxide in aqueous medium (11). This metabolic pathway would be unique to stilbene derivatives as compared to other carcinogenic aromatic amines. The stilbene double bond, however, in addition influences the distribution of the positive charge in the nitrenium ion formed from hydroxamic acid esters, and the β -carbon represents the most reactive site for nucleophilic attack (13). Accordingly, β -methylmercapto-4-acetylaminostilbene was the major product obtained from the reaction with methionine and upon the degradation of liver proteins from rats given N-hydroxy-4-acetylaminostilbene (13). However, the intermediate quinone-imide-methide (Fig.1) may not only stabilize by the rearrangement of a proton. It may also add water, and solvolysis of N-acetoxy-N-acetylaminostilbene leads to the same α,β -diol obtained through the epoxide pathway. In this case, however, a mixture of the threo and the erythro-isomer is formed (14).

The stilbene double bond thus represents a structural feature which by two different metabolic routes leads to the same reaction products, which in turn can be expected to be different from those obtained with other aromatic amines.

Scribner and coworkers (15) quite recently were able to identify some of the numerous reaction products between N-acetoxy-N-acetylaminostilbene and guanosine, adenosine and cytidine. The major adduct with guanosine is 1-(4-acetamidophenyl)-1-(1-guanosyl)-2-hydro-

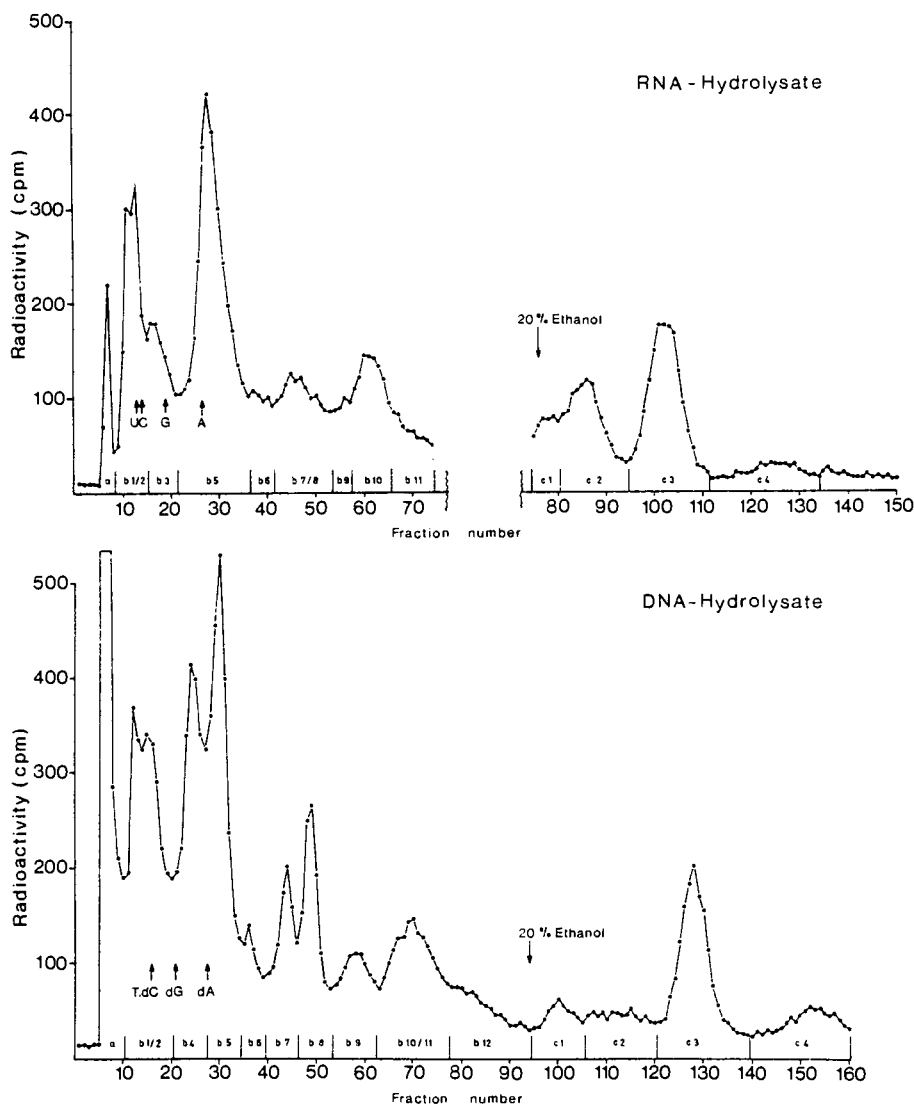


Fig.2. Separation of adducts from nucleic acid hydrolysates. (^3H)-trans-DAS (2.5 $\mu\text{mol/kg}$) was orally administered to female Wistar rats. After 5 h the livers of two animals were excised and the nucleic acids were isolated (20), enzymatically hydrolysed and the digests were chromatographed on Sephadex LH 20 (400 ml NH_4HCO_3 , 10mM, pH 7.5, 400 ml this buffer cont. 20% ethanol). Fractions of 5ml were collected and the radioactivity was determined.

xy-2-phenyl-ethane. The respective O⁶-derivative appears to be formed as a minor adduct. Adenosine yielded two major compounds: 1-(4-acetamidophenyl)-1-(N⁶-adenosyl)-2-hydroxy-2-phenyl-ethane and a compound in which N¹ of adenosine substituted the β-hydroxy group, leading to ringclosure: 3-(β-D-ribosyl)-7-phenyl-8-(4-acetamidophenyl)-7,8-dihydro-imidazo-(2,1-i)-purine. The reaction with cytidine finally leads to a deamination product: 1-(4-acetamidophenyl)-1-(3-uridylyl)-2-hydroxy-2-phenyl-ethane (16). Naimy et al. (15) assume that in all cases the β-carbon of the N-acetyl-N-4-stilbenylnitrenium ion is the site of the initial reaction and that further rearrangement gives rise to the described compounds.

Similar reaction products were also obtained when N-acetoxy-N-acetamidostilbene was reacted with homopolynucleotides, RNA and DNA in vitro (10). In addition, however, a number of other products was observed which were not seen in the alkylation of the monomers.

In vivo, however, still other reaction products appear to be formed predominantly from reactive aminostilbene derivatives. We have isolated rRNA and DNA from rat liver after oral administration of trans-DAS. Chromatography of the hydrolysates on Sephadex LH 20 revealed the presence of numerous adducts (Fig.2). The major constituents, like those designated b-5 and c-3 in Fig.2 could not be assigned to any of the in vitro products. Except for the N⁶-adenosine adduct, the in vitro products identified elute between fraction 55 and 80 on this column and radioactivity from the in vivo-hydrolysates has been provisionally assigned to them (17).

Dose-dependence of the metabolic activation of trans-4-dimethylaminostilbene

The metabolic activation of trans-DAS, as measured by covalent binding to cellular constituents, appears to be dose-independent over a wide range of doses (18). The lowest dose in our most recent experiments was as low as 5×10^{-10} mol/kg, which is equivalent to only 20 pg/animal of 200g. The highest dose was $2,5 \times 10^{-4}$ mol/kg, which is equivalent to the LD₅₀. Tissue exposure (Fig.3) was independent of the dose, except for high doses, where delayed absorption from the gastrointestinal tract, due to the low solubility of the compound, and a decreased uptake from the portal vein into the liver of absorbed material, lead to a decrease of the tissue index in the liver and a concomitant increase of the tissue index in some peripheral tissues like glandular stomach (19). This effect is more pronounced at 5 h as compared to 24 h after administration. Binding to proteins, rRNA and DNA in liver (Fig.4) follows rather closely the tissue exposure with total metabolites, indicating that enzymes involved in the metabolic activation are not saturated even at the high doses applied. This is underlined by the fact that the pattern of adducts in rRNA remained practically constant over the entire dose range studied (20).

Reactive metabolites are not only formed in the liver, they are also distributed in the circulation independent of the dose. This is indicated by the proportionate binding to plasma proteins and hemoglobin (Fig.4). From the linear dose-dependence of these and some other parameters measured so far we conclude that the kinetics of the metabolic activation are simple and linear at low doses. This implies that a pharmacokinetic threshold does not exist for trans-DAS in the

entire dose range studied. At high doses the binding index did not increase but rather decreased in contrast to the predictions from the model of nonlinear binding, proposed by Gehring and Blau (21).

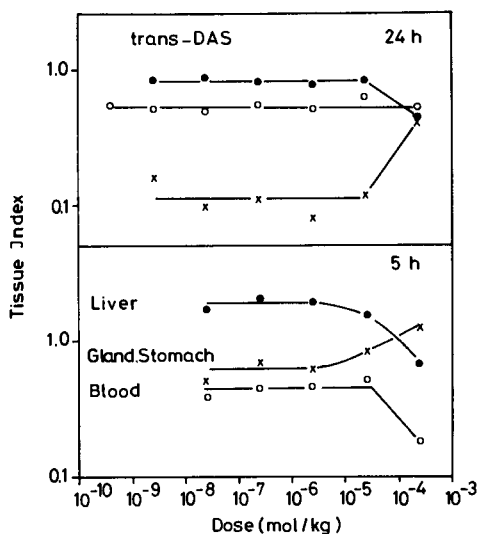


Fig.3. Dose-dependence of the tissue index after oral administration of trans-DAS. Index: concentration (nmol/g tissue) / dose ($\mu\text{mol/kg}$).

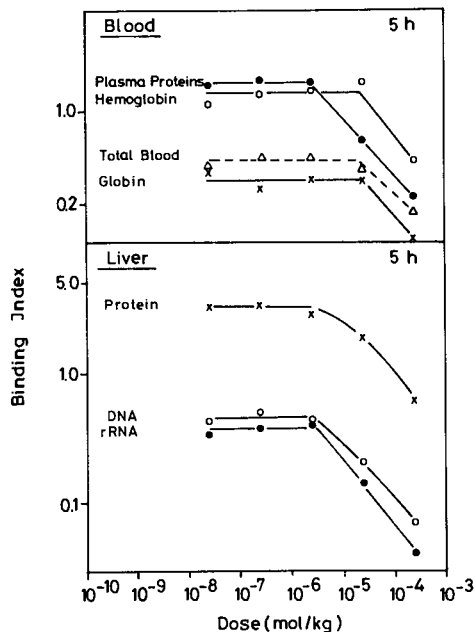


Fig.4. Dose-dependence of the binding index after oral administration of trans-DAS. Index: binding (nmol/g macromolecule) / dose ($\mu\text{mol/kg}$).

Metabolic activation of diethylstilbestrol

The synthetic estrogen diethylstilbestrol (DES) has been associated with the induction of tumors in young women who were exposed to DES prenatally (22). In addition, tumors were produced with DES experimentally in several animal species (23). These effects were primarily related to the hormonal activity of the compound. An alternative mechanism, however, could be that DES, like other carcinogens, is metabolically activated. This possibility occurred to us, when we found that epoxidation of the stilbene double bond may represent a major metabolic pathway. Thorough investigations of DES-metabolism disclosed numerous candidates consistent with this hypothesis (24-26).

4'-Hydroxypropiophenone was isolated from the urine of male rats dosed with DES and of female mice which received labeled DES-epoxide (25,26). It is most likely, therefore, that the proposed epoxide is indeed formed and is a precursor of this cleavage product. In addition, aromatic hydroxylation may proceed through arene oxides. Catecholes were identified as metabolites and may be further oxidized to the respective semiquinones and quinones (Fig.5).

Another major metabolite in most species results from the oxidation of the aliphatic side chain. Although ω -hydroxy- β -dienestrol is not expected to be reactive itself, as an allylic alcohol it might be sufficiently activated by esterification.

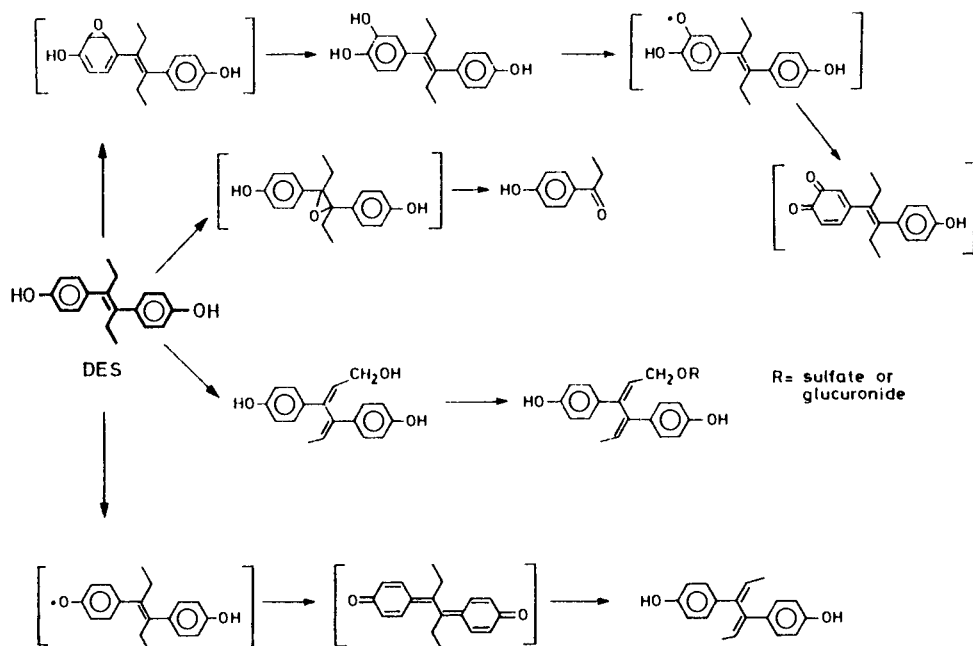


Fig.5. Metabolic activation of diethylstilbestrol (DES).

β -Dienestrol is also a major metabolite in most species. It is not formed from the DES-epoxide, as assumed originally (24). An alternate pathway received support from in vitro studies. The oxidation of DES can be mediated by peroxidase in the presence of H_2O_2 (27). This finding has now been reproduced, and it has been shown that horse radish peroxidase as well as peroxidase from mouse uterus catalyze this reaction and lead to the formation of the one β -dienestrol isomer (Z,Z-dienestrol) among three possible isomers, which is also found as a metabolite (28). Moreover, it has been demonstrated that intermediates of this reaction, i.e. the semiquinone or the quinone, irreversibly bind to albumin and salmon sperm DNA.

The biological significance of reactive intermediates in DES-metabolism cannot be ascertained at the present time. It appears interesting, however, that some observations may provide clues to the tissue specificity observed in DES-toxicity. Among the DES-metabolites only DES-epoxide exhibits pronounced estrogenic activity. The affinity constant for the cytoplasmic estrogen receptor from mouse uterus ($K_a = 1 \times 10^{-9} M^{-1}$) is only one order of magnitude lower than that of DES (29). The receptor affinity may thus assist the contact of a potentially reactive metabolite with a possible critical target by carrying the activation product to the cell nucleus.

The peroxidase mediated oxidation of DES may also contribute to the tissue specificity. Peroxidase activity has recently been demonstrated in estrogen dependent tissues (30-32) and is now even considered a marker enzyme for those tissues. Moreover, the enzyme can be induced by estrogens, including DES, even in immature animals. The possible significance of this may be underlined by the finding that the enzyme is found in the kidneys of male hamsters, which are susceptible to the carcinogenic effect of DES (33) and that the activity there is much higher than in the kidneys of rats and several strains of mice, which are not susceptible.

It will be interesting to see, whether peroxidase mediated oxidations are involved also in the metabolism of aminostilbene derivatives. 4'-Hydroxy-4-aminostilbene has been identified as a metabolite (34) and in vitro readily reacts with H_2O_2 in the presence of horse radish peroxidase (cf Fig.1).

Acknowledgements

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Metabolic Activation of Chlorinated Ethylene Derivatives

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ABSTRACT

Chlorinated ethylenes are activated metabolically to epoxides. Evidence for this first step reaction is obtained from the excreted metabolites, chlorinated ethanols and acetic acids. These can be derived from the epoxide rearrangement products, chlorinated aldehydes or acyl chlorides. An exception to this rule is encountered with trichloroethylene the epoxide of which rearranges *in vitro* to dichloroacetyl chloride whereas the metabolites are oxidation or reduction products, respectively of trichloroacetaldehyde. Since the rearrangement to the aldehyde can be forced by the presence of Lewis acids it is concluded that *in vivo* trichloroethylene epoxide is converted, by virtue of the trivalent iron of P450, at the site of formation in the hydrophobic premise to chloral.

According to results in a modified Ames test system vinyl chloride, vinylidene chloride and trichloroethylene exert mutagenic activity after activation by induced liver microsomes, whereas tetra- and 1,2-dichloroethylenes (cis and trans) are inactive. A molecular rule is derived from these findings that unsymmetric chlorine substitution induces, by an imbalanced electron withdrawing effect, the epoxides highly electrophilic, as a prerequisite for mutagenicity and carcinogenicity.

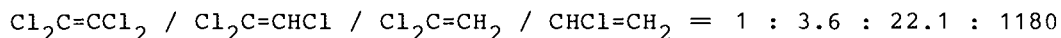
INTRODUCTION

Chlorinated ethylenes have attained increasing interest since the reports that vinyl chloride produces malignancies (hemangiosarcomas) in exposed workers (1) and in a variety of experimental animals (2-4). Some compounds of this class of chemical analogues are used as plastic monomers (vinyl and vinylidene chlorides), as anaesthetic gases (trichloroethylene) or as organic solvents and degreasing agents (tri- and tetrachloroethylenes); some are used as intermediates in organic synthesis (1,2-dichloroethylenes). Right after the disclosure of the carcinogenic potential of vinyl chloride suspicions have been raised that on account of their structural similarity all members of the group might exert carcinogenic activity. We have performed a systematic study on the whole series of chlorinated ethylenes in order to identify the structural requirements for the oncogenic effects, and which alternatives lacking the active structure were at hand, by starting with a theoretical consideration of the

chemical reactivity as a means to predict the type and amount of metabolic transformation, identify the metabolites, test their chemical and biological reactivity using in vitro mutagenicity test systems, and finally come to conclusions on structure/activity relationships.

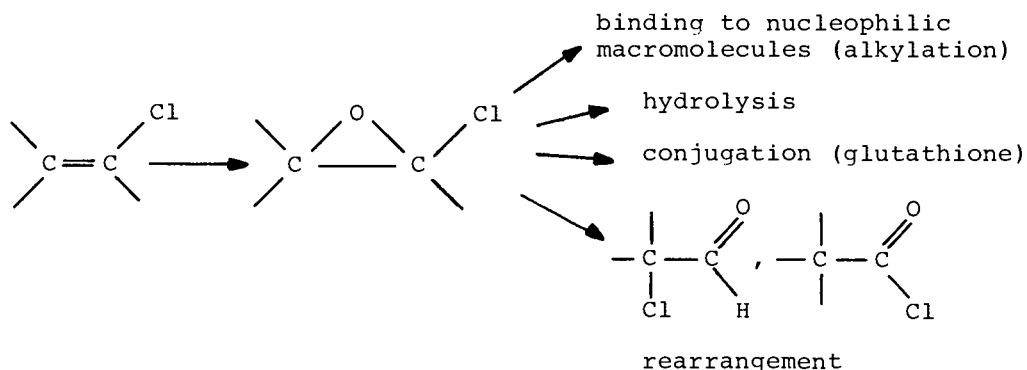
CHEMICAL REACTIVITY

In chlorinated aliphatic compounds, the peculiar properties of the C-Cl bond decisively influence chemical stability. In general, the electron withdrawing effect of the chlorine residues dominates the mesomeric donator effect of the involved carbon atoms. This results, in the case of alkanes, in a destabilisation of C-C bonds, whereas in olefinic structures the decrease in electron density exerts a stabilisation of the double bond. In addition, the bulky chlorine atoms provide a steric protection against electrophilic attacks. Thus, chemical reactivity is increased with the number of chlorine substituents. In the series of chlorinated ethylenes, this has been demonstrated by the reaction with ozone which increases, in terms of relative rates, as follows (5):



METABOLIC CONVERSION

Epoxidation as a first step of metabolism of chlorinated ethylenes has been postulated since long. The formed epoxides may undergo a variety of reactions in vivo:



Alkylation of nucleophilic sites in cellular macromolecules is regarded the primary lesion for acute as well as for chronic toxic and mutagenic and carcinogenic effects whereas hydrolysis and conjugation (both with and without enzyme catalysis) and intramolecular rearrangement represent deactivation mechanisms. A high tendency of rearrangement, mostly by migration of chlorine atoms, is common to all chlorinated ethylenes. The formed chlorinated aldehydes or acyl chlorides are far less reactive as compared to the epoxides. Therefore, the formation of chlorinated ethanols and acetic acids, by subsequent reduction, oxidation or hydrolysis of the rearrangement products, should be expected as excretable metabolic endproducts.

To test this, we have prepared the epoxides of the whole series of chlorinated ethylenes (except 1.1-dichloroethylene oxide which seems too unstable, and resists all attempts of synthesis by conventional methods) and studied the rearrangement mechanisms *in vitro* (in non-polar solvents) (6). The results are presented in fig. 1. With three

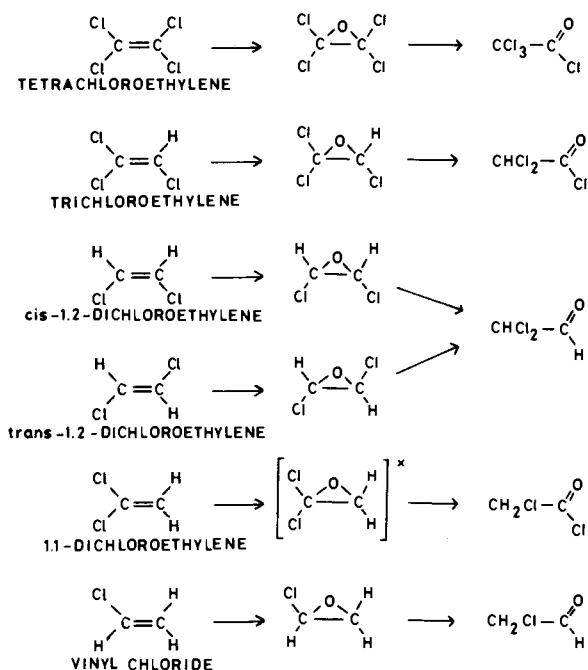
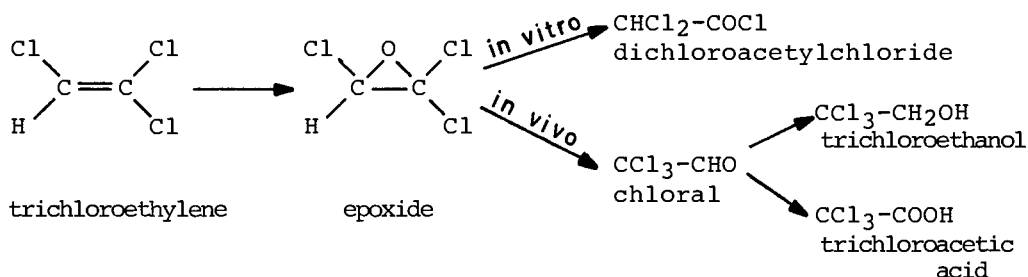


Fig. 1. Expoxidation and thermal rearrangement of epoxides in the series of chlorinated ethylenes.^x The expoxide of 1.1-dichloroethylene has not been described up to now.

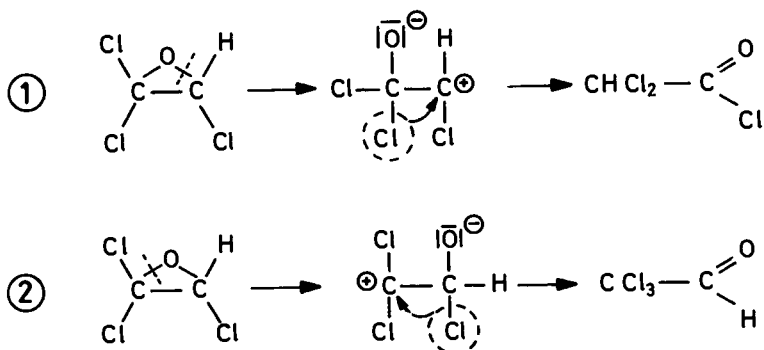
compounds, we find chlorinated aldehydes (1.2-dichloroethylenes *cis* and *trans*, vinyl chloride), in the rest acyl chlorides are formed; with vinylidene chloride (1.1-dichloroethylene) chloroacetic acid chloride is detected when trying to obtain the epoxide by UV-irradiation.

These results were compared with the metabolites formed *in vivo*. This was done with the isolated perfused rat liver preparation (6). As can be seen from table 1, the identified metabolites are in good agreement with the expectation, as derived from the rearrangement studies (fig. 1), in the cases tetra-, *cis* and *trans*-1.2-dichloroethylenes, 1.1-dichloroethylene, and vinyl chloride; with the latter two compounds, conjugates with glutathione and subsequent conversion products prevail, which has been confirmed by studies in whole animals by several authors (7-9).

There is, however, one important exception: trichloroethylene. This compound is rearranged in vitro to dichloroacetylchloride whereas in vivo not even traces of dichloroacetic acid can be found; this has been confirmed recently in a microsomal metabolic conversion system (10). The only rearrangement products, and subsequent metabolites are chloral(hydrate), trichloroacetic acid, trichloroethanol and its glucuronide, as oxidation and reduction products, respectively:



We can offer an explanation for this different behaviour in vitro and in vivo. The thermal rearrangement mechanism can be theoretically deduced if one assumes the formation of ion pairs as intermediates:



In the case of ① the single Cl-substituted ionized carbon has more stability than the double Cl-substituted in ②, thus the formation of dichloroacetyl chloride ① has higher probability.

One can, however, force the rearrangement to chloral, according to ② and to the behaviour in vivo, by the addition of Lewis acids, like FeCl_3 or AlCl_3 , to the epoxide (11). It is conceivable that such a Lewis acid like catalysis can occur under in vivo conditions at the site of the formation of the epoxide in the catalytic center

of P450; the trivalent iron would act as a Lewis acid. This would imply that no epoxide has a chance to escape the hydrophobic premise of that enzyme. Some confirmation for this assumption is provided by recent findings that the epoxide hydrolyses rapidly, in aqueous solutions, to CO, HCOOH, COOH·CHO and HCl; therefore, if the epoxide could react outside the catalytic center, some CO formation should be expected. However, there is no carbon monoxide detectable, neither *in vivo* nor in microsomal preparations, after trichloroethylene exposure (12). Thus, the rearrangement of trichloroethylene oxide to the non-reactive chloral by the activating enzyme itself represents a curious example for a protective mechanism.

BIOLOGICAL REACTIVITY OF METABOLITES

From the above deductions it should be expected that the epoxides of chlorinated ethylenes confer the alkylating and carcinogenic effect. This opens the question on the influence of the number and position of the chlorine residues upon the reactivity of these oxiranes. According to the -I effect of the chlorine atoms, unsymmetrically substituted oxiranes should be expected more reactive than the symmetrical ones. This could best be tested with an *in vitro* microbial mutagenicity system, with and without addition of activating enzyme preparations.

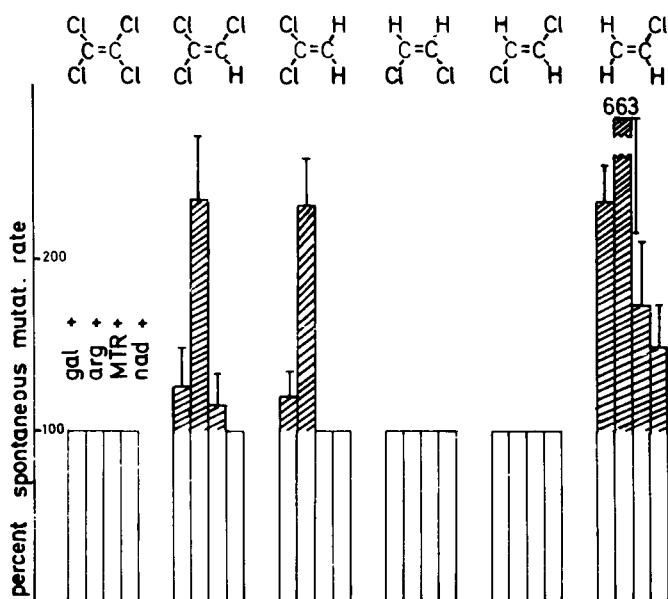
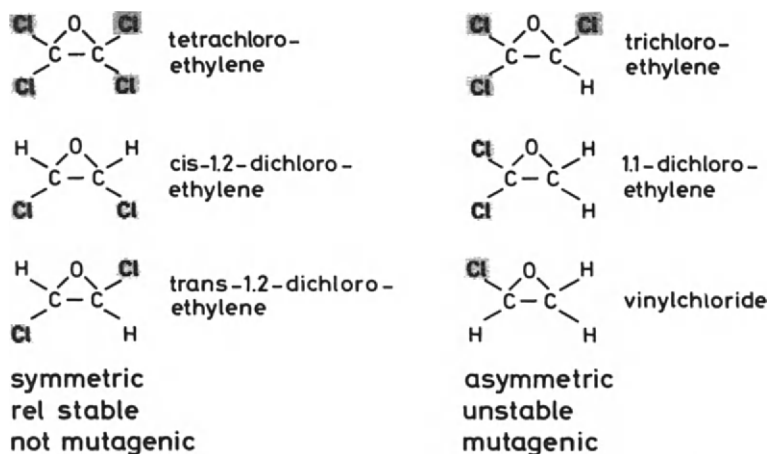


Fig. 2. Mutagenicity of chlorinated ethylenes in a modified Ames' test system, using stimulated mice liver microsomes, and E. Coli K 12 as a test germ. Filled columns: increase in spontaneous back mutation rate. Substrate concentrations: 0.9 to 10.6 mM to obtain at least 70% survival of test organisms (13).

The results of such a study are demonstrated in Fig. 2 (13). *E. Coli* K 12 was used since the high primary toxicity exerted by the compounds prohibited the use of *S. typhimurium* strains. Mutagenic activity was detected only after addition of microsomal preparations. There was high activity with vinyl chloride, less with vinylidene chloride and with trichloroethylene. Tetrachloro- and 1,2-dichloroethylenes *cis*- and *trans*- were inactive in this system.

From these findings we have extracted the following tentative molecular rule of the structural requirements for a mutagenic and/or carcinogenic activity:



The unsymmetric chlorine substitution renders the metabolically formed epoxides, by virtue of the unbalanced electron withdrawing effect, rather unstable. They exert a higher electrophilicity as compared to the symmetrically substituted analogues. Confirmation for this has been obtained recently by studying the half life of the epoxides in neutral buffer solutions (with 10% acetone): 12" for trichloroethylene oxide, and 1'20" for tetrachloroethylene oxide (14). The difference in strength of the electrophilicity correlates well with the mutagenic potential: the symmetric molecules are inactive whilst all unsymmetrically substituted compounds exert some mutagenic effect.

The *in vitro* mutagenicity testing results seem, at first glance, in good accordance with findings on the carcinogenicity in whole animals. Vinyl chloride is definitely identified as a carcinogenic agent (1), vinylidene chloride is active in rodents (15). Trichloroethylene has been reported to induce, after 18 months oral administration of extremely high doses, liver cancer in B3H6F₁ mice (16). It could be demonstrated, however, that the technical sample of trichloroethylene used in these studies contained considerable amounts of epichlorohydrine and epoxibutane which were added as stabilizers; both compounds are strong mutagens (17). Thus, the

carcinogenic potential of trichloroethylene has still to be demonstrated. Our above reported findings on the mechanisms of metabolic transformation confer considerable probability that trichloroethylene epoxide which is formed by P450 may well be completely converted, by a Lewis acid like catalysis of P450 iron, to the non-carcinogenic chloral within the hydrophobic premise of the enzyme. This exceptional behaviour could explain the low mutagenic potential in the Ames-test as well as the assumed non-carcinogenicity in intact animals. A recent comparative study with vinyl chloride and trichloroethylene in intact rats confirmed the occurrence of pre-neoplastic lesions in the liver after vinyl chloride, and the absence of such changes after identical doses of trichloroethylene (18).

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Carbenes and Free Radicals of Haloalkanes as Toxic Intermediates

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ABSTRACT

Polyhalogenated hydrocarbons are known to be reduced by cytochrome P450 under hypoxic conditions. The corresponding C-radicals, the primary reaction products, may induce lipid peroxidation or may be further reduced to yield carbene complexes with cytochrome P450. In the case of polyhalogenated methanes these carbenes may hydrolyze to give CO. All other carbene complexes are also believed to be unstable especially in the presence of oxygen and have a potential activity for covalent binding to macromolecules.

INTRODUCTION

Haloalkanes are widely used as solvents and anaesthetics. Their fate in the body is largely dependent on the structure of the haloalkane. Investigations on the structure-activity relationships seem interesting in view of the variety of haloalkanes and their manifold reactions in the body. Some of them have been reported to be highly toxic, others are without effect. It is clear that many of the haloalkanes exhibit toxicity under certain conditions. The definition of these conditions and the elucidation of the mechanisms involved is one of the challenging fields in Biochemical Pharmacology.

The extensive literature on the disposition and metabolic fate of this class of compounds allows certain generalisations to be made. Their lipid solubility characterises them as potential substrates for metabolic conversion by the microsomal drug metabolising system. If the molecule contains CH bonds the unspecific microsomal system is able to hydroxylate this bond. In those cases where halogens are substituents of the same carbon atom, an unstable intermediate is formed which eliminates a proton and an halide ion to form a carbonyl function. By this mechanism carbon monoxide results from monooxygenation of methylene chloride (1) and phosgene from chloroform (2,3).

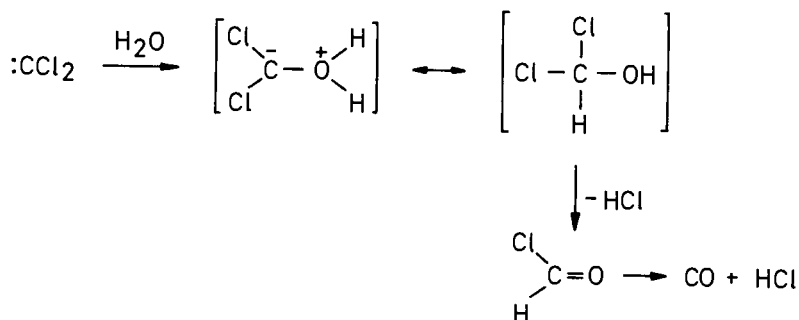
A recent study on trihalomethanes (4) has demonstrated that these compounds can also give rise to carbon monoxide in the presence of liver microsomes, an NADPH generating system and oxygen by a mechanism which is as yet unknown.

Studies on the metabolism of polyhalogenated methanes in vivo have shown that reduction of a C-halogen bond occurs. This reduction in most cases is mediated by cytochrome P450 and proceeds similarly to the reduction of nitro- or azocompounds by this enzyme. This seems surprising as reduced cytochrome P450 has a high affinity for molecular oxygen and would be expected to react exclusively with oxy-

gen. However, it has been suggested that the oxygen tension in cells at the centre of the liver lobules is low (5). At these low concentrations of oxygen the haloalkanes can compete effectively for the electrons of cytochrome P450 (6).

The nature of the products of this reduction process present an interesting problem. Since cytochrome P450 is a one electron donor a C-radical species must be formed first upon release of a halide ion. Indeed, in the well-known case of carbon tetrachloride the CCl_3 -radical has been postulated (7) and proved by means of spin trapping (8), and by demonstrating the formation of the dimerization product hexachloroethane (9). The toxicity of CCl_4 resulting in liver necrosis has been based on the lipid-peroxidation induced by a chain reaction starting from the CCl_3 -radical (10). Cytochrome P450 most likely causes the formation of this radical (11).

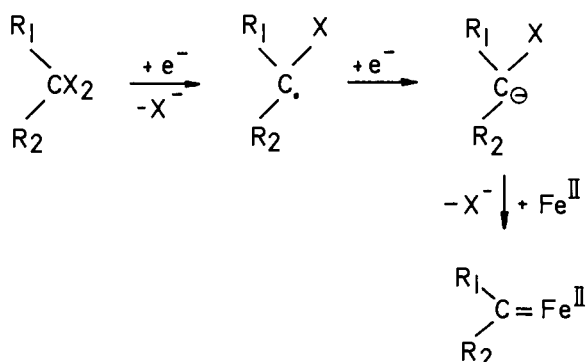
A report by Uehleke *et al.* (5) suggested that carbon tetrachloride can form a complex with reduced cytochrome P450 having a Soret absorption at about 450 nm. According to our studies we believe this complex to be a metabolite which is bound as a ligand to reduced cytochrome P450. Since carbon monoxide is also found as a product in this reaction, it was postulated that the carbene CCl_2 is the intermediate, which could hydrolyse to give CO (12). This may occur according to the following reaction:



A carbene species was also postulated as the spectral intermediate produced from halothane in the presence of reduced liver microsomes. In this case the carbene could also be formed by adding 1,1,1-trifluorodiazaoethane to reduced liver microsomes by an independent pathway (13). The halothane-carbene complex could be photodissociated by light which further proved the carbon ligand nature of the halothane metabolite (14).

From these experiments we have postulated that aliphatic compounds with more than one halogen at one carbon atom can be reduced by cytochrome P450 first to a radical and then to a carbanion which stabilizes by release of a halide ion yielding a metal-bound carbene (as shown on the next page).

Since free carbenes are known to be highly reactive species in organic chemistry we have been interested in their role as potential toxic agents in the body. This paper describes further experiments on the reductive dehalogenation and discusses the mechanisms by which haloalkanes could produce damage to the living cell.



RESULTS AND DISCUSSION

1. CO-formation from polyhalogenated methanes

We have previously reported that a series of polyhalogenated methanes yield ligand-type complexes and also form CO under anaerobic conditions (Table I).

TABLE 1 λ_{max} Values and CO Formation Obtained on Interaction of Various Halogenomethanes with Ferrous Cytochrome P450 in the Presence of Sodium Dithionite

Compound	P450 ligand complex nm	CO formation nmol/min/nmoles P450
CCl ₄	459	0.48
CBr ₄	465	0.20
CCl ₃ F	453	0.15
CCl ₃ CN	468	0
CHCl ₃	464	Trace
CHBr ₃	464	0.15
CH ₃ I	464	0.08

Experiments carried out after addition of haemoglobin to test and reference cuvettes

From the table it is evident that a close relationship exists between the formation of the postulated carbene complex and the release of carbon monoxide. Although the anaerobic conditions exclude lipid peroxidation as a possible source of this carbon monoxide, it was important to establish that the CO carbon atom was derived from CCl₄ in order to support the concept of the CCl₂ carbene as the key intermediate. This was achieved using labelled ¹⁴CCl₄ by trapping the CO formed and analysing for radioactivity after oxidation to CO₂. The preliminary results of these experiments are given in Table 2.

TABLE 2 Formation of ^{14}CO from $^{14}\text{CCl}_4$ in a Microsomal Suspension in the Presence of Sodium Dithionite

Experiment	d.p.m of ^{14}CO , detected as $^{14}\text{CO}_2$ in the effluent gas	
	1st litre	2nd litre
Microsomes	10,300	4,200
Acid-precipitated microsomes	360	1,500 ⁺

The anaerobic reaction mixture contained 90 mg microsomal protein, 30 μmoles $^{14}\text{CCl}_4$ (1 μCi) and 25 mg dithionite in 30 ml 0.1 M Tris-HCl buffer, pH 7.6. After the incubation the mixture was acidified and the volatile products driven with N_2 through a cold trap, a silica gel column and a Hopcalite catalytic column into 1 l evacuated flasks containing ethanolamine.

⁺

The counts in the control are attributed to low levels of $^{14}\text{CCl}_4$ slowly escaping from the silica.

2. The reduction of CCl_4 and CCl_3F

According to Table 1 both CCl_4 and CCl_3F form carbene intermediates. Interestingly, however, in contrast to carbon tetrachloride, the freon is essentially nontoxic (15). This correlates with the observed lipid peroxidation which is only apparent with CCl_4 and not with CCl_3F (16). On the other hand both compounds show covalent binding to microsomal lipids and proteins when incubated under conditions where the carbene complex should be formed (Table 3) (17).

TABLE 3 Covalent Binding of $^{14}\text{CCl}_4$ and $^{14}\text{CCl}_3\text{F}$ to Microsomal Protein and Lipid in Vitro under Anaerobic Conditions

Compound	Covalent binding (nmole/30 min/mg)	
	Protein	Lipid
CCl_3F (1 mM)	7.1	28.4
CCl_4 (1 mM)	15.1	58.5

Values for CCl_4 taken from Uehleke and Werner (18)

From these results we would conclude that covalent binding *per se* does not lead to necrosis of the liver cell, but that the chain reaction of lipid peroxidation initiated by formation of the CCl_3 -radical from CCl_4 is the critical event.

The question then arises why CCl_3F in spite of its very close chemical structure to CCl_4 cannot induce lipid peroxidation.

One reason may be the weak electron acceptor properties of this compound in microsomes, as indicated in Table 4.

Under strictly anaerobic conditions CCl_4 takes up electrons from NADPH causing a decrease in absorbance at 340 nm. CCl_3F acts similarly but the decrease amounts to only about 30-40 % of that caused by CCl_4 . It can be shown that the decrease is not

TABLE 4 NADPH Consumption by Anaerobic Rat Liver Microsomes in the Presence of Haloalkanes

Substrate	NADPH Oxidized ⁺ (nmol/mg protein / min)
CBr_4	4.75
CCl_3Br	2.46
CCl_4	1.31
CCl_3F	0.66
CHCl_3	0.33
CH_2Cl_2	0.00

⁺typical experiment

linear, indicating the formation of the carbene ligand which is likely to inhibit further reduction of the haloalkanes. On the other hand the postulated carbene complex is not stable since a continuous CO-production is observed (Fig. 1) due to hydrolysis of the corresponding carbenes.

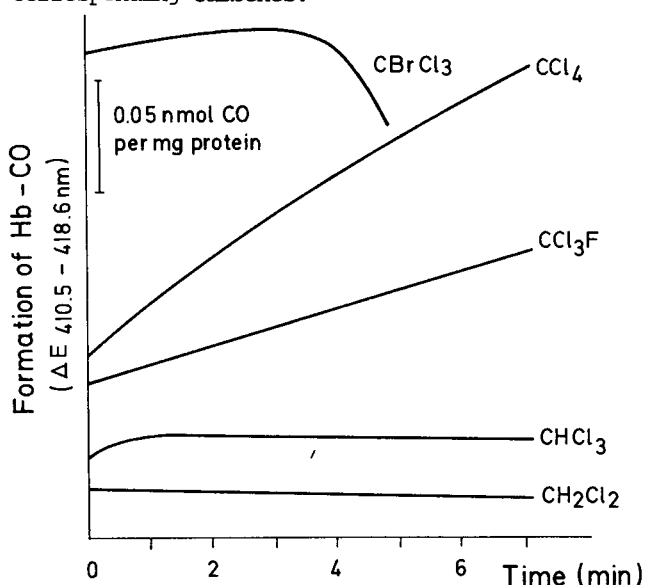


Fig. 1 Formation of carbon monoxide from halogenomethanes under anaerobic conditions

Incubations in closed cuvettes contained microsomal protein (1 mg/ml) and haemoglobin (2.4 μM) in 0.1 M Tris-HCl buffer made anaerobic by repeated evacuation and gassing with N_2 . Substrates (final concentration 1 mM) were added and the reaction started by the addition of NADPH (34 μM).

The diminished electron acceptor properties of CCl_3F compared to CCl_4 or even CBr_4 cannot exclusively explain the inability of this compound to induce lipid peroxidation. A second reason could be the stability of the CCl_2F radical. It could be suggested that this radical intermediate is less stable than the corresponding CCl_3 and CBr_3 -radicals.

3. Generalized scheme of reductive haloalkane metabolism

From our results and those reported in the literature a generalized scheme for the reductive metabolism of haloalkanes can be formulated. A number of factors contribute to the reduction of the haloalkanes and their subsequent fate in the body and these are now discussed.

3.1. Influence of the oxidation potential

In order to accept electrons from the various redox systems in the cells the haloalkane must possess a suitable oxidation potential. This usually rises from I to Br and Cl. The C-F bond resists any direct reductive cleavage at least by biological redox systems. An additional influence is the number of halogen atoms which tend to raise the potential with increasing numbers. Also any other inductive effects which decrease the electron density at the carbon will have the same influence. An additional factor is the chemical nature of the reducing component since stabilization of the transition state of the reaction will lower the activation energy and thus enhance the reduction. Metal complexes are especially suitable in this respect and most of the reducing electrons are provided by such metal complexes. In the cases of CBr_4 , CCl_3Br or CCl_4 reduction may also occur by other reducing agents like flavoproteins or sulfhydryl groups. It seems, however, from inhibition studies with carbon monoxide, that even with these haloalkanes the reduction in liver cell homogenates proceeds mainly by cytochrome P450. This enzyme has the ability to bind lipophilic compounds, including haloalkanes at the active site which is in close proximity to the haem iron. Substrate saturation is usually obtained at 10^{-4} – 10^{-3}M concentrations which allows the cytochrome to compete effectively with other reducing systems which can only react with second order rate constants.

3.2 Influence of steric factors

When a haloalkane has been bound to cytochrome P450 the electron transfer to a C-halogen bond requires a direct interaction with the reduced iron. This could be a function of the steric arrangement of the halogens. Where steric hindrance of the halogens by adjacent substituents occurs this interaction would be decreased. Similarly, the stereochemistry of the environment of the cytochrome P450 active site could be a determining factor. This could be especially important as several forms of cytochrome P450 exist with different substrate specificities. In the case of haloalkanes of high molecular weight a rather rigid orientation of the substrate may occur which prohibits the appropriate binding of the halogen to the iron atom.

3.3 Stabilities of the C-radical intermediates

As pointed out before the radical intermediates resulting from a one electron transfer to a C-halogen bond may vary in their stability. If it is stable enough to be released from the active site it can react with unsaturated fatty acids and the process of lipid peroxidation may be initiated. What may also happen, however, is a transient stabilization at the ferric haem by spin coupling, followed by transfer of a second electron. This complex can be written as $\text{Fe}^{\text{II}}-\dot{\text{C}}\text{X}_3$ or as $\text{Fe}^{\text{III}}-\text{CX}_3$. The latter resonance structure may be favoured and can react by two mechanisms: i) by protonation, yielding the reduced haloalkane and ii) by elimination of a second halide ion to yield a carbene which can bind to the iron by its lone electron pair to form a tight complex with the reduced haem.

3.4. Influence of the stability of the carbene complex

The unique structure of cytochrome P450 allows binding of the carbene to the sixth coordination site which by its highly hydrophobic character can further stabilize the carbene complex. The main stabilization results from the electronic interaction of the carbon and iron atom. It seems premature to describe these bond characteri-

stics in detail, but the synthesis of the corresponding haem carbene complexes by Mansuy et al. (18) may soon provide a better understanding of this interaction. A major factor in the decomposition of the carbene complex is its reactivity with components of the system that can reach the active site of cytochrome P450. One of these reactants could be molecular oxygen which could act as an oxidant and attack either the ferrous haem or the carbene or both. Oxidation of the iron would lead to an unstable complex which could release the carbene. This would most likely react with water or oxygen or could bind to surrounding proteins and lipids, leading to covalent binding. The electronic configuration of the carbene may have the greatest influence on the stability of the complex but it also may depend on the species of cytochrome P450. Our studies have shown that after induction with polycyclic hydrocarbons the resulting species of cytochrome P450 are only weakly complexing agents for the haloalkane-derived carbenes in contrast to the forms induced by phenobarbital (12).

In conclusion, it is clear that the halogenoalkanes can give rise to various intermediates produced by reduction at cytochrome P450. The initial product is a C-radical which depending upon its stability and chemical characteristics, may induce lipid peroxidation or may be further reduced to yield another intermediate, the carbene. This species is known to be highly reactive but the contribution that this makes to the hepatotoxic action of the haloalkanes is as yet unknown.

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Concluding Remarks

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The speakers at this Symposium have discussed many of the problems that arise in attempting to elucidate the mechanisms of toxicity caused by foreign compounds. Although many of the mechanisms are not entirely clear, much progress has been made. Indeed several general conclusions now seem warranted. I shall summarize many of these and illustrate them mainly with work from my Laboratory.

It has become increasingly evident that chemically reactive metabolites may be formed by many different reactions catalyzed by several different enzymes. However, an enzyme may catalyze several different reactions and may transform not only a parent foreign compound but also many of its metabolites. Thus it may catalyze not only those reactions which lead to toxic chemically reactive metabolites but also to those that lead to innocuous metabolites. Moreover, an enzyme may convert one compound to a toxic chemically reactive metabolite and another compound to innocuous metabolites. For example, pretreatment of rats with 3-methylcholanthrene increases the hepatonecrosis caused by acetaminophen (1), but decreases the hepatonecrosis caused by bromobenzene (2). Moreover treatment with phenobarbital increases the acetaminophen-induced hepatonecrosis in mice (1) but decreases it in hamsters (3). Thus the hope of preventing toxicities by treating subjects with inducers and inhibitors of the various forms of cytochrome P-450 appears no longer tenable, because such treatments may decrease the toxicity of one compound but increase that of another.

It is also evident that similar, if not identical, chemically reactive metabolites of a foreign compound may be formed by different pathways. For example, it is now known that a chemically reactive metabolite of phenacetin may be formed by at least three pathways (Fig. 1): 1) Phenacetin is oxidatively dealkylated by a liver microsomal cytochrome P-450 to acetaminophen (4), which in turn is transformed to a chemically reactive metabolite that combines with glutathione (5). 2) Phenacetin is directly activated by a liver microsomal cytochrome P-450 to what appears to be phenacetin-3,4-epoxide, which loses its ethyl group and combines with glutathione to form the acetaminophen-glutathione conjugate (6). 3) Phenacetin is metabolized to N-hydroxyphenacetin (7), then is converted to its N-O-sulfate and N-O-glucuronide conjugates (8), which in turn decompose to chemically reactive metabolites that combine with glutathione to form the acetaminophen-glutathione conjugate. It is noteworthy that the source of the phenolic oxygen in the acetaminophen-glutathione conjugate differs with the pathway: In the first pathway, all of the phenolic oxygen comes from phenacetin (9,10); in the second pathway, 50% of it comes from phenacetin (10) and 50% of it comes from atmospheric

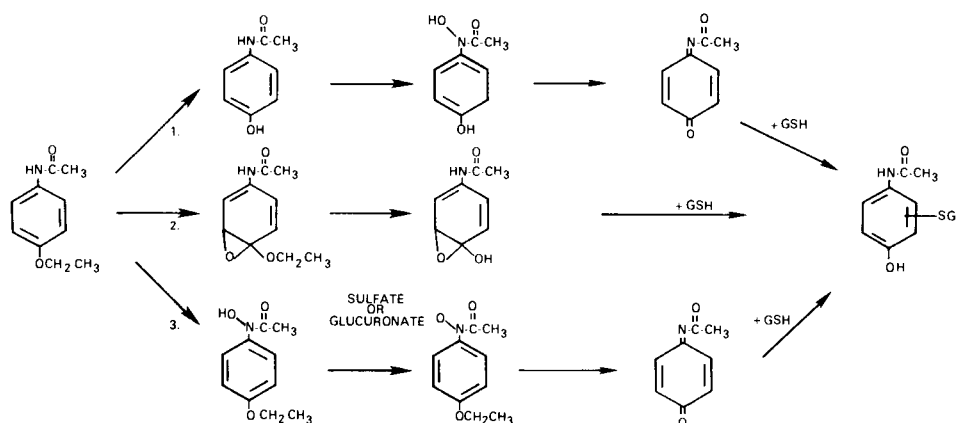


Fig. 1. Pathways of Phenacetin Metabolism

oxygen (6); in the third pathway, all of it comes from water (10).

Inspection of these pathways of phenacetin metabolism reveals that increases in the activities of uridine diphosphoglucuronyl transferase and phosphoadenyphosphosulfate sulfotransferase would not affect the formation of the chemically reactive metabolite by pathway 2, but may increase its formation by pathway 3 by promoting the formation of the N-O-glucuronide and N-O-sulfate conjugates of phenacetin and would decrease its formation by pathway 1 by promoting the formation of the phenolic glucuronide and sulfate conjugates of acetaminophen. Thus the effect of changing the activity of the conjugation system will depend on which pathway predominates in a given animal species. In hamsters, pathway 1 is dominant (10).

Studies on the formation of chemically reactive metabolites have shown that a substance frequently decomposes to several different products and that the formation of some of these products may occur through the sequential formation of several chemically reactive intermediates. For example, when ^{14}C -acetyl labeled phenacetin N-O-glucuronide is incubated in Tris buffer, pH 7.4, it decomposes to phenacetin, phenacetin-2-glucuronide, acetaminophen and acetamide (and presumably quinone) (Fig. 2) (11). When protein is added to the mixture, however, the amounts of acetaminophen and acetamide are decreased and considerable amounts of radiolabel are covalently bound to protein. Thus a portion of the phenacetin N-O-glucuronide is converted to phenacetin and phenacetin-2-glucuronide by mechanisms that are not mediated by the chemically reactive metabolites which ultimately become covalently bound. But at least some of the acetamide and acetaminophen is formed from the same intermediate that reacts covalently with protein. Addition of inorganic phosphate to the buffer results in the formation of phenacetin-3-phosphate at the expense of acetaminophen and acetamide (12). But at an infinite concentration of phosphate, 20-30% of the acetaminophen is still formed while no acetamide is formed. In the presence of excess serum albumin, an infinite concentration of phosphate would totally block the formation of both acetaminophen and acetamide, but even this concentration of phosphate does not completely block the covalent binding of label to protein. These findings thus suggest that in the presence of low phosphate concentrations, phenacetin-N-O-glucuronide can decompose to at least

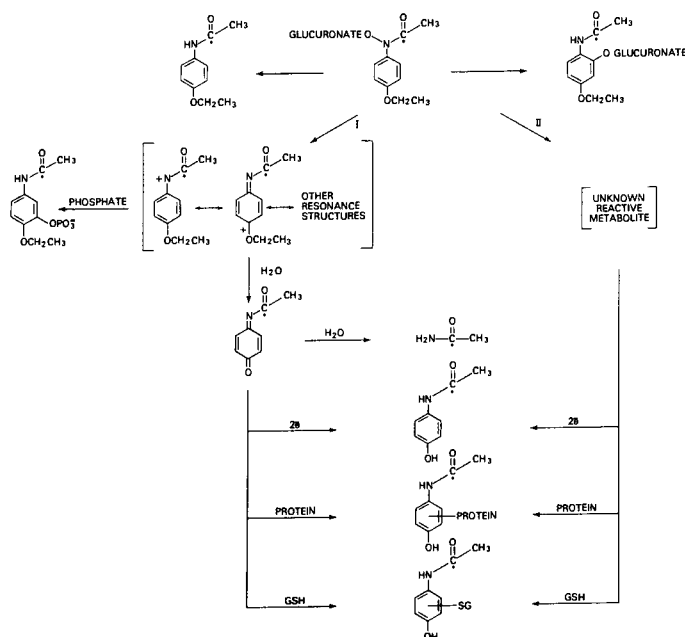


Fig. 2. Decomposition Pathways of Phenacetin and Glucuronide

three unidentified intermediates. At least two of these react with protein or form acetaminophen, but only one is hydrolyzed to acetamide and quinone. Whatever these intermediates may be, it is evident that the decomposition of chemically reactive metabolites can lead to several different products that are usually thought to be formed by more direct mechanisms.

Another important aspect is that a given kind of reaction does not always lead to metabolites that are unstable under physiological conditions. For example, N-hydroxyacetanilide and N-hydroxy-p-chloroacetanilide as well as N-hydroxyphenacetin and N-hydroxy-2-acetylaminofluorene (NOH-2AAF) may be converted to N-O-glucuronides

at similar rates but only the glucuronide N-hydroxyphenacetin appears sufficiently unstable to react rapidly with proteins (8). Moreover, even when reactive metabolites are formed by the same kind of reaction, the metabolites may react with different nucleophiles at remarkably different rates. Indeed a comparison of the patterns of adducts formed from different chemically reactive metabolites frequently may be used to distinguish between them. For example, the chemically reactive intermediates formed from N-O-sulfate and N-O-glucuronide conjugates of phenacetin react almost exclusively with the thiol group of cysteine (11). They do not readily combine with methionine groups in protein or adenyl or guanyl groups in DNA (11). By contrast the chemically reactive metabolite formed from 2AAF-N-O-sulfate is known to combine with all four substances (11,13,14).

Clearly, the formation and fate of chemically reactive metabolites of foreign compounds in enzyme preparations depend not only on the enzyme composition of the preparation but also on the cofactors the investigator chooses to add to it. For example, when ^{14}C -acetyl labeled NOH-2AAF is incubated with rat liver soluble fraction that has been passed through a Sephadex G-25 column to remove small molecular weight substances, it is slowly reduced to 2AAF by an unknown mechanism and hydrolyzed to acetate and N-hydroxyaminofluorene (NOH-2AF) (15) presumably by a combination of esterases and a transacetylase present in the soluble fraction (Table 1) (Fig. 3) (14,16-19). The transacetylase also catalyzes the formation of N-acetoxy-2AAF and N-acetoxy-2AF which on decomposition react with protein and DNA (16-20). The addition of phosphoadenylphosphate and p-nitrophenyl sulfate, which

TABLE 1. Metabolism of (^{14}C -acetyl) NOH-2-acetylaminofluorene

System	NOH-2AAF Remaining	Products			
		Acetate	Conjugates*	2-AAF	Protein Bound
nmoles/60 min/2 mg protein					
Buffer	16.0	0	0.0	0.0	0.0
+ Ascorbate (mM)	15.5	0	0.0	0.5	0.0
Soluble Fraction (SF)**	13.8	1.5	0.7	0.0	0.02
SF + Ascorbate (1.9 mM)	11.7	2.3	0.0	2.0	0.02
SF + PAPS	3.8	0	10.4	1.3	0.64
SF + PAPS + Ascorbate	3.8	0	2.2	10.0	0.11
SF + NADPH (1.0 mM)	11.4	-	-	1.1	-
SF + PAPS + NADPH	3.1	-	-	11.4	-

*Conjugates include a dimer of 2AAF, 2AAF-7-phosphate and a p-nitrophenol conjugate of 2AAF.

**100,000 x g rat liver supernate passed through Sephadex G-25.

Data taken from reference (15).

serve as a phosphoadenylphosphosulfate (PAPS) generating system in the soluble fraction, increases the rate of disappearance of NOH-2AAF by an order of magnitude and increases the amount of 2AAF formed. In addition, NOH-2AAF is converted to a dimer of 2AAF and to 2AAF-7-phosphate and a p-nitrophenol conjugate. Moreover, considerable amounts of radiolabel are covalently bound. But under conditions in which most of the NOH-2AAF is metabolized, the amount of acetate formed no longer can be measured presumably because the sulfotransferase system competes with the esterases and transacetylase for the substrate. Thus, there is no evidence that significant amounts of NOH-2AF is formed in the presence of a functional sulfo-

transferase system under conditions in which virtually all of the NOH-2AAF is metabolized. Addition of ascorbic acid to the system decreases the formation of the dimer and the phosphate and p-nitrophenol conjugates presumably by reducing the nitrenium ion to 2AAF. But the amount of acetate formed remains below the sensitivity of the analytical method. Similar results were obtained when NADPH, instead of ascorbic acid, was added to the system (L.S. Andrews and J.A. Hinson, unpublished results). Moreover, ascorbic acid and presumably NADPH decreases not only the covalent binding to protein but also that to exogenously added DNA (Andrews, Fysh, Hinson and Gillette, unpublished results). Hence the pattern of products formed from NOH-2AAF in hepatocytes *in vivo*, which contain PAPS, NADPH, NADH and ascorbic acid, may differ markedly from those formed by *in vitro* preparations that lack PAPS or NADPH, NADH and ascorbic acid. Thus, the relevance of many *in vitro* studies to the metabolism of foreign compounds *in vivo* frequently remains obscure.

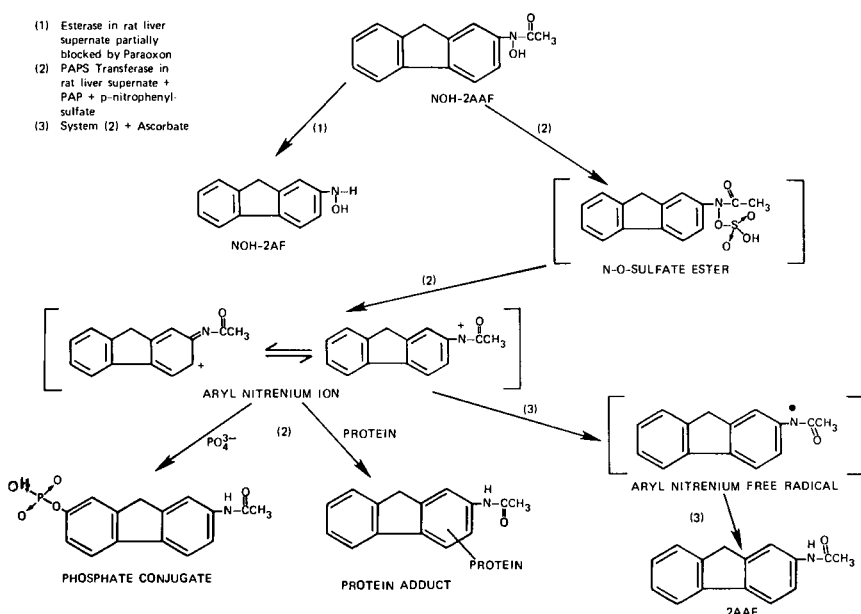


Fig. 3. Pathway of Metabolism of N-Hydroxy-2AAF

Studies during the past several years showing correlations between carcinogenesis and the covalent binding to DNA have led to the almost universally held view that chemically reactive metabolites initiate cancer by becoming covalently bound to target purine bases in DNA to form adducts that cannot be properly replicated. Indeed most of the studies reported at this Symposium reflect this view. Moreover, Dr. Ames (21) and many other geneticists have indicated that reactive metabolites will cause mutations not only in mammalian cells, but also in bacteria by similar mechanisms and thus have suggested that mutagenic studies may be used to predict carcinogenesis in animals and man. Indeed, the finding of a 90% correlation between carcinogenesis in animals and mutagenesis in various mutant strains of *Salmonella typhimurium* is most impressive (22). Nevertheless recent studies on the mutagenic effects of NOH-2AAF with the strain TA 1538 raise some fundamental

questions.

Incubations of NOH-2AAF with rat liver soluble fraction passed through Sephadex G-25 markedly increases the mutagenesis caused by NOH-2AAF (Table 2) (23). It

TABLE 2. Effect of Ascorbate and NADPH on NOH-2AAF-induced Mutagenesis

Additions*	Revertants/10 ⁸ TA 1538 Cells		
	Buffer	Supernatant	Supernatant + PAPS
None	65	1750	252
Glutathione (1 mM)	85	1700	225
Methionine (10 mM)	92	-	280
NADPH (1.0 mM)	-	2600	2800
NADH (1.0 mM)	-	2380	2490
Ascorbate (1.0 mM)	92	2896	2910

*Assays contained 3 μ g NOH-2AAF.

Data taken from reference (12).

has been suggested that this increase in the mutagenic activity might be due to the presence of a transacetylase which would convert N-hydroxy-2AAF to either N-O-acetate-2AAF or N-O-acetate-2-aminofluorene (16-20). Indeed the incubation of N-hydroxy-2AAF with a highly purified transacetylase (20) markedly increases both the mutagenicity and the covalent binding of ring labeled N-hydroxy-2AAF. However, the addition of guanosine monophosphate or 2AF, which markedly decrease the covalent binding of N-hydroxy-2AAF to nucleic acids, did not change the number of reversions (20). Thus, it seems likely that the increase in mutagenic activity caused by the transacetylase is due to the formation of NOH-2AF, a substance known to be a potent mutagen with TA 1538 (19,22,24).

When the PAPS generating system was added to the rat liver soluble fraction, however, the number of reversions of TA 1538 was markedly decreased (23), even though covalent binding to protein and DNA is markedly increased (13,14,23). Moreover, when the system includes NADPH, NADH or ascorbic acid in concentrations similar to those occurring in hepatocytes, the covalent binding is markedly decreased, (15, Andrews, Hinson and Gillette, unpublished results) but mutagenesis is increased (15). Since no acetic acid is formed during these reactions, it seems likely that the mutagenic metabolite is not NOH-2AAF, but a free radical formed from the nitrenium ion of 2AAF. Whatever it is, the mutagenic is short lived. Preincubation of NOH-2AAF with PAPS, the soluble fraction and ascorbic acid for different times before the addition of the bacteria decreases mutagenicity in parallel with the decreases in NOH-2AAF (15). Other studies have shown that addition of ascorbic acid to the sulfotransferase system markedly decreases covalent binding to exogenously added DNA as well as to protein (Andrews, Fysh, Hinson and Gillette, unpublished results). Thus, damage to bacterial DNA apparently may occur even when covalent binding of a mutagen to DNA is low, if not absent.

These findings place us squarely on the horns of a dilemma. De Baun *et al.* (13) have noted a correlation between the hepatocarcinogenicity of NOH-2AAF with its covalent binding to liver protein *in vitro* and liver sulfotransferase activity *in vivo*. Moreover, Weisburger *et al.* (25) have shown that administration of substances that deplete the liver of sulfate decreases the hepatocarcinogenicity of NOH-2AAF and that the simultaneous administration of these substances partially

prevents the decrease. If carcinogenesis is caused solely by the formation of covalent adducts between DNA and the chemically reactive metabolites derived from the sulfate ester of NOH-2AAF, then the mutagenic data suggest that mutagenesis and carcinogenesis may be caused by entirely different metabolites of the same carcinogen. If this is the case, much of the correlation between mutagenesis and carcinogenesis thus far observed, may be fortuitous. But if mutagenesis and carcinogenesis are caused by the same metabolite, then there may be mechanisms by which metabolites can damage DNA without becoming covalently bound to it.

Clearly, the data are not sufficient to resolve the dilemma but it is perhaps useful to point out a mechanism which is consistent with both the mutagenic and the carcinogenic data. During the reduction of the nitrenium ion of 2AAF to 2AAF, a free radical is presumably formed. This radical may be able to react with DNA to form 2AAF and a DNA free radical similar to that observed with ionizing radiation. Thus, the metabolite could cause an alteration in DNA without being covalently bound to it. Since the generation of the nitrenium ion still depends on the sulphotransferases in hepatocytes, there should be a correlation between the activity of the sulfotransferase and covalent binding of the nitrenium ion, the formation of the free radical and carcinogenesis. Moreover, the administration of substances that deplete the body of sulfate would decrease not only the formation of the nitrenium ion but also the free radical. Thus the lack of sulfate would result in a decrease in carcinogenesis and the administration of sulfate with the sulfate depleting substance would partially prevent the decrease in carcinogenesis.

Occasionally NOH-2AAF is carcinogenic in animal species that have low sulfotransferase activity. But perhaps NOH-2AAF in these species is preferentially hydrolyzed to NOH-2AF which in turn serves as the carcinogenic metabolite.

Much work needs to be done to resolve not only the dilemmas arising from research with NOH-2AAF but other dilemmas which arise in attempting to extrapolate in vitro data to living animals. For example, we still need to know considerably more about the pharmacokinetic relationships between biological stabilities of chemically reactive metabolites, their rates of diffusion out of cells, the rates at which they are carried by the blood to other tissues and their rates of excretion by kidneys. It might well be that toxicities caused by a given foreign compound in different tissues may be caused by different chemically reactive metabolites of different chemical and biochemical stabilities and that the pharmacokinetic disposition of the metabolites determines the target organ of the toxicants (26-28).

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The Agenda of Behavioral Toxicology

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New disciplines coalesce from fragments of old ones, but, if they are to survive, they must acquire a unique identity of their own. Behavioral toxicology is a term currently in fashion for which I admit some responsibility(1). Still, I am willing to consider whether the term truly represents a coherent discipline, or mimics instead a movement propelled by common tenets, or remains a disordered array of fragments.

A coherent discipline is exemplified by atomic physics. Unified theories can be formulated, then tested by acknowledged methods, typically requiring a new large accelerator. We couldn't pass that test. Although we socialize actively and skitter from one meeting to another, we can't attach ourselves to comprehensive theories or agree on optimal, or even suitable, methods. The sciences from which we borrowed so much - psychology, pharmacology, and toxicology--are not, however, that much further advanced; nor will they ever achieve the esthetic elegance of physics. New problems and issues keep popping up like targets on a shooting range.

The criterion of common tenets fits us somewhat better, but hardly meets the standards of Parisian haute couture. The criterion is too skimpy in some places, and hangs in limp folds in others. We are united only by the belief that behavior is significant; then dissent begins to pry us apart. Some of us find individual organism assessment a compatible style. We study subjects intensively, over long periods of time, almost like clinical cases. Some of us see greater profit in more succinct and superficial assessments embracing many subjects. We debate the advantages and disadvantages of innate as opposed to learned behavioral baselines. We argue about the virtues of highly automated systems as contrasted to the more global, comprehensive, sometimes more subtle evaluations offered by trained observers. I would deny, however, that the vigor of the internal debate reflects disorder. We may churn up a lot of turbulence, but flow in the same direction.

I said earlier that we are united by a common belief in the significance of behavior as an index of toxicity. Perhaps it is because most of us who practice this new discipline are not toxicologists by training. Most of us can be identified as psychologists, some of us as pharmacologists. Most of us passed through a phase as behavioral pharmacologists, a passage that made us wary of easy solutions, quick answers, and glib hypotheses. After nearly 25 years of attempts, often fruitless, to develop efficient screening methods for central nervous system drugs, we are not prepared to cede the feasibility of breakthroughs in screening for toxic effects. This background is responsible, too, for our commitment to experimental behavior science as our source of technology (besides the sublimated suspicion that most of the drug-induced behavioral changes we have observed in the past are simply toxic or adverse effects). When one sees an animal, that, by casual observation seems perfectly normal, but that performs oddly in an experimental situation, one quickly is convinced of the flaws of simple observation. One becomes a dedicated convert to experimental analyses of behavior.

Behavioral toxicology does stand apart from behavioral pharmacology, however. It is embarked on another evolutionary path. It is developing a culture and style of its own. What marks the style and approach is its immersion in the central question posed to toxicology today: How do we develop assays enabling us to set standards for contaminants in the environment when the predominant model is low-level exposure extending over the total or a major part of a lifetime? And, to further multiply the frustrations, how do we incorporate functional criteria? I do not need to remind you, by mentioning saccharin, that even cancer is not an easy criterion on which to achieve agreement. Behavioral pharmacology does not face as acutely as behavioral toxicology the hazards of extrapolation from a laboratory finding. Were we only in the business of screening, that is, merely detecting agents with potentially useful central nervous system effects, more alternatives would be available to us. Sensitivity would be secondary, selectivity primary. We would be content with any index of CNS action that gave us a direction to pursue. For a compound to be used as a medication, moreover, what difference does potency make? Dose can always be raised. In toxicology, sensitivity must come first. Interpretation and debate about the significance of such a finding is a later step.

Pharmacology also emphasizes restoration of the normal chemical balance of the body. It deliberately manipulates prevailing physiological status. Short-term rather than remote actions represent its dominant theme. And it is a science that searches for mechanisms. Toxicology's targets are more elusive; thresholds and no-effect levels are traditional terms and concepts. Its scope, moreover, encompasses the entire lifespan. What may beguile the pharmacologist as a phenomenon of early development may haunt the environmental health scientist as a legacy for a lifetime.

The participants in today's symposium reflect some of the key issues now perturbing behavioral toxicology. A comprehensive list of those issues represents a long agenda. Let me tersely review that agenda; it will provide a context in which you can evaluate the implications of this symposium.

How Do We Recognize An Adverse Behavioral Effect?

Death, of course, is the ultimate endpoint. Step back from that edge and certainty begins to dissolve. Even morphology may be a debatable guide. Pathologists can argue over the appearance of tissue. Do certain cellular changes represent a tumor? Or merely hyperplasia? Consider how much more difficult it is to establish a functional criterion. What might be the significance of an elevation in heart rate, or a decrease in rate of respiration, or an elevation in the activity of mixed-function oxidase? Are these merely adaptive or compensatory processes of the kind that the natural environment continuously elicits? Or are they unusual stresses, driving organisms closer to the limits of physiological capacity and ultimate breakdown? If questions proliferate over such relatively unitary phenomena, what about behavior? Behavior is so multifaceted, so much a tangle of interacting systems, so keyed toward adaptive and compensatory processes, so variable because variability is one of its main functions, that any displacement from baseline can evoke a dispute. What would be the toxicologic significance say, of a 10% fall in the rate at which a rat presses a lever, or a 10% increase in reaction time? Consider the problem posed a recent study by Levine (2), who discovered that carbon disulfide exposure reduced the rate with which trained pigeons pecked a response key. Performance improved because, to earn access to grain, the pigeons' pecks had to be spaced by a minimum interval. How could this be called a toxic effect? Only by showing, as did Levine, that the decline in rate generalized across many situations, even those in which it was penalized.

Another problem is a statistical one. Conventional statistical evaluations are based on recognized mathematical functions such as the Gaussian distribution.

Ascertaining probability by tests of significance, even with non-parametric techniques, assumes an underlying model. Suppose the consequence of a treatment, however, is not to change the mean performance of the treatment group, but merely to isolate or displace a small, particularly susceptible subgroup. Such effects seem often to be observed, for example, as an aftermath of lead exposure. The number of these deviant cases may not be enough to sway the statistical properties of the group, and conventional tests of significance will not confirm such a sequel. Perhaps newer approaches to quantitative assessment, as exemplified by Exploratory Data Analysis(3), will allow toxicologists to escape the constraints of traditional statistical models.

How Can We Specify the Non-Specific?

I have framed this question so as to convey what I believe may be the most crucial, difficult problem facing behavioral toxicology. The earliest behavioral indications of toxicity are typically vague, subjective and non-specific psychological and somatic complaints. They are easily dismissed by clinical skepticism. Too often, however, they serve as precursors to more severe, overt intoxications.

Table 1 is a list of symptoms associated with metal poisoning. Such lists can be multiplied endlessly for many different categories of substances. Gauging their

TABLE 1 Symptoms Ascribed to Metal Toxicity

ANOSMIA	JITTERINESS, IRRITABILITY
APPETITE LOSS	MENTAL RETARDATION
CONVULSIONS	PARALYSIS
DEPRESSION	PARESTHESIAS
DISORIENTATION	PERIPHERAL NEUROPATHY
DIZZINESS	POLYNEURITIS
DYSARTHRIA	PSYCHIATRIC SIGNS
FATIGUE, LETHARGY	SOMNOLENCE
HEADACHE	TREMOR
INCOORDINATION, ATAXIA	VISUAL DISTURBANCES
INSOMNIA	WEAKNESS

reality challenges our technical ingenuity. How do we detect and, especially, quantify them? Is it only our ignorance of appropriate test procedures that extends the mantle of non-specificity? Do we need more clearly to define these parameters of early clinical illness, just as we have such actions in psychopharmacology? Psychology offers a rich repository of possibilities. After all, psychology has spent much of its resources on exactly such questions. Further, how shall we translate such subjective terms into procedures feasible for the animal behavior laboratory? Will behavioral toxicology duplicate the success that schedules of reinforcement and operant techniques achieved in behavioral pharmacology? Do we have to examine behavior more microscopically in the search for early changes in performance? Is it a better strategy simply to assay specific impairments, working on the assumption that non-specificity only expresses the crudeness with which we typically examine such questions?

Are Standardized Test Batteries Feasible?

How should we respond to the requests, if not demands, that we provide such test batteries? The Toxic Substances Control Act mandates premarket testing of new chemicals. It also specifies behavior as one of the endpoints by which adverse effects are to be gauged. Is it our function to provide standardized test batteries that might be frozen into practice? Or, is it a sensible strategy to demand that manufacturers provide evidence of functional integrity from tests that they

develop themselves, and whose relevance the regulatory agencies can then weigh? What constitutes an optimal testing strategy? Is it more efficient than standardizing a test battery to proceed one step at a time? To select tests to assess more global functions, such as locomotor activity, then proceed to more specific functions such as sensory discrimination? Can we make any estimates about the relative costs and efficiency of these two strategies(4)?

Chronicity

We tend to be exposed to environmental contaminants over many years, even throughout a lifetime. Is there any substitute for chronic exposure? Is there any substitute for lifetime testing? We are well aware, from our sub-discipline of behavioral teratology, that the consequences of prenatal exposure may not even be obvious until the organism is well into advanced age, perhaps even senescence; that early exposures may be dangerous, not in their immediate effects, but in what they portend for the future(5). Special problems of husbandry arise in longitudinal, lifetime tests. Establishing dose-effect relationships multiplies the cost enormously. Aside from these problems, how do we choose parameters appropriate from birth until old age? How do we deal with the special problems posed by repeated testing to which organisms may adapt so well that only severe challenges can disrupt performance? How do we intergrade behavioral testing with the special demands of modern animal husbandry such as barrier controls?

Neurochemistry, Neuropathology, Neurophysiology

None of us believe that behavioral data relevant to standard setting and to toxicologic appraisal ought to be acquired in isolation. What kind of ancillary information should we elicit from other neuroscientists? These are disciplines in their own rights, with special problems, special demands, special points of view. Are there economical means by which we can coordinate our appraisals? How deeply can these other sources of information be pursued before the pursuit becomes inefficient?

New Directions

So far, we have sampled only a small fraction of the behavioral technology evolved over the past 100 years. Where might we search for methods? What are the most promising areas of psychology and allied behavioral sciences still unexploited? Many issues now being debated, and the objects of intense research in psychology, seem to possess enormous potential for the assessment of adverse chemical effects. But their utility can be determined only empirically. Is there some way to develop a strategy by which we can map this huge potential onto the significant issues that assail behavioral toxicology?

You should not be surprised, nor should we feel burdened, by the lengthy catalog of questions encompassed in this agenda. It merely reflects the richness of behavior. After all, to press a lever, a rat might use its left paw, or its right paw, or its snout, or its shoulder, or jump and down on it. If it confuses you to see us reaching and poking and jumping and squirming, it is only that we have adopted different strategies for making same response.

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Duration of Exposure: An Important Variable in Behavioural Toxicology*

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ABSTRACT

A very difficult problem currently confronting researchers is how to define the consequences of long-term, low-level exposures to any of a variety of chemicals. Exposure duration is a fundamental variable in behavioral toxicology. Toxic side effects may appear only after chronic administration of drugs. Many environmental contaminants accumulate in the body with chronic exposure to small doses that are well below the threshold for acute effects. Experiments using behavior as the endpoint are especially useful in revealing critical events related to exposure duration. The repeated measurement of behavior over long exposure durations poses new methodological problems that were seldom encountered in behavioral pharmacology. However, the possibility of similarities between the gradual behavioral changes evoked by toxicants, the slow neural diseases and the aging process, represents an exciting and potentially important role for behavioral research. This paper illustrates the importance of chronic exposures and points out some new methodological problems that seldom occur in acute experiments.

WHAT IS THE IMPORTANCE OF CHRONIC EXPOSURE?

Behavioral pharmacology has developed, for the most part, as a science of acute effects. Recently, toxicology has brought to our attention a whole new realm of research problems concerning chronic exposures, exposures both to therapeutic drugs and to non-therapeutic chemicals such as environmental contaminants (Evans and Weiss, 1978).

Experiments aimed at the consequences of chronic, low-dose exposures are important for several reasons. First, such exposures are realistic; they most closely resemble the conditions of human exposure to a variety of therapeutic and non-therapeutic agents. Chronic exposure is particularly important with environmental toxicants, because unlike drugs, many of these chemicals have a long biological half-life, which allows substantial accumulation to occur before

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pharmacokinetic equilibrium is reached. In this regard, chronic exposure may be of greatest importance for human and non-human primates, since these species usually eliminate many chemicals more slowly than non-primates (Caldwell et al. 1977; Hardman et al. 1973; Burns, 1970). Furthermore, prolonged exposure, even after equilibrium has been attained, can increase the frequency and severity of the toxic signs.

The consequences of chronic exposures may not resemble the consequences of acute exposure, as is the case with methylmercury (Shaw et al. 1975; Berlin et al. 1973). Chronic low-level exposure may result in subtle changes which can only be documented, in humans, by behavioral measures.

Finally, chronic exposures can be a useful research strategy for observing the development of behavioral impairment in "slow motion", so to speak. Short-term, high-dose exposures to some toxicants will result in a rapid decline in behavioral performance. In such a situation, the experimenter is left with a severely intoxicated animal, a decidedly uninteresting subject for behavioral studies.

The possibility of similarities between the gradual behavioral changes provoked by toxicants, the slow neural diseases and the aging process, represents an exciting and potentially important role for behavioral research. However, the repeated administration of chemicals and the repeated measurement of behavior over long exposure durations poses new methodological problems that were seldom encountered in the acute studies which typify behavioral pharmacology.

BEHAVIORAL CONSEQUENCES OF CHRONIC EXPOSURES

Movement Disorders

Chronic treatment with major tranquilizing drugs can produce disorders of movement which are termed tardive dyskinesias (Crane, 1968). These dyskinesias illustrate a behavioral change unrelated to the therapeutic action of a drug. The problem was recognized only after there had been substantial experience with long-term administration. Weiss and Santelli (1978) developed an experimental model in which monkeys displayed dyskinesias after ten weeks of exposure to haloperidol.

Chronic exposure to methamphetamine also produces changes that are not predictable from acute exposures (Fischman and Schuster, 1975; Eibergen and Carlson, 1975).

Behavioral abnormalities may emerge as a consequence of chronic exposure to environmental pollutants such as manganese, which produces a Parkinson-like condition which can be treated with L-DOPA (Cotzias et al. 1971). Industrial hexacarbons such as n-hexane and methyl-n-butyl ketone induce a delayed neuropathy which appears only after repeated exposures.

Visual Impairment

An equally fascinating, yet even more frightening, aspect of chronic exposure concerns, not accumulation, but rather the minimum time for which the toxicant must reside in the body before consequences appear. In other words, duration of exposure is a co-determinant,

along with dose, of the consequences. This phenomenon is rather well documented with the chemical and cellular changes caused by slow degenerative diseases of the nervous system and by the long-delayed results of chemical carcinogenesis (Druckery, 1967).

When a dose is held constant, the duration of its administration can influence the behavioral consequences in much the same fashion as increasing the dose in an acute dose-effect study. This is illustrated by our studies with scopolamine and with methylmercury, two agents which impair visually-controlled behavior. This is not to suggest that the two agents share identical mechanisms of action at the cellular or molecular levels, however.

First, monkeys were trained to perform visual discrimination problems. Details of method and control data are described elsewhere (Evans, 1975a and Evans and Garman, in press). Figure 1 shows the results of acute dose-effect determinations with scopolamine. The accuracy of visual discrimination declined with increasing dose. The decline was more marked when the monkey was confronted with dim stimuli than when confronted with bright stimuli, an important predisposing condition for indicating neurotoxicity (Evans, in press).

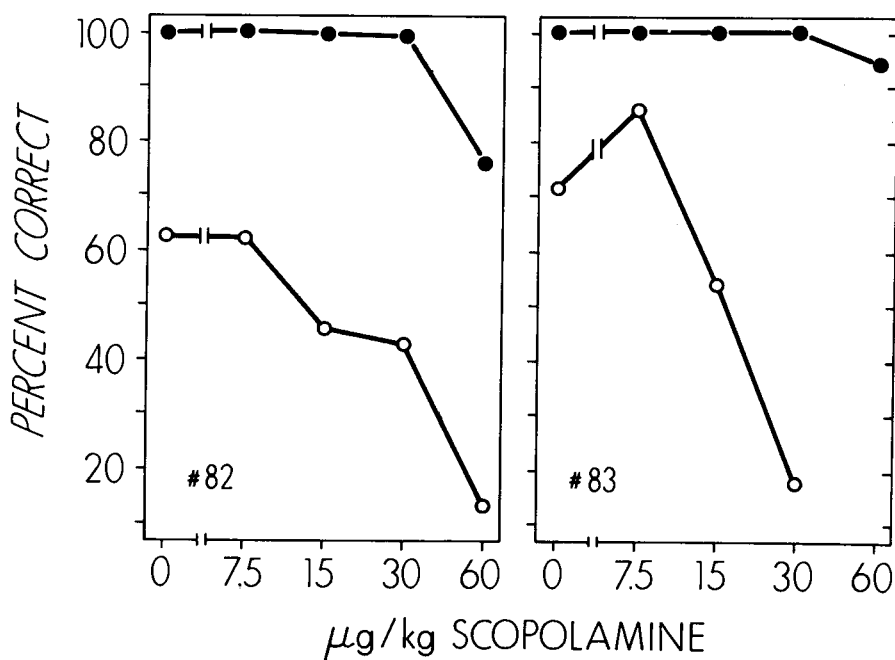


Fig. 1. Accuracy of form discrimination as a function of scopolamine dose. Luminance of the stimuli was bright for tests indicated by the filled circles and dim for tests indicated by the open circles. Data for monkey #82 are shown on the left, monkey #83 on the right. From Evans (1975a).

Chronic exposures to methylmercury, with the weekly dose held constant, produced a gradual deterioration in accuracy of discrimination shown in Fig. 2. As was the case with scopolamine, scotopic vision with "dim" stimuli was selectively impaired before discrimination with bright stimuli. The lower portion of Fig. 2 indicates that the blood Hg concentration remained steady until exposure stopped after the 20th week.

Had the exposure stopped after the 10th week, the only justifiable conclusion would be that there were no significant effects of a "dose" which produced blood Hg levels of about 3 $\mu\text{g/ml}$. Only with prolonged exposure did the behavioral consequences gradually emerge.

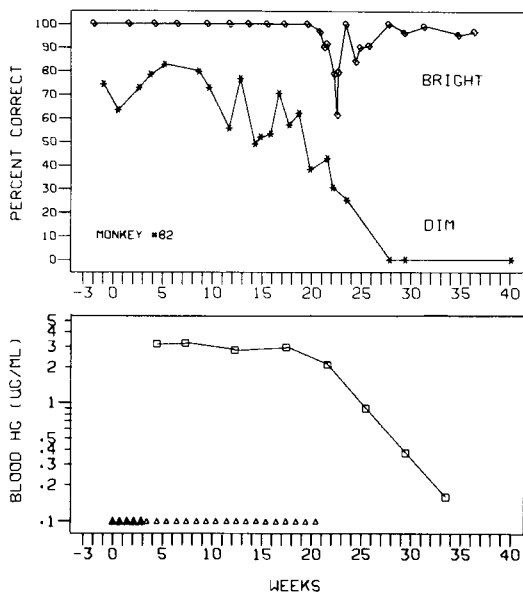


Fig. 2. Accuracy of form discrimination (upper graph) of monkey #82, during exposure to methylmercury. Data were obtained following the experiment with scopolamine illustrated in Fig. 1. Test conditions were the same in both figs. Note that scotopic discrimination did not recover after the exposure to methylmercury had stopped. The lower graph indicates the blood Hg concentration of this monkey. Triangles indicate the times at which the monkey voluntarily ingested methylmercury. From Evans (1975b).

Dose-response information about methylmercury could be obtained only with many more monkeys than we employed in the experiment with

scopolamine. This was necessary because the effects of methylmercury are not reversible (Fig. 2). Thus, the powerful repeated-treatments experimental design, employed with scopolamine, could not be employed to study the effects of methylmercury.

Additional weeks of exposure to methylmercury seemed further to impair discrimination in a manner similar to the additional impairment produced by an increment in the acute dose of scopolamine. This is further illustrated in Fig. 3, which shows the speed with which the monkeys responded to the discriminative stimuli (response time). Following a low dose of scopolamine (lower right part of Fig. 3) or a brief exposure to methylmercury (lower left) the monkeys were consistently rapid in their selection of the visual stimuli. Prolonging the chronic exposure period altered performance in the same manner as increasing the acute dose; in both cases the response times were longer and more variable from trial to trial. The duration of exposure to methylmercury was continued until very marked signs of intoxication appeared; thus the response time histogram for methylmercury (upper left) was more severely changed than those representing the highest dose of scopolamine (upper right).

The response time is usually less than 1.0 sec. The left half of Fig. 3 illustrates changes occurring during a chronic exposure to methylmercury. The right half of Fig. 3 shows acute dose-effect results with scopolamine.

METHODOLOGICAL PROBLEMS POSED BY CHRONIC EXPOSURE

Chronic experiments increase the risk of experimental error. Often it is impossible to recover from the consequences introduced by such error, as was the case with the chronic feeding studies of FD&C red dye No. 2 (Boffey, 1976). Therefore, one should consult references on good research procedures before undertaking chronic studies (Barnes and Denz, 1954; Anonymous, 1975). A few problems likely to occur in behavioral experiments are discussed below.

Choosing the Best Index of Dose

Although the daily amount administered to each animal is an important fact, the total amount administered over a chronic period may relate poorly to toxic effects. After absorption and excretion of the toxicant are in equilibrium, it may be of no further use to count the additional quantity of the toxicant administered. Toxic effects may be more clearly related to the content of some index media such as blood or brain, as illustrated in the studies of methylmercury described above.

Often it is necessary to speak in terms of the dose-rate, which takes into consideration not only the total amount of the toxicant administered to the animal, but the time span during which the administration is completed. Continuous inhalation of benzene causes toxic signs that were not seen with intermittent exposures, even though the animals were exposed to the same total amount of benzene in both chronic and intermittent regimens (Coffin, et.al. 1977; Gardner et al. 1977).

The importance of duration of exposure is illustrated by findings with perphenazine, a neuroleptic. Chronic treatment, for one year,

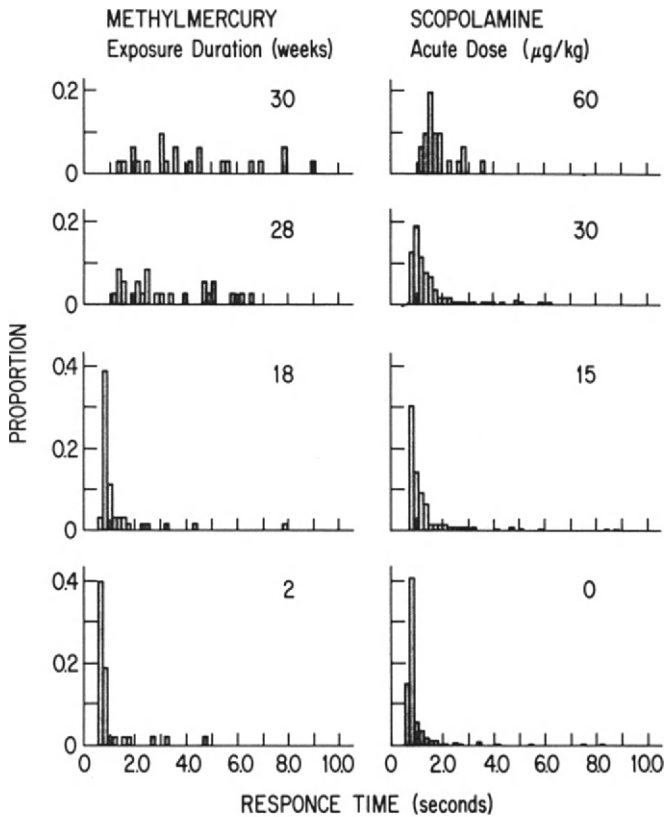


Fig. 3. Frequency distributions of response times for monkeys performing the same visual discrimination as described in Figs. 1 and 2. The Y axis indicates the proportion of all response times falling within the time bin indicated by each bar.

resulted in visible brain damage (Pakkenberg et al. 1973) but a ten-fold increase in the dose level for an exposure period of six months was without effect (Fog et al. 1977). This suggests that the response to a toxicant is a dual function of the concentration in the blood (C) as well as the length of time for which this concentration is maintained (T). C x T interactions are often observed in environmental toxicology.

One of the few illustrations of the C x T interaction, with behavior

as the experimental endpoint, emerged from our studies of methylmercury (Evans et al. 1977). The time of appearance to toxic signs was longer in the monkeys having lower blood Hg concentration. The latent period between the start of exposure and the appearance of observable behavioral and neurological signs depends upon the "dose" (Fig. 4). In these studies blood Hg concentration provided the index of dose. Toxic signs were approximately the same, however, regardless of the delay in their appearance.

Several of the animals having the lowest blood Hg concentrations did not develop toxic signs within the 1000 day period of the experiment. Does this reflect a threshold? Perhaps it does, but note the variability in extrapolating to low doses. Substantial resources and time would be required for a scientifically valid answer.

It is even more difficult to discriminate between a threshold and a cumulative effect in chronic experiments where a constant dose was not maintained but instead was varied during the exposure period (Berlin et al. 1975; Shaw et al. 1975; Luschei et al. 1977). If the dose varies greatly during the chronic exposure, it may be more useful to consider the peak or maximum, rather than the average, as the index of exposure.

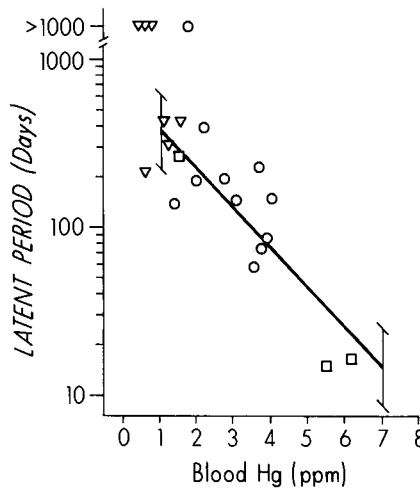


Fig. 4. The influence of blood Hg concentration upon the latent period preceding the appearance of overt toxic signs in monkeys. Each point represents one monkey's mean blood Hg concentration. All monkeys continued to receive methylmercury until overt signs appeared or until the end of the experiment (greater than 1000 days) if no signs appeared. From Evans et al. (1977).

Changes Related to Growth and Aging

Chronic studies are likely to encompass several stages of the life-

span. Toxic effects vary, depending upon the age at time of exposure (Benke and Murphy, 1975; Harbison, 1975). This reflects both physiological and behavioral changes associated with the maturation process. For example, the locomotor activity of young rodents increases with age and then begins to decline with the approach of maturity. Visual acuity of primates improves throughout infancy, and then declines in later years.

Progressive changes in the oral absorption of chemicals accompany both the maturation of the digestive processes as well as the dietary changes associated with maturation. Research on the behavioral toxicity of lead has underscored the importance of growth and nutritional factors (Bornschein et al. 1977).

Is it wise to increase the dose administered to growing animals during a chronic experiment so as to maintain a constant mg/kg proportion? Compared to body weight, the weight of the brain is relatively constant throughout growth and changes little, even in the face of substantial changes in body weight (e.g. Scharer, 1977). Neural tissue exhibits degenerative changes and declining neurochemical activity with advancing age (Gilmore, 1972; Greenburg and Weiss, 1978).

In these and other respects, the slowly-evolving changes in chronic intoxication may resemble the slow neurological diseases (e.g. Hotchin and Buckley, 1977). Exposure to neurotoxicants may cause an acceleration in the aging process (Weiss and Simon, 1975). Exposure to radiation or to industrial contaminants can result in degenerative changes in the nervous system which resemble those of aging (Kogel, 1977; Schaumberg and Spencer, 1978).

Changes in Baseline Behavior

Results from long-term studies can be complicated when control animals exhibit gradual changes in baseline behavior. The repeated measurements demanded by most chronic experiments exceed the capacity of most of the simple behavioral screens. Performance in an open field, in passive shock avoidance or on a rotarod, changes markedly as the animal gains familiarity with the situation. Tests of learning, frequently employed to study the pharmacology and toxicology of acutely-administered substances, may be less useful in chronic studies where the toxic effect may occur long after the learning process is completed.

Tests of previously-learned behavior, exemplified by operant conditioning techniques, may be useful for chronic studies because they generate a behavioral baseline that is relatively stable. However, no method may be entirely exempt from baseline drift.

Chronic effects may also be complicated by tolerance, adaptation or compensation, whether at the behavioral or physiological level (e.g. Teichner, 1967). A slowly-evolving neurological deficit might be masked by compensatory changes in the nervous system and by an animal's adoption of new response strategies. These changes resemble those known to behavioral pharmacologists as "behavioral tolerance". Finally, each increase in exposure duration increases the risk that intercurrent disease may alter the physiological baseline.

CONCLUSIONS

The combination of exposure duration, dose and other fundamental variables, represents a complex matrix with which future research must be prepared to deal. Although efforts to screen chemicals and drugs for behavioral toxicity must continue, these efforts must be reinforced with basic parametric analyses that can provide the foundation for the new science of behavioral toxicology.

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Changes in Performance, Personality and Subjective Well-being as Indicators of Long-term Exposure to Toxic Environments

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In the branch of behavioral toxicology that I represent, we study the behavioral, or psychological, changes caused by occupational exposures. The causal relationship between the toxic agent in the environment and behavioral changes is not, however, direct, but consists of several steps, many of which involve feedback mechanisms and intervening variables modifying the effect. This hierarchy especially exists for the slowly developing effects of long-term exposures, which are the primary concern of occupational toxicology and medicine.

The steps are as follows:

EXPOSURE

UPTAKE

CUMULATIVE UPTAKE IN CNS

BIOCHEMICAL EFFECT ON CNS

BEHAVIORAL RESPONSE

(interaction between individual and environment)

EFFECTS ON BEHAVIORAL OUTPUT

A toxic agent in the ambient air does not bring about behavioral effects without uptake in the organism; for long-term behavioral effects cumulative uptake of the agent or its metabolite into the brain is essential. The biochemical effect on the central nervous system (CNS) stimulates behavioral response, i.e. a change in some functional unit, or units, of the CNS. Due to the capacity of the CNS to compensate an impaired function with other functions, this primary behavioral response is often difficult to detect, but if it is strong or is longlasting, it affects the interaction between the individual and his environment and reveals itself as changes in behavioral output. The changes are modified by other factors that determine the interaction, e.g. the intellectual and emotional characteristics of the individual, his past history and his physical, psychological and social environment. Modifying factors increase the variability of the behavioral effects both quantitatively and

qualitatively.

The methodological problems involved in the study of early effects due to long-term exposure are associated with the availability of valid methods for measuring exposure or uptake, the availability of adequate referents, the control of modifying factors, and the choice of sensitive and reliable test methods.

Current knowledge about which functional CNS units are the most affected by toxic agents is deficient. The behavioral response is hardly very specific, but covers several functions. Moreover, in the interaction between the individual and the environment the behavioral changes evidently spread into larger areas of behavior. Therefore, if one wishes to evaluate the mental health hazards caused by toxic agents, one cannot be content with one or two test methods but must cover a large range of behavior.

In empirical studies, test methods have been used for performance functions, personality and subjective symptoms. The different aspects of performance are generally emphasized for several reasons, the most important being the objectivity, reliability and exactness of test methods for sensory, motor and sensorimotor performance, and also for cognitive or intellectual functions. Moreover, impairment in these functions can be considered as the least sensitive to modifying effects, and, therefore, as the most valid indicators for the toxic response of the CNS.

However impaired performance functions are not the only effects, and not necessarily the most important ones. Both biopsychiatric and psychopharmacological data suggest that the functional CNS centers involved in the origination and regulation of emotional and affective behavior can be even more sensitive to changes in brain biochemistry than the other brain centers are. The objective and exact measurement of emotions is, however, problematic. The complexity of changes in emotional behavior causes another problem; in addition to toxic response such change may reflect the activation of a compensatory mechanism and also secondary reactions to the primary effect. Moreover, they can be supposed to be more dependent than performance on the individuals primary personality and on his actual life situation.

Questionnaires on subjective symptoms provide the simplest method for gathering information on the effects on subjective health or well-being. However, not only toxic effects, but sometimes also the very knowledge of being exposed can produce symptoms, due to aroused anxiety. Individuals also differ in introspective ability and their proneness to experience and reveal mental discomfort and somatic symptoms. Therefore, subjective symptoms cannot be considered as very reliable indicators of toxic effects.

In this communication the results of two empirical studies are briefly presented as an illustration of both the quality of disturbances found in the domains of performance, personality and subjective well-being and the relationships between these types of effects.

EFFECTS OF EXPOSURE TO SOLVENT MIXTURES

In a study on long-term exposure to organic solvents 100 car painters were examined. Some of the results have already been reported (1). The exposure of the car painters consisted of a mixture of aromatic and aliphatic hydrocarbons, alcohols, esters and ketones. There were no marked differences in work conditions and level of exposure between the car repair shops where the subjects worked. The 8-h time weighted average of the total exposure was about one-third of the Finnish threshold limit value for solvent mixtures.

As there are no biological uptake tests for the exposure in question, the method of

group comparison was used. The reference group consisted of age-matched employees of the Finnish State Railways (locomotive engineers and assistants). The possible role of differences in the initial intelligence level was controlled with a separate comparison of 33 pairs of exposed and nonexposed subjects matched for age and intelligence level (measured during their military service).

The test battery included tests for intelligence, memory, psychomotor performance, and personality (1, 3). Data on subjective symptoms were gathered with a questionnaire with items formulated so that they covered frequently reported symptoms of workers with solvent intoxication or symptoms revealed in corresponding studies.

Figure 1 illustrates the main results of the group comparison of performances *. It presents the mean performances of the exposed group in standardized scores, the reference group having been used as the standard in the adjustment. With the exception of the reaction times, all the illustrated differences were statistically significant. The differences were the most marked for the Block Design test for visual intelligence and the verbal memory test. The extensiveness of the differences was somewhat unexpected since verbal functions are generally considered to be rather resistant to toxic effects.

In a corresponding manner fig. 2 illustrates the group differences in personality as revealed by the Rorschach personality test. In our earlier studies we developed a scoring system with five variables for this test. They are adaptability, emotionality, spontaneity, self-control and creativity. The interdependence of these variables varies according to the population being studied. In this investigation there was a negative correlation between emotionality and self-control ($r = -.54$), both of which displayed a statistically significant difference between the groups. The exposed subjects showed less emotional reactions and were more prone to control their thinking and behavior.

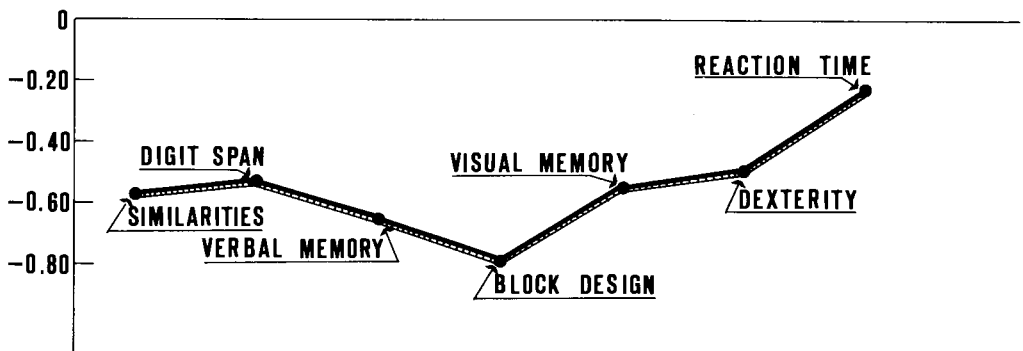


Fig. 1. Standardized scores for the mean performances of the car painters.

* Only a representative subset of the tests used in the carpainter study is included.

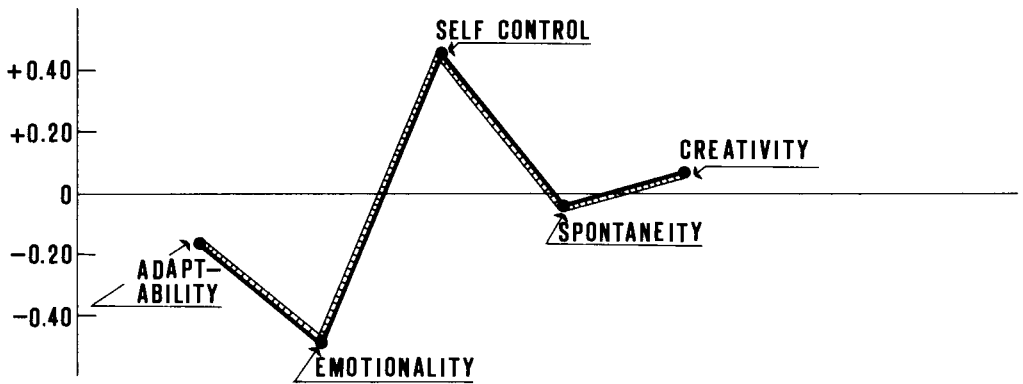


Fig. 2. Standardized scores for the means of the personality variables.

The subjective symptoms of the questionnaire represented five categories of disturbances in subjective well-being: sleep disturbances, fatigue, disturbances of memory and alertness, disturbances of mood, and somatic complaints of neurovegetative dysfunction. (The other questionnaire items are outside the scope of this paper.)

Figure 3 presents the differences in the symptom categories. The occurrence of sleep impairments differed only slightly between the groups. The most marked difference was found in disturbances of memory and alertness. These disturbances include forgetfulness, concentration difficulties and proneness to be lost in one's own thoughts. Also the symptoms of fatigue and mood disturbances were significantly more numerous among the exposed subjects when compared with the reference series. The latter category included mood lability, depression, irritability and restlessness.

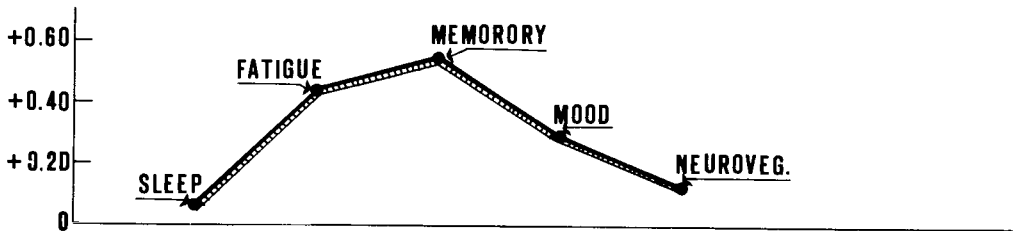


Fig. 3. Standardized scores for the means of the symptom variables.

In the cross-correlation between the performance and personality variables very few correlations were found. However, the personality variables emotionality and self-control correlated significantly with the results of the Block Design test and with the verbal memory tests. Impaired performance was related to loss of emotional reactivity and to accentuated self-control. No significant correlation was found between the performance or personality variables and symptom scores.

EFFECTS OF LEAD EXPOSURE

In a study on the behavioral effects of lead, 88 workers employed in either a storage battery factory or a railroad machine shop were examined. The following results

apply to the 82 workers who filled out the questionnaire developed for the study.

The most important basis for the data analysis of the study was an internal comparison of the relationship between lead uptake and behavioral variables, although a reference group was also used for comparison. All of the lead workers had been regularly monitored during their entire exposure time, or at least for the last five or ten years. The maximal, average and actual blood lead (PbB) concentrations were used as measures of uptake. The mean values SD for the three levels were 57.8 ± 16.8 , 42.2 ± 10.9 and 32.9 ± 10.0 $\mu\text{g}/100$ ml respectively *.

Thirty-six workers of an electronic plant served as the referents. They were volunteers, and their mean age was four years lower than that of the exposed group.

With the exception of some minor revisions the performance test battery and the symptom questionnaire were the same as those used in the car painter study. The Rorschach test was not used.

Figure 4 illustrates the performance profile of the lead-exposed subjects when compared with the referents. All of the test results of the exposed subjects were lower. The differences were significant for verbal intelligence, the memory tests, and reaction times. The possible role of the younger age of the reference group, or of other systematic differences between the groups, could not, however, be excluded.

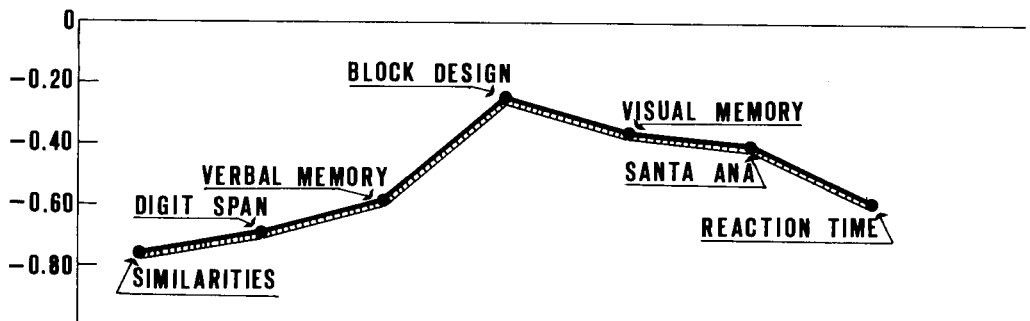


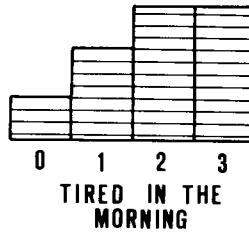
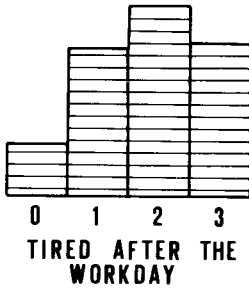
Fig. 4. Standardized scores for the mean performances of the lead workers.

When the test results were correlated with the measures of lead uptake within the exposed group, two tests showed statistically significant correlations with the average PbB level. These were Block Design and the Santa Ana Dexterity Test both tasks demand coordination of visual and motor functions. No correlation was found between the verbal tests and the average or maximal PbB levels, but memory for digits was almost significantly related to the actual PbB concentration.

The data yielded by the symptom questionnaire were treated in two ways. First the lead-exposed subjects were divided into three subgroups according to their maximal PbB values, and the occurrence of symptoms in these subgroups and in the reference group were compared. Second, the scores for the symptom categories were calculated and correlated with the uptake variables.

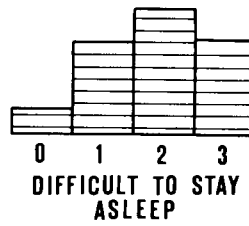
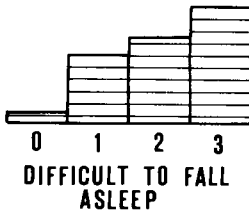
Figures 5-10 present the prevalence of two symptoms from each category in the four groups. On the right side of the figures, the correlation coefficients between the corresponding symptom category and the measures for lead uptake are given.

* The results dealing with workers with maximal PbB concentrations below 70 $\mu\text{g}/100$ ml will be presented elsewhere (2).



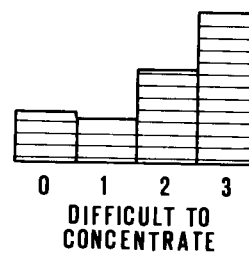
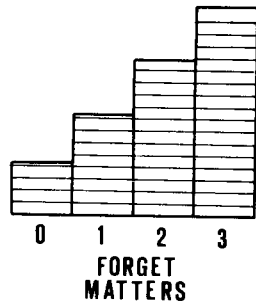
No significant correlations with PbB

Fig. 5. Fatigue



No significant correlations with PbB

Fig. 6. Sleep disturbances



Correlation with
Act. PbB .23
Max. PbB .29
Aver. PbB .24

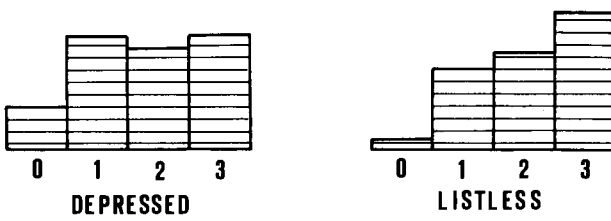
Fig. 7. Memory and alertness

Group 0: Referents

Group 1: Maximal PbB < 50 μg / 100 ml

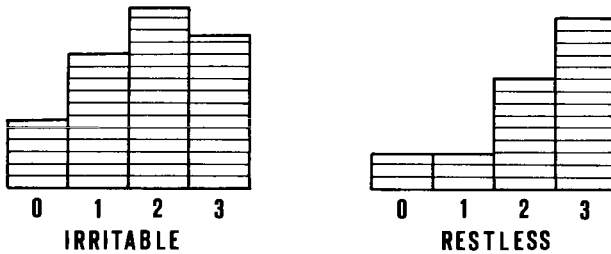
Group 2: Maximal PbB 50 - 69 μg / 100 ml

Group 3: Maximal PbB \geq 70 μg / 100 ml



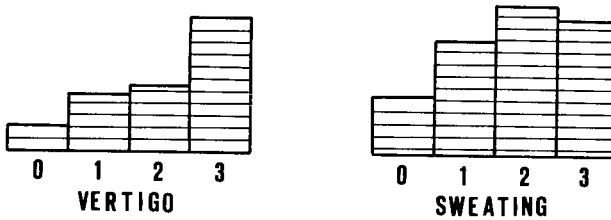
No significant correlations with PbB

Fig. 8. Depression



Correlation with
Max. PbB .20
Aver. PbB .21

Fig. 9. Mood lability



Correlation with
Aver. PbB .27

Fig. 10. Neurovegetative complaints

Disturbances of memory and alertness correlated significantly with all the uptake variables, and vegetative dysfunction with the average PbB level. In addition, the correlation of mood lability symptoms with the average PbB level was almost statistically significant.

None of the single symptoms of sleep disturbances showed any relationship with the uptake level. For the symptoms of fatigue, the peak was either in the group with the highest exposure level or in the medium group with a maximal PbB level of 50 - 70 $\mu\text{g}/100\text{ ml}$. Of the symptoms of depression, only listlessness and fearfulness were related to uptake. The correlation between mood lability and the average PbB concentration was due to one single symptom, restlessness, which increases sharply with the uptake level.

The number of symptoms reported by the reference group were low. The almost systematic difference between the reference series and the exposed subgroup with the lowest uptake level is somewhat perplexing.

It may mean that even such a low exposure level causes symptoms, but there may also be other explanations. There is a possibility that some part of the excess of symptoms reported by exposed subjects in this study, and maybe also in many other studies, can be explained by attitudinal factors. Those studied as referents may be prone to give a negative response and those examined as exposed subjects a positive one. It should also be pointed out that the referents of this study were volunteers. But such factors could hardly explain all the differences, and surely not the relationships found between the uptake level and symptoms. In the car painter study the risk for attitudinal bias was less because the questionnaire was administered to both groups as a part of a health examination.

In the cross-correlation between symptom scores and performance functions some statistically significant results were found. The psychomotor performances were slightly related to sleep disturbances and neurovegetative dysfunction, while the memory tests correlated with depression and subjective disturbances of memory and attention.

DISCUSSION

The data I have just presented show that the psychological effects of occupational exposures, manifesting themselves in the realm of performance, personality and subjective well-being, can be detected and quantified by means of psychological methods. However, it is not realistic to expect very high correlations between toxic exposure and behavioral variables, because of the large individual variability in (a) the psychological functions and features, (b) the ability to compensate impairments and (c) other factors which modify behavioral effect. Moreover, the psychological methods vary in their sensitivity in respect to toxic effects, as well as in their error of measurement, and much work is still needed in the search for the most valid methods detecting behavioral manifestations of toxic effects.

In addition, personality changes and subjective symptoms represent different aspects of behavioral manifestations, and they are not linearly related to each other. Previous studies on the effects of carbon disulphide (4) and styrene (5) have given similar results. The practical implication for both research and the detection of early toxic effects in the realm of occupational medicine is that the presence of one kind of effect cannot be predicted by findings of others. Therefore the use of measures of all three kinds of effects gives the best validity for the detection of toxic impairment.

Individual factors thus seem to determine which aspect of behavior is the most affected or the order in which the effects appear. The intellectual and emotional resources of the exposed subject, as well as his social situation, may be decisive for the manifestation of behavioral effects.

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Neurophysiological Characteristics of the Action of Toxic Substances on the Organism

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In the U.S.S.R., the method of functional electro-encephalography and methods of investigation into complex forms of animal behavior are widely used in the assessment of the toxic effects of chemical substances. Functional alterations of the central nervous system, in the case of poisonings, emerge precociously and prevent the appearance of disturbances of the other organs and systems of the organism.

The purpose of the investigations reported on here was to study the mechanism of the action exerted by chemical substances on the central nervous system as well as to find the electroencephalographic indicators of their harmful action. For this purpose, the researchers used the analysis method of the primary and secondary reactions of the generated potentials of the cortical optical substance of animal and human brains; this method makes it possible to characterize the condition of cortical inhibitions. They also used a method for analyzing the summary electrical activity of a series of structures of the animal encephalon that provide sensorial response to olfactive irritants (olfactory bulb, pyriform cortex), as well as a system analysis method enabling detection of the characteristics of the behavior and adaption (tonsils, hippocampus, reticular formation of the cerebral trunk). The last method used involved study of sensorial thresholds in the presence of noise stimulus and analysis of reactions of the rhythm change.

In parallel, there was investigation of complex animal behavior patterns subsequent to the action of chemical substances on the organism. Experiments were made with carbon disulfide, acetone, benzene, formaldehyde and leads in various concentrations, with investigation of their short-term and chronic actions.

The neurophysiological investigations were performed on rabbits weighing from 2,5 to 3 kilograms. For the generated potentials, the investigators used 0,8 mm-diameter constant electrodes. An active electrode was implanted in the maximum activity focus of the optical projection zone, and a passive

electrode was placed on the skull frontal bones. The generated optical potentials were stimulated by luminous signals emitted by the E.E.G. Orion photostimulator, using unilateral stimulation (energy of the flash impulse, 1,4 J. Time length, 1,2 ms). The lamp was located approximately 30 cms from the animal's head. The output of the generated potential was unipolar. After amplification, the average of the generated potentials was found by using an Atak 201 Nihon Kodens computer (two averages for 50 realizations each). The following components of the generated potentials were submitted to analysis : primary response (latent periods of the positive and negative phases, peak amplitude), slow negative wave (latency, maximum amplitude and time length).

The threshold of sensorial effect of a noise stimulus was determined by the occurrence of an Aronsal reaction on the occipital cortex in the form of a distinctly expressed theta rhythm. The investigators used a pure 500-Hz sound for five seconds, gradually increasing its intensity from 10 to 80 dB. The reaction was considered as being at threshold if a theta rhythm emerged distinctly in 5 out of 10 consecutive occurrences during the course of the noise action.

The animals were chronically submitted to the experiment in 200-litter rooms with exchanges of air by multiples of 30. The group of control animals was in the rooms, through which air circulated clean of dust and gases. The constant consistency of the concentrations indicates during the experiment was ascertained at least twice weekly via the colorimetric method (sensitivity 0,5 micrograms).

For the purpose of investigating the potentials generated in human beings, an active electrode was placed on the skin of the skull at the location 2 to 3 centimeters above the inion, exactly on the median line, with the neutral pole on the right ear lobe and the grounding on the back part of the lower third of the right forearm. The subject, eyes closed, was comfortably seated in an armchair in front of a cylinder through which passed pure air or an investigated gas, at a flow rate of 20 litres per minute. The photostimulator lamp was installed on a level with the subject's eyes, at a distance of 50 centimeters. Overall lighting in the room was 16 lx. After a three-minute adaption period, the subject was presented with a series of 100 aperiodical flash impulses at a frequency, 1 during 1 to 2 seconds, with an intensity of 0,27 J. After amplification on an UBP-03, the average was calculated by an Atak 201 computer, whose scanning startup was synchronized with the startup of the stimulator. During a ten-minute passage of pure air to sustain the active state, the subject was presented with a specific work program :

identification of geometric figures, solving of arithmetic problems, the sequence of presentation of which was determined by the investigator and was modified with each experiment. The time required for identification of the geometrical figures and for solving the problems, as well as the mistakes made, were recorded graphically on the E.E.G. tape. At the end of the work program, there was a recording of the average for 100 performances. This was followed by 10 minutes of exposure to gas in micro-concentrations (in the control experiments of exposure to pure air), during which time there was presentation of a work program similar to the previous one.

The factor of micro-concentration of gas in the observations of human subjects was ensured by an O.K. Aptukhin capillary diffusion cell (1964, modified jointly with its inventor by N.A. Dailidovitch et al. 1967), by thermostatzation. Determination of the concentration was done at the end of the electrophysiological experiments was by means of the modified "t" criterion (L.N. Bolchev, N.V. Smirnov 1965).

In the investigation of the complex animal behavior patterns, the investigators used one of the behavior toxicology methods (B. Weiss, 1973), the basic principle of which is the creation of a pattern of motivated behavior. As a dependent variable, during the experiment the investigators used animal behavior that was controllable by diet. The change in behavioral reactions served as the integral index of the action.

Investigations on rats were performed with standard equipment manufactured by Grasson-Stadler company (United States), in accordance with the complex "fixed-interval-fixed relation" program. During the chronic experiments on the rats, there was an investigation of the action of carbon disulfide inhalation and of the peroral action of lead in various concentrations. The following indices of behavioral reactions were recorded : number of right and wrong answers per program ; coefficient of work efficiency ; indicator of the rats 'activity during performance of the "fixed relation" program or during performance of "the fixed relation" program or during performance of the "fixed interval" program ; gestural activity, etc. Statistical processing of findings was done in percentages in relationship with the main body of findings and with the corresponding indicators of the control animal group (R.N. Birioukova, 1962). The findings of the neurophysiological investigation pointed to the use of extremely important selection of the action of the toxic agents studied. The character of the electro-encephalographic reactions and the succession of inclusions of the structures of cervical matter at the time of the action of the substances investigated, in low or high concentrations, indicate that the structures of the olfactive analyzer are highly sensitive structures, responding

faster than others to the action of toxic factors by pathological reactions (cortico-median nucleus of the tonsillary apparatus).

It was found that the chemical substances, in relation to the level of their active concentrations for short-term use, induce two kinds of reactions - an approximately scientific nonspecific reaction and a specific reaction. The first mentioned reaction is characterized by desynchronized activity of the neo-cortex and by acceleration of respiration. In the structures of the olfactory analyzer there was no concurrent occurrence of changes in previous activity. Depression of high-frequency inductive activity in the olfactory structures is the characteristic of the specific reaction.

The concentrations of chemical substances inducing an approximately scientific reaction are considered by the central nervous system as negligible stimuli, in the same way that the pattern of the response to these stimuli coincides with the electro-encephalographic reaction to other sensorial stimuli (light, noise). The specific reaction depends on the type of adaptation, since it emerges as a biologically significant reaction of the organism.

Study of the sensitization process during the repeated actions showed that in the animals that had been subjected to prior action of high concentrations of chemical substances, the use of low concentrations of these substances combined with a functional load (rhythmic light) induced the occurrence of paroxysmic activity of the structures of the olfactory analyzer, which emerges as the irrefutable indicator of the harmful action exerted by toxic substances on the central nervous system.

Investigation was made of the generated potentials of cervical matter under conditions of chronic action of atmospheric pollutions. Thus, during 6 weeks of round-the-clock exposure to carbon disulfide at a concentration of 0,2 mg per cubic meter, the parameters of the generated potentials were not statistically different from the indicators of control and basic data). In the 6th week of the action only, it was possible to note a barely perceptible trend toward increased amplitude of the primary response and of the slow negative wave ($124,6 \pm 1.1$ and $95,8 \pm 20,3 \mu v$, respectively).

Carbon disulfide in a concentration of 2 mg per cubic meter brought about a statistically meaningful reduction in amplitude of primary response of an average of 43% , as well as an increased duration of the slow negative wave for an unchanged amplitude.

In so far as the primary response of generated potentials becomes modified for a change in the activation-inhibition condition, the investigator's analysis of the primary and secondary components of the generated potentials enabled them to process the findings with great certainty. As we know, the ampli-

tude of the primary response depends on the functional condition of brain. (it decreases when there is an increase in tonic actions on the part of the reticular formation of the brain stem, and it increases when they decrease, or under the action of anesthetics. According to the most widespread viewpoint, the slow negative wave is the sign of hyperpolarization, developing synchronically in the pyramidal cells of the optical cortex, in such a way that the amplitude of the slow negative wave and slope of its growth are linked with the intensity of the cortical inhibition processes.

The decrease observed in primary response and the increased duration of the slow negative wave, without change in the latter's amplitude, at the time of the effect of the carbon disulfide in a concentration of 2 mg/m³ point to a weakening of the cortical inhibition processes. Extremely precise, stable changes were found in the primary response of the potentials expressed, which expresses the occurrence of stimulation in accordance with specific pathways, this gives reason for ascribing the high vulnerability of the optical analyzer to the action of a given substance.

This coincides with the data concerning lesions of the optical system in man and animals as the result of chronic carbon disulfide poisoning.

It is possible that concurrently with these nonspecific reticular effects, there may be disturbances in the receptive mechanisms and in the conduction tracts that condition the observed effect of decreased amplitude of primary response in the case of chronic carbon disulfide action in concentrations of 2 mg/m³. From the physiological standpoint, these mutations can be considered as harmful, in so far as neurophysiological analysis of the findings indicates deterioration in cortical inhibition processes. The changes also indicate the possible occurrence of paroxysmic forms of brain activity under longer stress or under the effect of stronger concentrations. This finding is meaningful for utilization of the global mechanism of the action of toxic substances on the synaptic organization of brain, for both low and high concentrations under prolonged use. The investigator's findings further show that the substances tested, which were of varying chemical structure and had different physico-chemical properties, act in their principles in the same manner (although in differing lengths of time) on the central nervous system.

Comparison of the findings of this investigation of the mechanism of the neurotropic action of carbon disulfide with the data collected in studies of ozone, formaldehyde and other chemical substances showed that neurotropic substances exert harmful action on the cervical processes -- the parameters of the primary and secondary reactions change in the same manner and prove the acceleration of activation of the weakening of the inhibition. Thus, experimen-

talstudy of a series of chemical substances brought to light a nonspecific indicator of harmful neurotropic action. Clearly, the method involving study of generated potentials, which is simple and repetitive, can be recommended as suitable for calculating the harmful action of chemical substances on the central nervous system.

Investigation of the generated potentials of the cortical optical substance in man revealed the high stability of their amplitude characteristics as well as their temporal characteristics, and the prospects for using this indicator in the assessment of micro-concentrations of chemical substances. Thus, carbon disulfide in a concentration of 0,09 milligram per cubic meter brings about decreased amplitude of the short-latency components and an increase in the long-latency components of the generated potentials in man.

Along with these neurophysiological methods for studying the central nervous system's functional condition for assessment of the toxic effect of chemical substances, methods were used involving the toxicology of behavior. Thus, the study of the chronic action of lead on rats' behavioral reactions was carried out in accordance with a complex "fixed interval-fixed relation" program--120/120. Solutions of lead (lead acetate) having a concentration of 0,1 : 10,0 and 100 mg/l were administered per oz five times a week for 3 months.

Results of the investigation showed that the character of the animals' behavioral reactions changes in relation to the intensity of the acting factor. Thus, for an action by lead in a concentration of 0,1 and 10 mg/l, there were found to be multidirectional changes of such indicators as the number of correct responses and efficiency in performing the "fixed interval-fixed relation" program. One characteristic was the nonoccurrence of change during the first week of stress of the animals, and the instability of the changes occurring in the second week and later. The animals were found to show either an increase or a decrease in the indicators investigated as compared with the corresponding indicators of the control animal group. The functional tests with veronal failed to reveal any deviations.

Changes in the animals' behavior were found in the first week, for a lead concentration of 100 mg/l. the changes occurred in only one direction in all the animals, and expressed a decrease in all the indicators investigated. Thus, efficiency in performance of the "fixed relation" program decreased on the average from $172,5 \pm 34,7$ in the groups to $139,9 \pm$ and in the group of experimental animals, the coefficient of "fixed-interval" efficiency decreased from $224,8 \pm 40,3$ to $155,3 \pm 36,6$.

Functional testing considerably increased the difference between the indicators of the control and experimental groups.

Analysis of the findings makes it possible to classify the 100mg/l concentration of lead among those concentrations exerting indisputable action on the character of animal behavioral reactions.

Comparison of the findings of the behavioral reactions study with findings concerning overall toxic action of lead and toxic action exerted on gonads by lead in the same concentrations under conditions of chronic action on animals for 6 months (Tcharyev, O.G. 1977) shows that the action of lead as regards toxic effect in expression, with urine having delta-amino-levulinic acid with porphobilinogen and others, occurs in the presence of lead concentrations one or two times lower than for changes in behavioral reactions. Comparative assessment of the action of carbon disulfide on the central nervous system and on behavioral reaction revealed the same regularity. Changes showing disturbances of the complex patterns of the animals' behavior were found for carbon disulfide concentrations of 20 mg/M3.

The changes found in behavioral reactions during the first two or three weeks of carbon disulfide action (20 mg/m3) and of lead action 100 mg/l) turned out to be unchanged subsequently. Chronic experimentation does not constitute a more sensitive method, but it does yield more significant mutations, thereby indicating the usefulness of shortening the investigation time to 3 or 4 weeks.

This, together with the investigation and extraction of basic indicators, adds up to about one month and a half.

The findings of the investigation on behavior justify the behavioral reactions study method being recommended for a prior assessment of the toxicity of chemical substances, and for the determination of minimum effective amounts in short-term experiments.

It should be emphasized that the combination of neurophysiological methods, and behavioral toxicology methods yields rewarding results, since it makes it possible to enjoy the advantages of both methods and to assess the effects and mechanism of the toxic action of chemical substances on the organism in every area.

Neurochemical Changes Associated with the Behavioural Toxicity of Organophosphate Compounds

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INTRODUCTION

The relations between behavioural and biochemical changes induced by anticholinesterase (antiChE) agents have been intensively investigated over a period of many years (for discussion and references see 1, 2, 3, 4). After an initial series of experiments dealing mainly with the relations between behavioural depression, inhibition of cholinesterases (ChE) activity, and increase of acetylcholine (ACh) contents in the brain, several specialized lines of work have been established, attempting to deal with various important aspects of the intoxication. For example, it has early been recognized that subdepressant doses of antiChEs can be a useful tool in neuropsychological and psychopharmacological research, due to the facilitated acquisition and/or performance in several tasks which require response inhibition (see Ref. 1, 3, 5, 6). Furthermore, attempts to analyze regional differences in the neurochemical effects of antiChEs have been stimulated by the finding that various responses (locomotor activity, feeding, drinking, avoidance, and various types of operant outputs) are not affected in the same way by a given treatment, particularly with respect to rate of recovery after maximal depression (see, e.g., Ref. 7).

The present paper deals mainly with possible mechanisms of behavioural desensitization or tolerance in the face of continuing depression of enzyme activity after either single or repeated treatments with organophosphate compounds. This problem has considerable interest both from a physiological viewpoint, and from a practical viewpoint. In fact, the available data have provided considerable insight on the dynamics of cholinergic system function, while long-lasting biochemical changes in exposed human subjects (such as serum or red blood cells ChE inhibition) particularly those who are

apparently free of specific symptoms of intoxication, poses considerable problems at the public health level.

METHODOLOGICAL ASPECTS OF STUDIES ON BEHAVIOURAL TOLERANCE

When dealing with behavioural desensitization or tolerance some preliminary distinctions must be made before a closer analysis of possible underlying mechanisms at a physiological-biochemical level. At one extreme of the continuum there are instances in which changes in the behavioural effects of a chemical agent appear to be closely related either to changes in the disposition of the agent itself (e.g., accelerated elimination from the body), or to changes in the responsiveness of a target system as measured by the same physiological-biochemical methods which are used in the first place to assess the effects of the treatment. At the opposite end of the continuum there are instances in which metabolic and/or physiological-biochemical evidence on tolerance mechanisms is not obtained, in spite of extensive investigations in different directions. More important than that — since the universal negative cannot be proven — behavioural evidence often shows that attenuation or disappearance of the original effect is not the result of treatment per se, but rather a specific consequence of behavioural testing in the drug state.

Several well-established methods are available to separate such "behaviourally augmented" tolerance (Ref. 8) from general tolerance phenomena. For example, two groups of animals can be treated repeatedly before or after testing sessions, respectively. When the group treated pre-test shows a substantial reduction or a disappearance of the effect, one can proceed to a sensitivity assessment in the other group, by shifting the animals to treatment before testing. If treatment effects are as large as those originally found in the other group (or at least, substantially larger than those measured after repeated pre-test exposure of the other group), then further efforts must be devoted to the identification of physiological-biochemical (or neuropsychological) mechanisms responsible for specific "behavioural compensation" (or "relearning to perform in the treatment state"), rather than to the search of metabolic or physiological-biochemical mechanisms responsible for general tolerance phenomena triggered by the continued presence of the foreign chemical per se.

An alternative approach to the understanding of specific treatment-behaviour interactions, as opposed to treatment effects per se, is to design experimental situations in which the same response change initially induced by the chemical has different consequences at the reinforcement level: for example, decrease versus no decrease in the amount of food or water obtained, or increase versus no increase in

the number of shocks received. If the attenuation of treatment effects takes place in the face of a high error cost, but not (or at least, substantially more slowly) in the face of a low error cost, then one has good prima facie evidence for a "compensation" or a "relearning" factor being responsible for at least an important part of the observed tolerance. Incidentally, the search for such a factor by an appropriate experimental strategy and its conceptualization for both theoretical and heuristic reasons is not tantamount to denying the existence of physiological-biochemical mechanisms responsible for it (see the considerable confusion in previous controversies on these matters; see, e.g., the discussion of a previous paper, Ref.3). The separation of behaviourally augmented tolerance from tolerance that takes place simply as a function of continuing exposure serves mainly the purpose of a more efficient search for underlying mechanisms. In fact, it is unlikely that the explanations which often account for the second type of phenomenon (e.g., changes in disposition) can also account for specific compensation phenomena.

Clear-cut cases at one or the other extreme of the continuum, as expected, are the exception rather than the rule. One such case is represented by the predominance of a compensation or relearning factor in the desensitization to repeated antimuscarinic exposure. (In fact, no desensitization takes place with post-test exposure or when the cost of altered responding is nil; or if tolerance takes place in these conditions, it requires hundreds of treatments, compared to the few ones which often suffice when animals are treated before testing in situations with a high error cost.) In all other known instances — e.g., in the case of stimulants of the amphetamine type, of hypnotic-sedatives and tranquillizers of the barbiturate and benzodiazepine types, of narcotics, and of cannabis derivatives — it has been shown that behavioural desensitization includes both a sizable general component depending on drug exposure per se, and specific components of behaviourally augmented tolerance depending on drug-test interactions. Organophosphate compounds can be included in this broad category. In fact, alternation and discrimination experiments with differential error cost, as well as avoidance experiments using pre- and post-session treatments, have shown that some of the tolerance observed can be ascribed to a compensation or relearning phenomenon, while a considerable portion seems to take place independently of drug-behaviour interactions (for discussion and sources of data see Ref. 3).

As emphasized previously, various response changes are not attenuated at the same rate during repeated organophosphate exposure, or even after single exposure. However, the behavioural and neurochemical data so far available (see e.g., Ref. 7) are not yet sufficient to assess the relative role of different factors in this phenomenon, for example, (i) differential biochemical-physiological recovery in

subsystems serving more one or the other of several behavioural outputs, versus (ii) different relations between tolerance developing per se and behaviourally augmented components, as a function of the response involved. This issue is further complicated by the fact that when a functional impairment after treatment is of a steady type (as is the case after repeated organophosphate injection), and the response studied is essential for the organism's survival — e.g., feeding, drinking — experiments carried out to separate general tolerance phenomena from specific treatment-behaviour interactions should include control procedures which have not yet been employed, such as intragastric feeding of some of the groups. (Otherwise, all measurements remain limited to conditions in which the animals are tested only in the presence of the functional disturbance and with a high error cost.) In spite of these uncertainties, however, one can now return to the problem of tolerance development as a function of treatment per se, since it seems that a substantial portion of the recovery observed in different situations cannot be ascribed to specific treatment-behaviour interactions.

ALTERNATIVE PATHS TO ORGANOPHOSPHATE TOLERANCE

The work on changes in cholinergic system functions which may help accounting for general tolerance phenomena has followed two main lines, as a consequence of some important differences in the early experiments from which these lines were originated. On the one hand, the group of Russell, Overstreet, and several other coworkers studied tolerance mainly in groups of rats treated with an initial dose of 1 mg/kg i.m. of DFP, followed by repeated doses of 0.5 mg/kg each at 3-day intervals (see, in particular, Ref. 4, 9). In these conditions low and stable brain ChE levels and high brain ACh levels were maintained throughout the period of behavioural desensitization. This suggested that the main factor in tolerance must be either a change in receptor sensitivity in face of an excess of the neurotransmitter, or a taking over of the functions of the cholinergic system by (an) other neurochemical-physiological system(s). On the other hand, our group focussed its attention on the finding that after the first 6-12 hr of acute intoxication following single organophosphate treatment the recovery of behavioural (avoidance) responses goes hand in hand with a return of brain ACh levels towards normal, in spite of a continuing depression of ChE activity. (Most of these experiments, however, showed some re-increase of ChE activity at 16-24 hr intervals, while behavioural tolerance was also observed with repeated treatments inducing a stabilization of ChE activity at 10-20% of the normal level: see the data reviewed in Ref. 3.) Consequently, the emphasis in subsequent neurochemical research was mainly on mechanisms allowing the elimination of excess ACh in the face of a lowered enzyme activity.

Evidence for Changes in Receptor Sensitivity

The experiments carried out by Russell, Overstreet and coworkers over the past decade, too extensive and sophisticated to be analyzed here in any detail (see, in particular Ref. 4, 7, 9 and work quoted therein), clearly favoured the receptor desensitization hypothesis at the expense of the neurochemical redundancy (substitutive system) hypothesis. In summary, it was shown by the use of several autonomic, consummatory and operant responses that behavioural desensitization during repeated DFP exposure was paralleled by a reduced sensitivity of the same responses to muscarinic stimulation by non-organophosphate agents (direct stimulation by carbachol and pilocarpine; indirect stimulation by physostigmine). Conversely, behavioural sensitivity to muscarinic blockade by atropine was increased, while challenges with monoaminergic system agents (alpha-methyl-para-tyrosine, amphetamine) showed normal sensitivity of behavioural responses to these drugs. As expected on the basis of maintenance of elevated ACh levels, the evidence on possible interferences with the synthesis of the neurotransmitter (measurements of choline-acetyltransferase activity and of choline uptake) was negative; in other words, the data were against an end-product inhibition hypothesis (Ref. 4, 9).

It must be noted at this point that experiments showing a modified sensitivity to muscarinic agonists and blockers, but not to agents influencing other neurochemical systems, cannot draw definitive distinction between (i) tolerance due to a change in receptor sensitivity, and (ii) behavioural compensation via taking over of function by non-cholinergic systems. However, the Russell-Overstreet group and other experimenters have recently obtained direct evidence for reduced binding of a highly specific muscarinic ligand (3-quinuclidinyl-benzilate) during organophosphate exposure, which obviously provides unquestionable confirmation of the indirect evidence on receptor modification so far provided (Ref. 10).

Evidence for Selective Modifications of ChE Isoenzyme Profiles

As already mentioned, the work carried out in the Rome laboratory dealt mainly with the mechanisms allowing a return of brain ACh towards control levels, in spite of a continuing depression of ChE activity after single exposure. Modifications of the rate of ACh synthesis were soon excluded as a possible compensation mechanism (Ref. 11). As already mentioned, it was also known that some recovery of ChE activity occurs at a relatively early stage (Ref. 3, 12).

Initially little attention was given to some experiments published by Davis and Agranoff in 1968 (Ref. 13), indicating a short half-life of some of the ChE isoenzymes in the rat retina. (The fast

recovery of such isoenzymes after DFP intoxication was shown to depend upon protein synthesis.) Subsequently considerable efforts have been directed towards the study of ChE isoenzymes and their changes as a function of several physiological and pathological conditions, e.g., developmental processes (Ref. 14), thermal acclimatization (Ref. 15), and brain lesions (Ref. 16). As concerns toxicological analyses of organophosphorus compounds, however, the studies of isoenzyme profiles have so far been limited to a restricted range of experimental conditions. For example, Vijayan and Brownson (Ref. 17), using polyacrylamide gel electrophoresis, showed in 1975 that after a high dose of parathion (2.5 mg/kg i.p.) various rat brain isoenzymes were affected similarly, while after a lower dose (1.25 mg/kg i.p.) the isoenzyme accounting for the largest portion of ChE activity was affected more than the others. Furthermore, Srinivasan and coworkers (Ref. 18) found in 1976 that synaptosomal and microsomal ChE isoenzymes from rat brain were differentially affected by intracerebral DFP treatment, and that the recovery of various isoenzymes after such treatment took place at different rates. Even more recently, Overstreet and coworkers (Ref. 19) using measurements at 4 hr after DFP (1.0 mg/kg i.m.) confirmed that different isoenzyme forms are not affected to the same extent by organophosphate exposure.

The experiments carried out over the past two years in our laboratory, dealing mainly with the time course of isoenzyme profiles from 3 hr to 25 days after single DFP treatment, will be reported at this point.

Materials and methods. Sprague-Dawley male albino rats (160-200 g) were treated with DFP at a dose resulting in a reduction of total ChE to about 20% of the control level after 3 hr (1.1 mg/kg s.c. in arachis oil). Separate groups of six rats were sacrificed at 3 hr, 18 hr, 2, 4, 6, 12, 18 and 25 days after DFP treatment. Appropriate groups treated with the vehicle were run in parallel.

Whole brain, including cerebellum, was removed, frozen and thawed three times. One hemisphere was homogenized using tris-HCl buffer (pH 8.5, 0.038 M; tissue:buffer ratio 1:6) to obtain the so-called soluble fraction of ChE, and the other using the same buffer containing 0.1% Triton X-100, in order to obtain the so-called solubilized fraction (see later). The homogenates were centrifuged at 100,000 g for 1 hr in a Beckman L 2 65B ultracentrifuge. The total ChE activity of the supernatants was determined according to the method of Ellman and coworkers (Ref. 20).

The separation of the isoenzymes was carried out by disc polyacrylamide gel electrophoresis. Cylindrical double layer gels were prepared in 12 cm glass tubes (diameter 0.5 cm). The complete gel consisted of a 9 cm layer of 7.5% separating gel and a 1 cm layer of 3%

spacer gel. A continuous tris-glycine buffer (pH 8.1, 0.05 M) was used for electrophoresis. Samples of supernatant (15 μ l corresponding to about 6 nmoles of acetylthiocholine hydrolyzed per min) were electrophoresed with a running time of 150 min at 1 mA per gel.

In order to carry out the enzymatic reaction and staining, the gels were incubated at 30°C with shaking in a solution of 0.2 M maleic acid, 0.002 M CuSO_4 , 0.01 M glycine and 5.2 mM acetylthiocholine iodide (pH 6.5), and washed in 30% Na_2SO_4 overnight. Finally, gels were placed in a saturated solution of dithioamide containing 1.64 g/l of sodium acetate and after washing in water scanned at 600 nm in a Gilford 2400 spectrophotometer. The peak areas were integrated using a Hewlett-Packard (9804 A) digitizer.

Preliminary experiments carried out on untreated rats and presented separately in a methodological paper at this meeting (Ref. 21) achieved a standardization of the procedure to yield highly reproducible results with either soluble or solubilized ChE. The enzyme fraction found in the supernatant prepared with buffer without detergent after ultracentrifugation was considered as soluble ChE. This fraction corresponded to about 18–20% of total ChE in crude homogenate. However, it is difficult to assess whether the so-called soluble enzyme is actually soluble or just weakly bound to membranes and released easily. Three main molecular forms were detected in this supernatant, accounting for about 63, 19 and 18% of ChE in the soluble fraction.

Solubilized ChE was defined as the fraction obtained through the homogenization of the tissue with the buffer containing Triton-X 100 at the ratio 1:6, as usually reported (see e.g. Ref. 22) and corresponded to about 40% of the total ChE in crude homogenate. Under these experimental conditions the isoenzyme pattern did not differ — either from a qualitative or from a quantitative viewpoint — from that of soluble ChE. Moreover, it was highly similar to that found by Vijayan and Brownson for the normal rat brain (Ref. 22).

It should be noted that in order to achieve a complete solubilization of membrane-bound enzyme, one should use a tissue:buffer ratio equal to 1:20 so as to ensure complete extraction of the isoenzymes firmly bound to membranes and slow-migrating. The resulting isoenzyme pattern, however, is different from that obtained with a 1:6 ratio; therefore, the latter was used throughout the present experiments in order to allow a comparison of the results with the available literature data.

Results and discussion. The results on ChE isoenzyme patterns in the soluble fraction in control conditions and at representative time intervals after a single dose of DFP (1.1 mg/kg s.c.) are presented in Fig. 1.

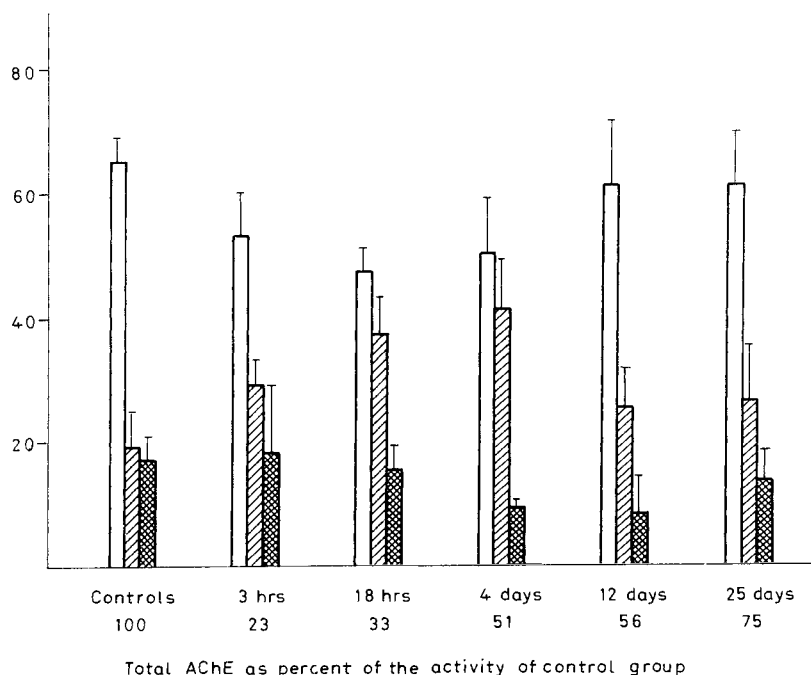


Figure 1. Percentage distribution of brain ChE isoenzymes (so-called soluble fraction) in untreated rats and after treatment with DFP (1.1 mg/kg in arachis oil s.c. N= 6 per group. Averages \pm standard deviation). White bars show the isoenzyme forms migrating slowly in polyacrylamide gel electrophoresis (A in text), shaded bars the forms migrating at intermediate speed (B), and cross-hatched bars the fast migrating forms (C). The recovery of form B, which in absolute values shows a return to pretreatment level within 4 days — i.e. at a time when total ChE activity is still reduced by about 50% — is faster than that of other forms; hence the considerable modification of percentage profiles at several time intervals after treatment.

It appears clearly that after 3 hr, when the inhibition of total ChE is close to 80%, one can already observe a shift in the isoenzyme pattern. The slow-migrating molecular forms (henceforth indicated as A) are reduced proportionately more than forms migrating at intermediate speed (B) while the percentage accounted for by the fast migrating isoenzyme (C) appears to be unaltered (i.e. the reduction in total amount measured is the same as the overall ChE reduction). In other words, the relative contributions of two out of the three

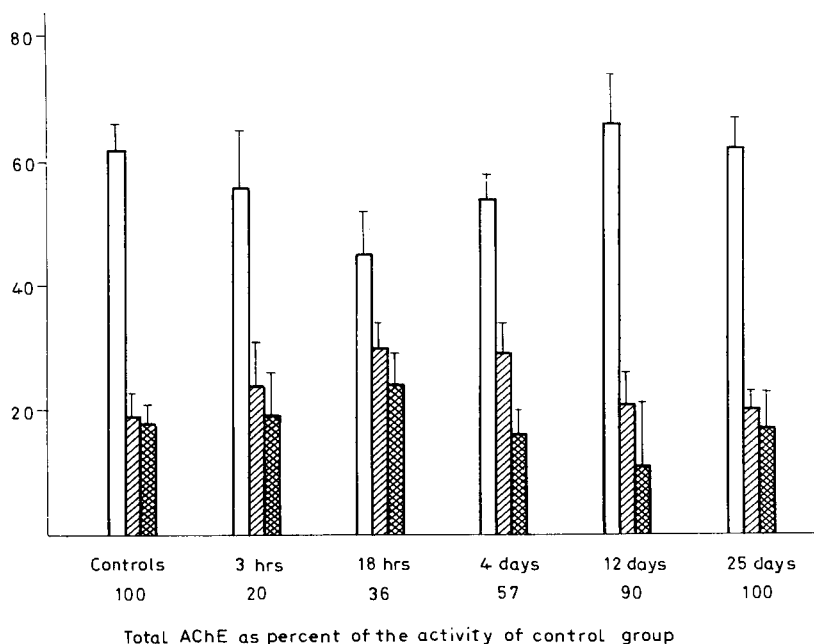


Figure 2. Data comparable to those of Figure 1 are presented here for so-called solubilized fraction of ChE. The changes observed at various time intervals after DFP are in the same direction as in the previous figure, although less marked (for a discussion of the significance of this difference see text).

isoenzyme forms to the total ChE activity still measurable in the intoxicated animals differ from those observed in untreated animals. Such alteration in the pattern tends to become more pronounced over a period of several days after treatment, which goes hand in hand with the initial portion of the recovery curve for total ChE activity. This means that after 18 hr, when total ChE activity shows a 1.5 fold increase over the level measured at 3 hr (from 23 to 33% of control level) the recovery is due mainly to a re-increase of form B. As a matter of fact, when absolute levels, rather than percentage distribution of enzyme activity due to various forms are considered, it turns out that form B, after having shown a reduction of 65% at 3 hr, and of 36% at 18 hr returns to the control level within the 96th hour.

In the late stage of the intoxication, the shift in isoenzyme

pattern is gradually reversed. However, after 25 days, the relative percentages of forms A and B are still somewhat different from those observed in the absence of treatment, while total brain ChE activity in the soluble fraction is still 25% below control level.

The results of the isoenzyme pattern in the so-called solubilized fraction obtained from the brain of the same rats are presented in Fig. 2. It appears that the changes in profiles over time occurring in this fraction are similar to those found in the soluble fraction, although less pronounced. An other difference resides in the fact that both total ChE and percentage distribution of isoenzyme forms shows complete recovery at 25 days. In the attempt to interpret these findings it should be kept in mind that an incomplete solubilization obtained by the use of a tissue:buffer ratio of 1:6 may have released some molecular forms which are less selectively affected by DFP treatment. Thus one must suspect that some masking effect can be observed in studies on the solubilized fraction, which suggests that the profiles obtained with the soluble fraction are more informative than those obtained with the solubilized fraction.

Any attempt to make in-depth comparisons on selective inhibition and/or recovery of ChE isoenzymes with previous work in this area would be premature at this moment, because of the differences in experimental conditions, such as compound used — in the case of Vijayan and Brownson's work with parathion (Ref. 17) — or mode of administration of a particular compound — in the case of the work by Srinivasan and coworkers (Ref. 18) with the intracerebral DFP treatment — not to speak of other factors such as age of the animals and type of technique employed for electrophoresis. It is noteworthy, however, that the results obtained in the early stage of the intoxication by DFP (3 hr) in the present experiments are similar to the aforementioned data by Overstreet and coworkers, using the same antiChE agent, a similar interval (4hr) and comparable separation methods (Ref. 19).

As concerns the physiological significance of differential isoenzyme inhibition and recovery, and of differences between soluble and solubilized fractions, one could hypothesize that in vivo the bound ChE forms are less selectively responsive to organophosphate treatment, due to their complexing with membrane components which may be responsible for conformational changes of protein molecule, and thereby determine constraints on the organophosphate-enzyme interaction. At this point, it might also be speculated that the bound portion of the enzyme has a limited capacity to hydrolyze ACh, and that cytoplasmatic (i.e., soluble), rather than vesicular, ChE is important for neurotransmission — hence the interest of the data on selective recovery of some isoenzyme forms in the soluble fraction for the understanding of both biochemical changes, such as return of brain ACh towards control levels, and behavioural changes, such

as desensitization preceding overall restoration of ChE activity.

GENERAL DISCUSSION AND CONCLUSIONS

The data reviewed in previous sections and the more recent experimental findings summarized above, as well as several other lines of evidence which cannot be included in the present analysis, convincingly show that three important factors play a definite role in the organism's coping with behavioural imbalances induced by organophosphate exposure. These factors are (i) the poorly understood "compensation" or "relearning" phenomena not due to exposure per se, i.e., those suggested by the differences in tolerance sometimes observed with pre- versus post-test exposure, or as a function of error cost (behaviourally augmented tolerance); (ii) modification of receptor sensitivity in the face of prolonged depression of ChE activity and prolonged elevation of ACh levels, apparently a consequence of continued exposure per se, rather than of treatment-behaviour interactions; and (iii) selective recovery of particular isoenzyme forms at relatively short intervals (at least compared to the duration of overall depression of ChE activity after exposure to so-called irreversible inhibitors), which at least under certain conditions may contribute to the return of brain ACh towards control levels in spite of the overall reduction of enzyme activity. In any case the aforementioned data by Davis and Agranoff (Ref. 13) can be confirmed and extended to a wider range of situations, the latter phenomenon may eventually be ascribed to a remarkably wide range of half-lives of the different isoenzyme forms.

Organophosphate treatments for which sufficient biochemical and behavioural data are now available represent a small portion of the compounds and exposure schedules with both physiological-biochemical interest and public health implications. Therefore, one cannot venture into any generalization concerning the relative role of each of the aforementioned factors, nor make guesses about possible interactions between these and several other important factors. Although the present discussion cannot be extended further, it should be recalled at this point that (i) neurochemical changes induced by a given treatment can show important differences from one brain area to another (however, no definite working hypothesis can be made concerning the relations between such variation and the differential treatment effects on several behavioural responses); (ii) organophosphate treatments can modify metabolic phenomena which in turn influence the sensitivity to the treatments themselves, e.g., biotransformation of phosphorothionate insecticides to yield the ChE inhibiting oxygen analogs (oxons), and disposition of active products or metabolites via less active (or inactive) transformation products; (iii) at least part of the behavioural effects of several organophosphates are due to an interference with peripheral, rather

than with central cholinergic mechanisms; (iv) at least some of the behavioural effects of one or the other organophosphate have been shown to take place independently of cholinergic system changes; and (v) substantial differences in mechanism of action must sometimes be ascribed to one or the other of several organismic factors ranging from the more obvious ones such as species, developmental stage, and diet, to those which have so far received limited attention, e.g., sex (Ref. 19) and strain. As concerns treatment-strain interactions, some of the data obtained by Stavinocha and coworkers several years ago (Ref. 23) appear to be directly relevant to the area covered by the present paper. In fact, they suggested that behavioural tolerance to the effect of repeated demeton-S (disulfoton) exposure is due to one or the other of the mechanisms which have been analysed here depending on the strain employed. Overall chronic reduction of ChE activity was comparable in Holtzman and Charles River animals (75-80%), but the former showed faster behavioural tolerance which was paralleled by a return of brain ACh to control levels, while the latter took more time to develop tolerance, which occurred in spite of a continuing elevation of neurotransmitter levels. It is obviously tempting to ascribe the former phenomenon to selective recovery of particular isoenzyme forms (especially since data from several laboratories seem to exclude substantial organophosphate effects on various steps of ACh synthesis), while the second type of tolerance seems to confirm the role of changes in receptor sensitivity under certain circumstances.

The bewildering array of possible main factors and interactions resulting from the long list of different phenomena of proven importance still represents an underestimate of the problems one can be faced with in the real world. In fact, at least three types of interactions between organophosphates and other treatments have been omitted from the present discussion, namely, those between antiChE compounds of the organophosphate and non-organophosphate (carbamate) types, those between organophosphates and different types of antidotes (mainly, but not exclusively, anticholinergics and oximes), and those between organophosphates and a wide range of other chemicals to which humans can be exposed at the same time including, e.g., non-antiChE pesticides, drugs, and various additives and pollutants).

At this point some distinction should be made between future work aiming at a better definition of model physiological-biochemical changes with relevance for the understanding of behavioural changes (e.g., trends of isoenzyme profiles in different brain areas after treatments; studies of the effects of localized treatments), and work to be carried out to meet the needs of preclinical toxicology. Since there are innumerable combinations of treatments and organismic conditions, on the one side, and possible behavioural and biochemical measurements, on the other, it is obvious that predict-

ive toxicological studies based on in-depth analyses of model situations can make sense only if the trend is towards a limitation of the number of compounds which are used. Furthermore, schedules of admissible human exposure also to be limited to a restricted range of typical schemes, must be periodically re-assessed in the light of the best available knowledge both on the risk of intoxication due to ChE depression and on other risks such as delayed neurotoxicity.

As concerns the former, the more urgent task seems to be to fill the gap on experimental models of biochemical and behavioural vulnerability of chronically exposed organisms which have reached asymptotically a steady state condition, and may respond in an unpredictable fashion to changes in the features of the exposure itself (e.g., shifts to different doses of a given compound, or to different compounds). In fact, both desensitization (tolerance) phenomena and sensitization phenomena are known to occur, but the available data are mostly unsystematic, therefore insufficient for rational extrapolation from animal to human data.

At the present state of knowledge, it appears clearly that strategies ignoring the constraints outlined above — i.e., not worrying about excessive proliferation of compounds and exposure schedules in the face of an increasing complexity of interactions between treatment and other factors — must inevitably downgrade the role of general toxicology, and behavioural toxicology in particular, to a post hoc rationalization in the laboratory of epidemiological data collected in the wild.

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Approach to a Behavioural Screen in Toxicity Testing

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ABSTRACT

In this paper I will suggest that the toxicological screening of new chemicals in animals may be improved by the addition of relevant behavioural methods, and I describe the use of a particular test which may be suitable. The principles of testing animal behaviour will then be briefly discussed, to set this method in context.

INTRODUCTION

Behavioural toxicology is usually applied to one aspect of a known problem. It has been known for two hundred years or more that a high dose of mercury or lead or the vapour of a solvent like ether has some sort of toxic action on the nervous system. Given that some human exposure is probably inevitable, it is important to discover the effect of a low dose: how much exposure must be regarded as harmful and in what circumstances? This is where behavioural methods are at present most useful, for while biochemistry and histology are necessary to measure how much of a chemical enters an experimental animal's body, behaviour often provides the best measure of whether it has had any actual effect.

Many toxicologists have a different task. A previously unknown candidate drug or pesticide has to be screened and any effects likely to be harmful have to be discovered. It may be assumed that every drug has side-effects, the preferred task of the toxicologist is to demonstrate that these are so small as not to present a hazard making the drug unacceptable in use; failing that, if the hazards are potentially serious, the toxicologist should discover them as early as possible, before undertaking the time-consuming and expensive process of long-term toxicity testing. A side-effect in the screen is worth ten in the clinical trial.

A toxicological screen therefore has to be sensitive to any effects of a chemical at low doses, comparable to those to which human beings might be exposed. At the same time it should not be excessively sensitive, there should not be too many false positives. The screen also has to be 'wide-spectrum', sensitive to as many different potentially harmful effects as possible. Finally a screen has to be simple and easy to operate, in order to cope quickly with a large number of candidate compounds.

It is suggested in this paper that suitable behavioural tests are a worthwhile

addition to toxicity screens. They can provide information not available from conventional toxicological methods in a substantial proportion of the compounds tested, and can do so at an acceptable cost in money, time, and effort. Experience with one behavioural method will be described. Some principles of designing tests of animal behaviour will then be briefly discussed to help toxicologists develop the method further and to appreciate the potentialities of different behavioural methods in measuring different pharmacological effects.

Objectives of a Behavioural Test in Toxicity Screening

In an acute toxicity screen, the highest doses of interest are those which turn out to span the LD50. Behavioural observations at these doses add little new information, unless convulsions or coma give a clue to the chemical's mode of action, and a formal behavioural test would serve no purpose. At the highest non-lethal dose, we might still expect the dosed animals to be ill, and indeed the illness might be obvious on the crudest observation, yet a formal behavioural test would be justifiable. The subjective observation that the animals were reluctant even to stagger round the cage would be made objective and quantitative. The test would help indicate the time of onset of illness and of recovery. Thirdly, behavioural tests operate by stimulating some action and their most interesting benefit might be to show when animals that are apparently ill perform nearly as well as controls, in other words to show how far the animals can overcome a handicap.

The benefit of a behavioural test is found especially where a significant behavioural change occurs at a dose or doses showing no effect by conventional methods. Quantitative changes can be found where on subjective, "clinical" observation, behaviour is normal and no trace of harm is found on haematological screening or at post-mortem dissection and pathological examination.

A suitable behavioural method would also reveal what may be called specific behavioural effects, those which a CNS-active drug might produce. It should distinguish such selective effects from the secondary consequences of the animals being "ill" or in pain.

Finally it is important to note that behavioural observations are reliable, and that behavioural variation is not the same as random fluctuation. As with other methods, there is a threshold dose for behavioural effects, below which no effect is apparent.

METHODS

An "Exploration-thirst" Test Applied in Acute and Subacute Screening

The "exploration-thirst" test (Refs. 1, 2) was applied to the animals undergoing an acute intraperitoneal toxicity screen on 20 consecutive compounds and then on 10 consecutive compounds in a subacute inhalation-toxicity screen. The compounds were a mixed collection of general industrial chemicals, either candidate products where the present screens were intended as the first stage of long-term testing, or intermediates in their manufacture, where toxicological information was needed for industrial hygiene purposes.

This test has two main components. If a rat is placed in an unfamiliar place, an open field, a maze, or even a strange cage, it moves systematically round the space available to it. This exploration can be measured as motor activity. If

the rat is deprived of water and water is available, it will eventually drink. In the acute toxicity screen, the procedure was therefore to inject the animals intraperitoneally in the morning, three rats of each sex at each dose. They were observed in the usual way for deaths and signs of obvious toxicity, and control rats and the survivors (unless they were clearly too ill) were deprived of water overnight. This deprivation (about 18 h) was sufficient to reliably induce drinking but not to add unnecessary stress. The behavioural test was a 5 min trial in an unfamiliar cage, with water available, 24 h after injection. The rat was then offered water in the home cage and deprived again for a second trial 48 h after injection.

In the inhalation toxicity screen, rats were exposed for 6 h daily for three 5-day weeks. Exploration-thirst trials followed the 12th and 13th days' exposures; the 12th was slightly abbreviated to allow time for drinking before the second night's deprivation.

In each trial, exploration was observed as pauses in walking or in bouts of sniffing, and analysed statistically as the number per minute until the rat found water and started drinking. Human observation runs the risk of subjectivity, of course, but even inexperienced observers are remarkably self-consistent and in a few cases turned out not to be a significant source of variance. Two experienced observers watching the same rats correlated at $r = .92$. One of them also correlated significantly with a single-channel electronic meter of motor activity.

The other measure was of time until the first lick of the water-bottle. Once started, drinking is usually continuous; sometimes (rarely in trial 1, 50% in trial 2) rats explore further after the first lick or two, before returning to drink. Times are recorded in seconds (transformed to the square root for statistical analysis). The first lick shows that the rat has learned the location of water; if the rat explores further before drinking, the most likely of several possible explanations is that the animal needs some kind of further information about the unfamiliar environment. The change in time from trial 1 to trial 2 gives prima facie evidence of learning, as it does in the first few trials in a maze.

In the very simplest case, a drug could alter behaviour along two dimensions in this test. Dosed rats could walk faster or slower than controls, and a change in the observed rate of exploration is independent of whether they drink earlier or later. Some of the various possible effects have been produced with various drugs, and are shown in Table 1.

TABLE 1 Examples of Drug Effects in the Exploration-Thirst Test

Observed rate of exploration	Time to the start of drinking		
	Earlier	Same as controls	Later
Faster	Pentobarbitone low doses		d-Amphetamine
Same as controls	Trichloroethylene	(drug has no effect)	
Slower			Chlorpromazine

(For explanation, see text on next page)

Trichloroethylene was administered by inhalation (100 - 1000 ppm, 6 h/day) and tested after 15 - 37 days, the effect was usually seen in trial 2 (Ref. 3). The other drugs were given by i.p. injection before trial 1, pentobarbitone Na at 5-10 mg/kg 1 min before, d-Amphetamine at 0.5-2 mg/kg 15 min before, and chlorpromazine at 1-4 mg/kg. Of these, only chlorpromazine had residual effects in trial 2.

Thus rats given chlorpromazine explore more slowly than controls on average, and drink later; they may delay drinking also on trial 2 without further injection. Those given d-Amphetamine (0.5 mg/kg) tend to walk faster than controls but (at 0.5 or 2 mg/kg) may not drink at all in the 5 minute trial. Rats inhaling trichloroethylene vapour drink earlier than controls on trial 2 while walking at the same rate.

RESULTS

Acute Toxicity Screen

The effects of the 20 compounds from the acute toxicity screen are shown in Table 2. Four compounds had no significant effect in the exploration-thirst test, although one of them had caused convulsions and another led to dyspnoea shortly after injection: after 24 h the rats had presumably recovered. Thirteen compounds both lowered the rate of exploratory walking and delayed drinking in at least one of the two trials; ten did so in both. In seven of these 13 compounds, and also in the three where one measure was significantly slower but not the other, the rats showed post-mortem evidence of peritonitis, suggesting that in these cases the behavioural change may have been secondary to pain from a local irritant action of the injected chemical.

TABLE 2 Effects of 20 Compounds from an Acute Toxicity Screen in the Exploration-thirst Test

Rate of Exploration	Time to the start of Drinking		
	Earlier	Same	Delayed
Faster			
Same		4	1
Slower		2	13

The remaining six compounds may have had a specific effect on the central nervous system. On the one hand, there was no post-mortem or clinical evidence of pain or gross toxicity. On the other hand, behavioural changes were observed which could account for the objective effects of delayed drinking and fewer exploratory movements. Two of the compounds caused the rats to spend long periods grooming themselves or gnawing, reminiscent of the stereotyped movements induced by dopaminergic drugs. Rats given the other four compounds did not walk slowly, they alternated between total immobility and fast running to the next corner of the cage; this is certainly not characteristic of non-specific "illness" and such "stop-and-go" movements have also been seen in rats given opiates such as pethidine.

A statistically significant behavioural effect of one sort or another was therefore seen in 16 of the 20 compounds in this series. The important point to establish the method's sensitivity is to compare the doses at which these effects were observed with those found by conventional means. Table 3 shows that for thirteen of these 16 compounds, one or more doses were given where a behavioural effect was significant but no effect was detected by clinical observation or post-mortem histological examination. In the other three cases, both methods were equally sensitive.

TABLE 3 Dose-sensitivity: Incidence of Behavioural and Conventional Effects

Number of compounds where an effect was detected by:-	<u>Acute</u>	<u>Subacute</u>
	lowest active dose	lowest concentration tested
conventional methods alone	4	1
both methods	3	1
behavioural method alone	13	4
no effect detected		4
	<u>20</u>	<u>10</u>

Comparison of the dose-sensitivity of the exploration-thirst test with that of clinical observation, post-mortem dissection and histological pathology and, at the subacute stage, with haematological and urinary-biochemistry screens. The table shows the effects for the lowest active dose in the acute screen, as several of the compounds were also given at doses where no effect was detected by either method; in the subacute screen, four compounds were only tested at an inactive concentration.

Subacute Inhalation Screen

In an acute screen, sensitivity is not a prime consideration in any case, but at the subacute stage it becomes important. A primary objective is to find a "no-effect" dose or concentration, however provisional the estimate may turn out to be, whether it is to act as a guide for industrial hygiene purposes or merely to set dose-levels for long-term toxicity testing. In the present series, only ten compounds were tested, but Table 3 shows that nevertheless behaviour was more sensitive than all other methods used in four cases. In one of these four, the same effect was found in two separate experiments when the compound was screened again at a lower concentration. In four other compounds a "no-effect" concentration was found with no effect by either behavioural or conventional means, and only in one case did clinical observation reveal an effect (nasal irritation) where there was none in the behavioural test.

The four compounds where behaviour was more sensitive and the one where both methods were equal, all appeared to have a specific action on the central nervous system. One was a solvent with effects only on the time to drinking, presumably mediated by traces of an anaesthetic effect. Three compounds caused the rats to

spend long periods grooming, so that the mean rate of exploration was low and drinking was delayed. For two of these compounds, a "head-only" method of exposure was used, the rats were confined in tubes in such a way that only the nose projected into the air carrying the compound. Their grooming was therefore not a direct response to dust particles in the fur. Control rats breathing clean air also groomed themselves thoroughly, but only did so for the first minute or two after removal from the exposure chamber into the cage for behavioural observation. Grooming by the exposed animals therefore suggested stereotyped behaviour, on the criterion that it was a continuation or exaggeration of what the animals are stimulated to do in any case.

The remaining compound, p-Hydroxyphenylacetonitrile, selectively increased the rate of exploratory movement, actually doubling it in trial 2. Despite the small sample (6 exposed rats and 6 controls) divided between three observers, the increase was significant at $P < 0.001$. The time to drinking was not changed, there was no evidence of anorexia or stereotyped motor activity and no other sign of toxicity, though a higher concentration caused proteinuria. The material was tested as a solution in methyl iso-butylketone but, unfortunately, no vehicle-controls were available. The observed effects may therefore have been due to the solvent rather than the test compound.

DISCUSSION

Behaviour in Toxicity Screening

The exploration-thirst test was applied to two consecutive series of compounds subjected to routine toxicity testing. In half of them it revealed a behavioural change, distinguishing several types of effect, at a dose where no effect was detected by any conventional means. While a sample of 30 compounds is small in this context, the method seems promising.

The cost of the exploration-thirst test is best expressed in terms of time, since the direct financial cost is small. Actual observation until the start of drinking in two 5-min trials averaged about 7-8 min/rat. The overheads of preparation, tabulating the results, statistical analysis, and writing a routine report add up to a similar time, so that the marginal cost of adding the test to a routine toxicity screen was about 0.25 man-hours/rat, 3 or 4 man-hours/compound. In a preliminary acute screen, this could be thought excessive, but at the sub-acute stage when an estimate of a "no-effect" dose is required, this cost seems well worth while. Early warning is, after all, the objective of toxicity testing.

Principles of Animal Behaviour as an Applied Science

It will be useful to compare this method briefly and perhaps in an over-simplified way with other ways of testing animal behaviour (Ref. 2).

Behaviour inevitably fluctuates, for it is the way a homeostatic organism relates to a fluctuating environment. An experimental method therefore has to allow the animal to remain sensitive to the independent variable, i.e. the drug, without being over-sensitive to incidental environmental stimuli. Not every technique in the literature manages to achieve the optimum balance of reliability and sensitivity, but it may be that no single optimum exists suitable for all possible drugs.

Any method utilising animal behaviour has to start by solving two related problems. The first is that behaviour has evolved to suit the animal, and a way has to be found of inducing the animal to perform some action reliably at a time of the experimenter's choice. The second is to define that action so that measurement can be both objective and quantitative.

In many experiments the second problem is solved by conditioning the animal to perform some essentially artificial action, like pressing a lever, which can be recorded automatically and objectively. The animal is then conditioned to press the lever according to a definite schedule by, for example, depriving it of food and offering food if the animal performs within the limits that the experimenter sets. Operant conditioning needs careful and extended training but induces behaviour that is very reliable and can be tailored to test, fairly selectively, a specific hypothesis about a drug effect.

In a subacute screen, however, the toxicologist is not yet in a position to make a specific hypothesis. He does not yet know whether he has a problem. If a method is both sensitive to a wide variety of different possible behavioural effects and can distinguish between them, the toxicologist can afford to lose a little precision; but when screening a large number of compounds, he cannot afford the time to condition animals to perform anything not in their basic repertoire.

The alternative is therefore to attempt to recognise units in the animal's spontaneous behaviour, and to find a situation where the animals can be relied on to perform these actions at a sufficiently high rate. The most reliable situation is the disturbance due to an unfamiliar environment, and we can easily observe the motor activity that is the visible sign of exploration. Now exploration may or may not be constructed of visible units like a wall is built of bricks; units are certainly easier to distinguish in some other kinds of behaviour, grooming, drinking, and social behaviour, so that it is entirely legitimate to short-circuit the search for units of exploration by automating the system with an 'animex' type of machine or photocells. However the observation we have used attempts to define units by showing when they come to an end: an exploratory unit ends when the rat pauses in walking or changes the place it is sniffing at; and the whole period of exploration ends, usually, when the rat starts to drink.

Now even inexperienced observers find no difficulty in recording this exploration consistently, and even if it is decided to make automatic records of the formal measures of exploratory activity and time to drinking, there are still important advantages in human observation. My last point is to explain this.

Behaviour is inevitably complex if it is to help the animal earn its living in the real world, and it remains complex even in a laboratory situation that the experimenter considers simple. In most of science it is possible to simplify by studying one variable at a time. The classical pharmacologist can isolate a tissue in an organ bath and isolate one drug receptor by selectively blocking others. But only an intact whole animal can behave. We can selectively amplify one kind of behaviour, we can increase the probability of drinking by depriving the animal of water and offer it the opportunity to drink; but we cannot, in general, reduce a particular kind of behaviour selectively. We can ignore the complexity but we cannot make it go away, and in toxicological screening the complexity is in fact positively beneficial.

Suppose the compound being studied were to reduce the rate of exploration in the exploration-thirst test, or of motor activity, and only this single measure is taken. There are many possible explanations: the activity might have been

inhibited by pain or other gross toxicity, or the animal might be spending the time grooming, or even drinking, and while some of the possible explanations would imply a harmful effect, the others might not. An attempt is made in the exploration-thirst test to distinguish between different effects by taking two independent measures. It becomes possible to distinguish some kinds of selective action, making the rats drink earlier or later without altering exploratory activity or vice versa, from an unselective action, slowing down both measures. The real importance of the human observer comes from the different ways in which an apparently non-selective effect can be brought about. A general slow-down of the rate of walking could be secondary to pain, but if the animal grooms for long periods, or if it crouches immobile in one corner and then dashes quickly to another, then it is reasonable to postulate some kind of specific effect on the central nervous system. Only a human being can evaluate the unexpected.

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Alertness Impairing Substances and Transport and Occupational Safety: Laboratory Assessment of Hazard

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ABSTRACT

The suitability of a model of vigilance performance in monotonous conditions is demonstrated, and in experiments on 243 volunteers basic control data have been received on the effect of two reference substances, ethanol and pentobarbital, each in three doses. Regression of effect on the dose forms the basis for the solution of problems of practical importance concerning the effect of foreign substances, industrial chemicals and drugs on working efficiency, requiring the upkeep of vigilance in monotonous situations.

The results warrant the following conclusions: 1) A decrement in vigilance in an experimental situation is a relatively good prediction of human ability to remain alert in real conditions, especially in subjects prone to "near-accidents" for falling asleep (so called microsleeps). 2) The majority of Ss was unable to resist a decrease in vigilance under the drug effect, not even at a stepped up compensation effort (pentobarbital). With ethanol the risk is enhanced by insufficient or paradoxical perception of its effect (significant decrease in the feeling of sleepiness at a significant increase in the incidence of microsleeps).

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The technical and social development gives increasingly rise to situations in which a decrease in alertness entails the risk of accidents with unproportionate health and material damage. Obviously in such situations the accident hazard is enhanced by the hypnotic and sedative effect of foreign substances. This applies to substances to which the human is involuntarily exposed, especially in the workplace, as well as to those which he takes voluntarily, like alcohol and drugs. Moreover, the combination of the above types of substances may potentiate the undesired effect.

For industrial chemical agents with a prevalently hypnotic and sedative effect, for example the majority of solvents, it is the ability to remain alert which forms the criterion for the establishment of hygienic standards of maximum allowable concentrations in the working environment (MAC values). Similar problems arise with respect to drug prescription to not work disabled patients; the system of contraindications and warnings is as yet not satisfactory. For a rational application of these preventive measures, criteria are needed for a quantitative comparison of this kind of side-effects of the indicated drugs which, for various technical and ethical reasons, are difficult or almost impossible to obtain from accident statistics or field experiments. There are remaining only laboratory model situations the results of which can be indirectly validated by comparison, with ethanol as a reference substance (Horváth, Frantík 1970 and 1973; Klein 1969).

In our laboratory we have been working systematically with a model of vigilance performance under monotonous conditions which we regard as suited for the laboratory assessment of the potential accident risk of the use of sedatives, exposure to industrial solvents, etc. (Horváth, Frantík, Kopřiva 1976). After many years experience we abandoned batteries of tests, and prefer long lasting repetition of a single task without disturbing changes and interventions. An essential component of the presently used modification is discrimination of acoustical clicks (duration about 4 msec) applied into both earphones in the same or in different intensities, appearing thus as spatially localised. According to instructions the subject responds, for example, to acoustical stimuli from left and right with pressing a key and ignores stimuli in the middle position which form about 50% of all stimuli. The model includes a secondary

optical feedback task of maintaining the position of a dot on the oscilloscope by permanently pressing a key with the lefthand finger. The criteria of the monotony and drug effect are: 1) parameters of behaviour, reaction time and number of errors, 2) physiologic characteristics of vigility, especially EEG, general motor activity, cardiovascular functions, 3) psychological characteristics of the subjective state.

The test program is automated, as well as data recording on a punch tape. Tests are performed in sound proof, weakly illuminated boxes under maximally monotonous conditions.

The forenoon tests include training by means of tape recorded instructions. If criteria are satisfied a control program is presented consisting of 2 blocks of 50 stimuli each with 5-9 sec intervals, 3 blocks of a "rapid" program with intervals around 1 sec and of the "monotonous" part (5 blocks of 50 stimuli each with intervals of 5-25 sec, mean 15 sec). In this last part of the experiment performance decreased rapidly (Fig. 1), the per cent of omissions increased (from 4 to 15%), the number of microsleeps, coinciding with maximum theta activity, rose. Deterioration in performance was evident in about 9/10 of Ss. There were also significant changes in subjective feelings, increments in the rating scale of sleepiness, passive mood, depression, angriness, loss of energy and cheerfulness, etc. (Fig. 2).

In several series of experiments we have studied the effect of ethanol for the purpose of comparing it with equipotential doses of pentobarbital as a secondary, more suitable reference substance.

Pentobarbital in doses of 1.35, 2.00 and 2.65 mg/kg orally impaired significantly vigilance performance: mean percentage of errors of omission in the monotonous part (Fig. 3C), percentage of errors in the worst block (Fig. 3A) and the number of 2-minute sections with a high incidence of omissions (Fig. 3B) increased. Mean pentobarbital blood levels were 96, 169 and 180 $\mu\text{g}/100$ ml serum - after excluding subjects with extremely low levels who apparently absorbed the drug very slowly. Without this exclusion the mean values were 65, 107, 126. Other characteristics of performance (discrimination ability, speed of reaction) did not change strikingly under the effect of pentobarbital, except a slightly increased number of false positive responses (about 15%) after the lowest dose of pentobarbi-

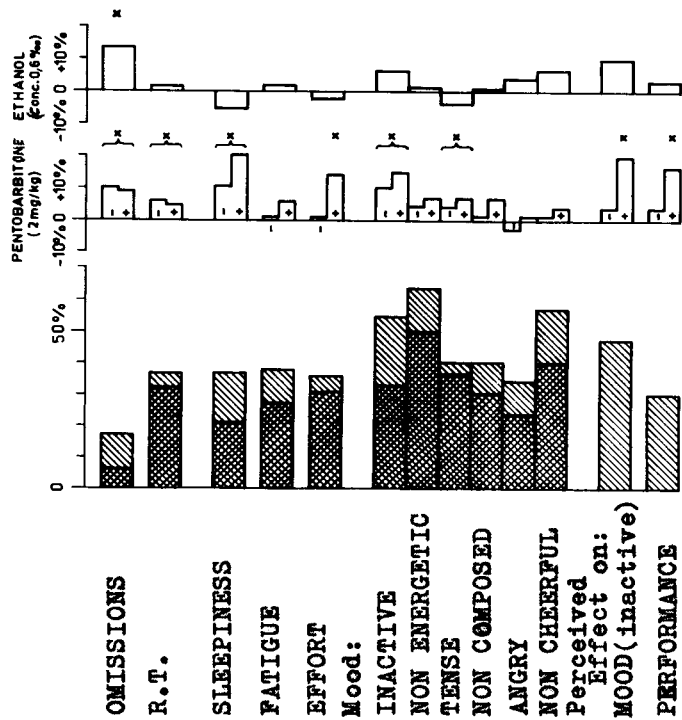


Fig. 2. Subjective state and performance in the vigilance test (Scales in percentage of maximum possible score)

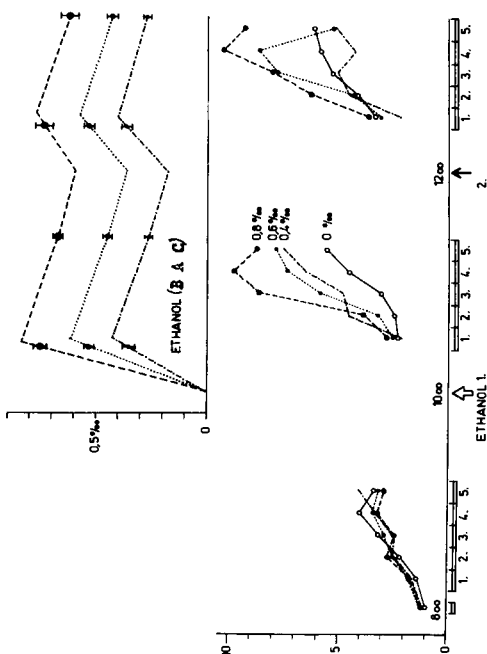


Fig. 1. Time course of vigilance decrement, i.e. the increase in the percentage of detection omissions (lower part), and interpolated time course of blood alcohol concentration (group means with 95% confidential limits) in the first, second and third session (5 blocks per 50 stimuli each) in placebo and three ethanol subgroups (on axis x - hours)

tal. Perception of the drug effect on mood and performance differed already after the lowest dose and was not dose-related (Fig. 4). In the subjective rating of sleepiness, fatigue, effort and mood, subjects with the lowest dose of pentobarbital did not differ from the placebo group, whereas the highest dose distinctly enhanced the feeling of sleepiness, fatigue and effort and shifted the mood to passivity.

Ethanol in doses calculated so that blood concentration reached 0.4, 0.6 and 0.8% produced a similar effect as pentobarbital (Fig. 1), whereby the medium dose of pentobarbital had a greater effect than a concentration of 0.4 and a smaller effect than 0.6% of ethanol (Fig. 2). To test whether maintenance of ethanol concentration in blood for many hours changes its effect on vigilance, 140 minutes after the first dose a maintenance dose was given. Figure 1 demonstrates the time course of vigilance decrement, i.e. rise in the per cent of omitted reactions in the first (control) test, in the second test (20-85 min. after the first dose) and in the third test (20-85 min. after the maintenance dose of ethanol). The effect of the two higher doses was significant in the second and third test, the effect of the lowest dose was significant in the second test only. Reaction time and fine discrimination of signals were not significantly affected by any dose.

Figure 1 also shows that even the highest dose of ethanol caused no deterioration from the beginning - the number of errors rose significantly only in the third or fourth fifth of the experiment; that is, only after interaction with the long stay in the monotonous environment. The highest dose (corresponding to a concentration of 0.8% blood alcohol) increased the per cent of Ss who fell asleep during the experiment, from 8% in the control test to 55%. Thus, this concentration affected considerably the ability of most Ss to remain alert. Maintenance of the level for several hours did not further increase the effect but neither did it eliminate it (with the exception of the lowest dose).

A substantial difference between the two substances is in the perception of effect - the marked effect of ethanol on performance contrasts (in comparison to pentobarbital) with its slight reflection on mood, and perception of the effect on mood and performance (Fig. 5); in the two lower doses ethanol rather lowered the S's own

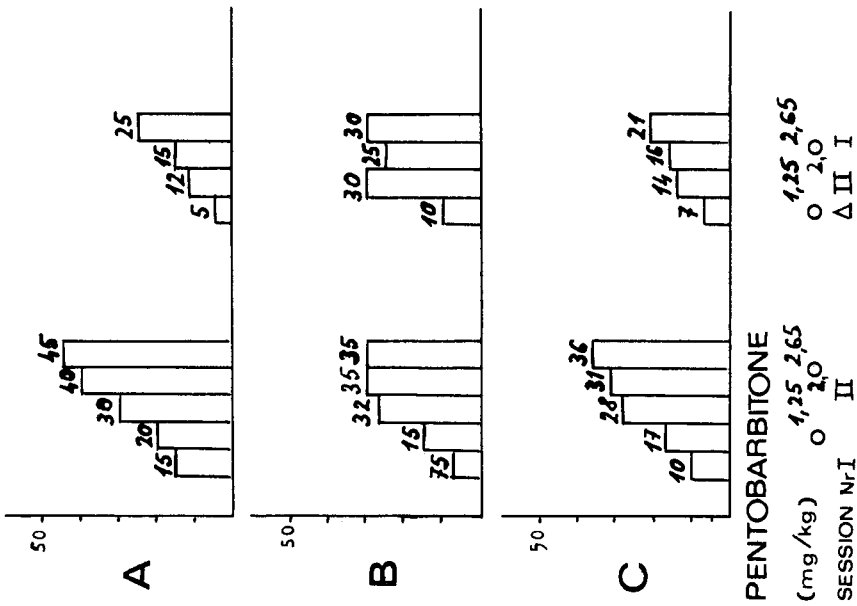


Fig. 3. (For explanation see text)

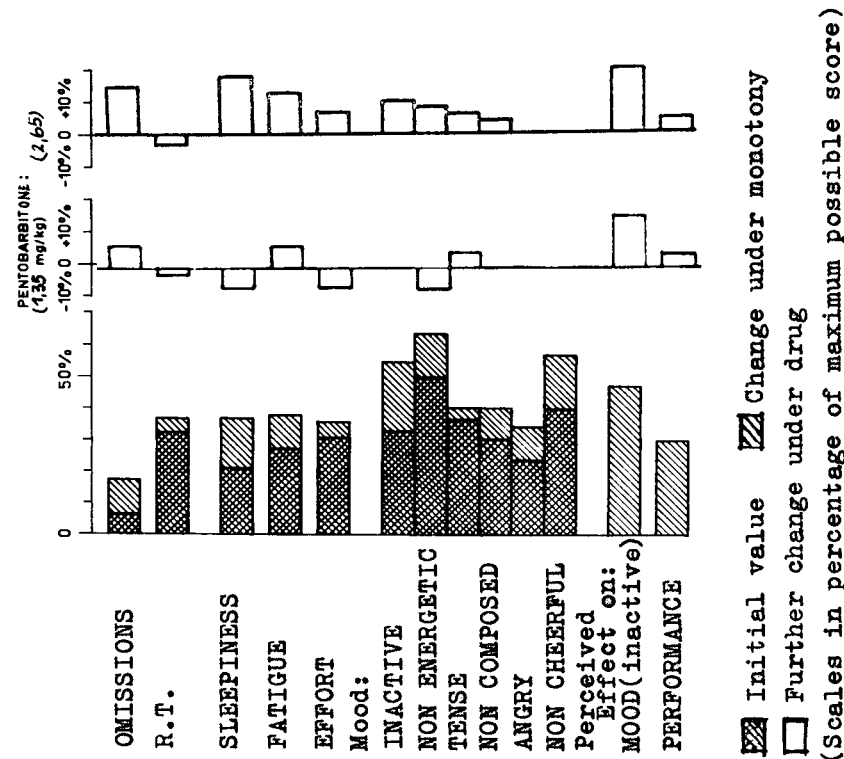


Fig. 4. Subjective state and performance in the vigilance test

rating of sleepiness and performance decrement (Fig. 6) (Horváth, Frantík, Peprník, Beneš 1978).

Even when excluding mathematically the effect of different blood levels of the effective drug this still does not explain at least 60% of the considerably high variance in individual performance decrement. We have tried to analyse the cause for this large interindividual difference in the effect of monotony alone and in combination with drugs from three aspects: 1) The actual state of activation influenced by the deliberate effort of the subject to compensate the drug effect, 2) individually different ability to maintain alertness, 3) other personality traits, especially "neuroticism" in Eysenck's conception.

In the test series with a pentobarbital dose of 2 mg/kg the effort for compensating the depressant effect of the drug was manipulated by the information that the drug may in some subjects depress alertness and thus impair efficiency. The Ss were also advised that on the basis of information of the control apparatus they would be told in time whether the drug had any effect or not by a lit-up signal "effect" or "no effect", after the first block of the monotonous program.

Instruction for compensation did not influence the effect of pentobarbital on performance (Fig. 2). At the same time the answers in the questionnaire revealed that the instruction influencing compensation had been accepted by the Ss in the way intended. The groups given a positive instruction for compensation showed significantly higher scores in effort, motivation, and compensation effort. Instruction for compensation affected also the index of discrimination criteria: at positive instructions for compensation the Ss tended rather to respond than not to respond to hardly discriminable stimuli. This finding is related to the S's compensation effort to resist the depressant effect of the drug which he anticipated on account of the positive instruction.

A validation interview was performed with all subjects (drivers) prior to the second session, in which they responded to questions on their ability to remain alert in various types of monotonous situations (lectures, mental work, night driving, etc.). The mean per cent of omissions in the monotonous program correlated with the number of given symptoms of reduced ability to maintain alertness.

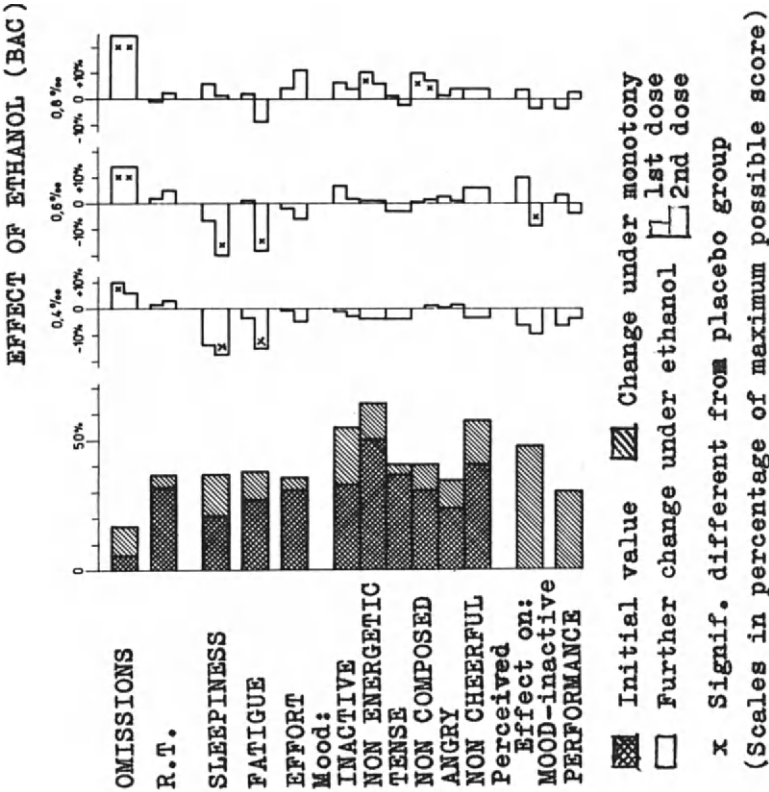


Fig. 5. Subjective state and performance in the vigilance test

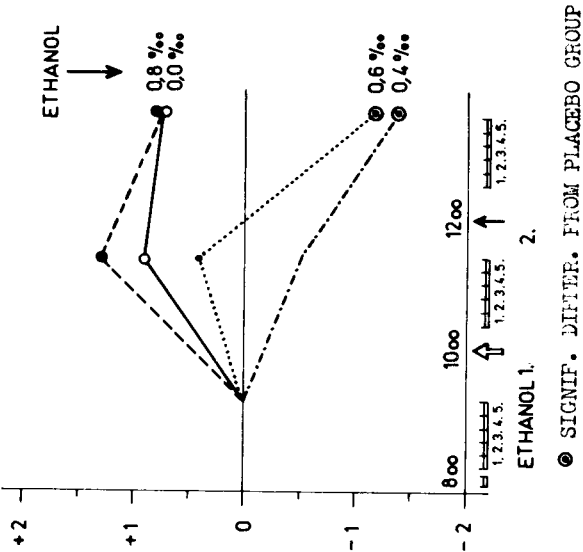


Fig. 6. Changes of subjective rating of sleepiness in relation to ethanol dose

We tried, therefore, to estimate the usability of this method in the selection of drivers using the criteria of near-accidents due to falling asleep. We found that in the reduced sample we could, on the basis of omissions to easily discriminable signals, identify 78% of Ss who had reported near-accidents for falling asleep.

The hypothesis as to lower ability for deliberate compensation of reduced efficiency in more neurotic Ss was borne out by the results. The regression coefficient of omission increments to easily discriminable signals on neuroticism was significantly positive at a positive instruction for compensation and differed only slightly from zero at a negative instruction. The difference indicates that a warning that performance might deteriorate under the effect of the drug produced in more instable (more neurotic) Ss a further worsening, while in more stable Ss an improvement of performance.

Concerning validity of the experimental models it is noteworthy that in the subgroup of more neurotic Ss we could not prove the above mentioned correlation between statements made in the validation interview and reduced vigilance in the laboratory experiment: the more neurotic Ss mentioned greater sleepiness in various situations but, apparently due to the situation tension, they had a lower vigilance decrement in the laboratory situation.

The effects of reference substances form a basis for the assessment of similar effects of drug groups important for work and traffic safety (e.g. antihistaminics), solvents and their combinations. Up to now, dithiaden (2 and 4 mg) was compared with clemastin (1 and 2 mg) and the experiment with toluene (inhalation in concentrations of 0.2-0.8 mg/l) was started.

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Principles of Percutaneous Absorption *

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ABSTRACT

Percutaneous absorption is a passive diffusion process. The main obstacle is the horny layer (stratum corneum). The entire stratum corneum functions as penetration barrier. The result of this barrier function is the creation of the reservoir, i.e. the deposition of substances on and within the barrier. The reservoir is responsible for the unique type of pharmacokinetic in and through the skin. If the horny layer is injured, i.e. in diseased skin the barrier function is disturbed and reduced and percutaneous absorption is enhanced. In normal skin the size and localization of the application area play a dominant role for the absorption rate. Metabolism, binding to proteins or to cellular components and accumulation in subcutaneous fat may occur, but in general these phenomena have little influence on the percutaneous absorption rates. Those quantities of foreign material which have passed the stratum corneum barrier will be taken up almost quantitatively by the cutaneous blood and lymph system, i.e. they are resorbed.

INTRODUCTION

Pharmacokinetic investigations on topically applied drugs have basically three aims (1):

1. To find the optimal conditions for penetration of the drugs into the skin. Contrary to pharmacokinetic studies of systemically applied drugs, investigations on diseased or artificially injured skin can and should be included, since penetration rates may be higher by orders of magnitude in diseased as compared to normal skin, and, in most cases, the drugs in question will be applied to damaged skin.
2. To obtain information concerning the layers and structures of the skin through which drugs will penetrate, in order to detect the target for the drug. This aim, however, is difficult to realize at present, since most targets react and are saturated with very low amounts of the substance, while the greater part of the drug is either bound in an unspecific manner or diffuses freely. Thus the structure to which the greatest amounts of the drug are attached is not necessarily the target of this particular agent.
3. To determine the velocity of the drug flow into and through the respective skin layers, the period of the steady state concentration in these layers, eventual metabolic deactivation and the velocity of flow out of the tissue into the general circulation. These parameters are of practical and theoretical interest. Thus information is needed on intervals of drug application, in order to establish

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continuous pharmacological action. A pharmacokinetic comparison of the same drug after local and systemic application may be of interest too and may yield valuable information as to the advisability of a certain therapeutic procedure.

The use of radioactively labeled drugs is a valuable tool in these investigations, although there are technical problems associated with this experimental approach, as well as those arising from the morphology of skin itself.

In order to avoid semantic misunderstanding, the following terms are defined: penetration is considered to be the process of entrance into one layer; permeation is the migration through one or several skin layers; resorption is defined as the uptake by the cutaneous blood- and lymph-vessels and absorption is the sum of all these processes.

MATERIAL AND METHODS

Labeled drugs. For most experiments, tritiated or carbon-14-labeled drugs were used, labeling with sulfur-35 and in one case with cadmium-115 was also employed. A specific activity of 3-5 mCi/mg for the tritiated drugs or 0.5 - 1 mCi/mg of ^{14}C or ^{35}S was sufficient when a concentration of 0.1% drug in the preparation was employed. Before use, the radiolabeled substances were checked for the presence of impurities and labile label by thin layer chromatography.

Preparations with labeled drugs. Even distribution of the drug in the vehicle is an essential prerequisite for meaningful penetration data. Since radiolabeled preparations are expensive and are prepared in small quantities only, simple but effective incorporation techniques must be employed. In most cases we have incorporated the label in our laboratory. A prerequisite for this procedure is that the unlabeled substance should be completely dissolved in the vehicle. In the case of incomplete dissolution, the labeled and unlabeled drugs have to be recrystallized together in order to obtain a uniformly labeled substance which can then be incorporated into the vehicle. Complete solubility as well as uniformity of crystal size and distribution were checked as required with a light microscope. Until use, the labeled preparation was stored in small, sealed glass vials to avoid evaporation of water or other constituents.

Application to the skin in vitro. Fresh skin from mastectomy or cadavers was used; subcutaneous fat was removed without damaging the dermis. An application area was marked and 1-3 mg/cm² of ointment or 2-5 $\mu\text{l}/\text{cm}^2$ of solution were applied and gently distributed over the skin surface. The skin specimen was then inserted into the penetration chamber, which was kept at 32°C. The lower part contained saline which was stirred slowly. The upper part was fitted with an open tube for air circulation in order to avoid hydration of the horny layer. When permeation into the saline was high enough to cause significant back-diffusion, the solution was changed during the penetration experiment.

Determination of drug concentrations in the skin layers. After defined periods, excess drug remaining on the skin surface was carefully removed by gentle wiping with dry cotton swabs. The skin specimen was then fixed onto a rubber stopper of suitable size and the horny layer removed by about 20 strippings with adhesive tape; it should be kept in mind, however, that total removal of this layer is not possible and some 'isles' of horny layer remain attached to the epidermal surface (2-5). The strips were each placed in the scintillation cocktail and counted for radioactivity. The skin specimen was then frozen and one or more round samples, 6 mm in diameter, were punched out and refrozen on a freeze microtome. In order to achieve slicing parallel to the skin surface, a steel ring was positioned around the specimen, having the thickness of the skin specimen. A glass slide was placed upon this ring, lightly touching the skin surface, parallel to the microtome deck surface and to the slicing direction of the knife. On warming the carrying

glass with the fingertip, it could be drawn off, the ring removed and a plane surface was achieved.

16 slices of 10 μm thickness were cut; these were regarded as epidermis. The rest of the specimen, the dermis, was cut into 40 μm slices; the thickness of the skin was determined by the number of slices. The slices were placed each into a counting vial and were hydrolized with 0.3 N NaOH for 5 hours at 80° C. After cooling, the vials were filled with scintillator cocktail containing acetic acid to neutralize the NaOH. The samples were counted in Beckman liquid scintillation counters LS 150 or LS 230.

Calculations. Taking the amount of drug applied, its specific activity and the thickness d of the layers (horny layer = 20 μm , epidermis = 160 μm , dermis = 40 μm x number slices), the concentrations of drug in the single slices and skin layers were calculated using a Wang 2200 computer. The values obtained were expressed as percent of the applied amount, as $\mu\text{g}/\text{cm}^2 \times d$ of the skin layer, as $\mu\text{g}/\text{ml}$ of the respective tissue and as μmolar concentration in the tissue.

Application in vivo. When the *in vitro* results had to be confirmed, the drug was applied *in vivo* to a skin area designed for surgical excision (mostly in the vicinity of a tumor). The time of application was so chosen that the intended penetration period could be completed before surgery. Just before excision, the surplus of drug was thoroughly removed with dry cotton swabs and the horny layer was stripped off. Immediately following excision, the treated area was frozen and a 6 mm biopsy specimen was punched out. Separation and measurement of the layers was performed as described above. Serum and urine samples were analyzed to obtain information on the amount of drug absorbed and excreted.

RESULTS AND DISCUSSION

Distribution in the Horny Layer

Barrier Function. The horny layer is the main barrier against free diffusion of substances into the organism. Because of this 'barrier' function, only small amounts of substances succeed in permeating into the living layers of the skin and the organism, even when large amounts come into contact with the skin surface. This permeating is a passive diffusion process, there is no proof for any active transport mechanism being involved in percutaneous absorption. The whole of the horny layer is regarded as being a rather homogenous penetration barrier.

This view of the penetration barrier as a property of the horny layer as a whole is supported by the following experimental results. When the amount of substance is measured in each single tape strip, logarithmic curves are obtained for most substances (Fig. 1) yielding in a half logarithmic presentation straight lines with a steep slope. Thus, the amount of penetrating substance decreases with increasing depth in a logarithmic fashion, ranging mostly over 1.5 decades (5). This logarithmic concentration gradient can be represented by a convergent geometric series with a constant multiplier of less than 1; its precise value depends on the substance and the vehicle used.

Applied to the horny layer, this implies that each single layer offers the same resistance to penetration, the effect being cumulative throughout the stratum corneum layers.

If such considerations are valid, then such a system should show the same resistance to penetration from both directions. This result was, indeed, obtained when the same substance was applied to the lower (subcutaneous) side of the skin and allowed to permeate through the skin and the horny layer, inverse penetration, see

below. In the dermis and epidermis an almost even distribution was found after an extended penetration period (Fig. 2). In the horny layer, however, a logarithmic gradient is observed as before, only now it is in the reverse direction (Fig. 3). (The accumulation in the upmost layers of the stratum corneum in Fig. 3 can be explained by swelling of the latter through contact with the saline solution). In this hypothetical view the horny layer is regarded as a multilayer system of alternating lipophilic and hydrophilic layers. The structure of the stratum corneum -- flat layers of corneocytes with their lipophilic cellular membranes still present, and a hydrophilic keratin matrix -- is in agreement with this proposed model.

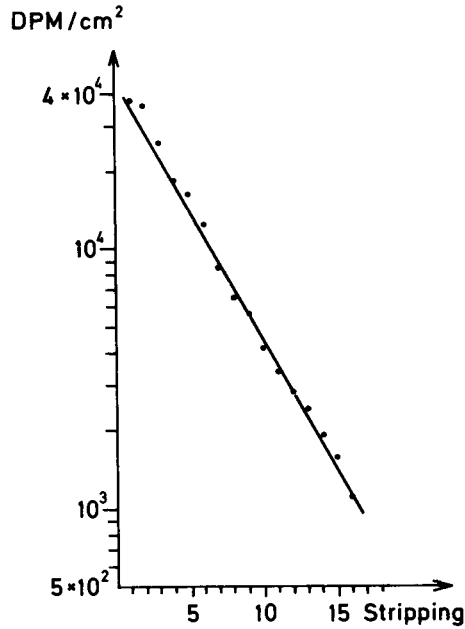


Fig. 1. Distribution of radioactivity in human horny layer after topical application of a radiolabeled (4-chlorotestosterone acetate) in vitro.

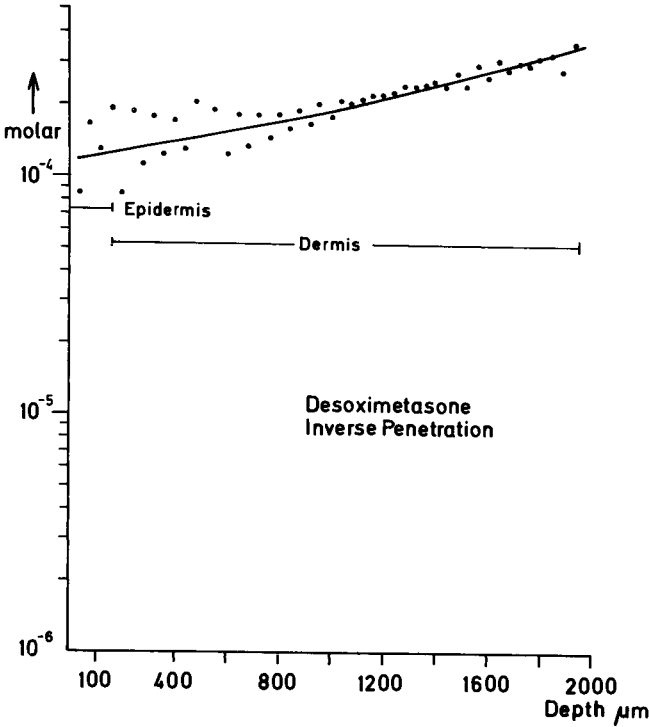


Fig. 2. Molar distribution of a corticosteroid after penetration in vitro into human skin from the subcutaneous side.

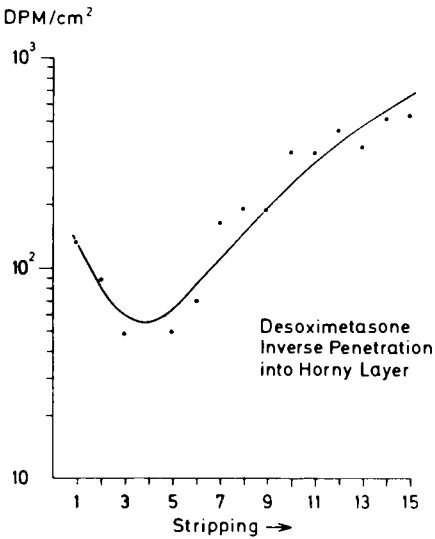


Fig. 3. Distribution of radioactivity in human horny layer after penetration of a labeled steroid in vitro through the dermis and epidermis from the subcutaneous side.

Similar stripping can be performed on psoriatic skin, i.e. on diseased skin showing distinct alterations of the stratum corneum. However, in these cases fewer (3 - 10) strippings are sufficient to reach the epidermis. The concentration gradient obtained is no more linear in the half logarithmic presentation (Fig. 4), but still demonstrates a certain barrier function, i.e. even in psoriatic skin there is no free diffusion of substances, although the resistance to penetration is much reduced.

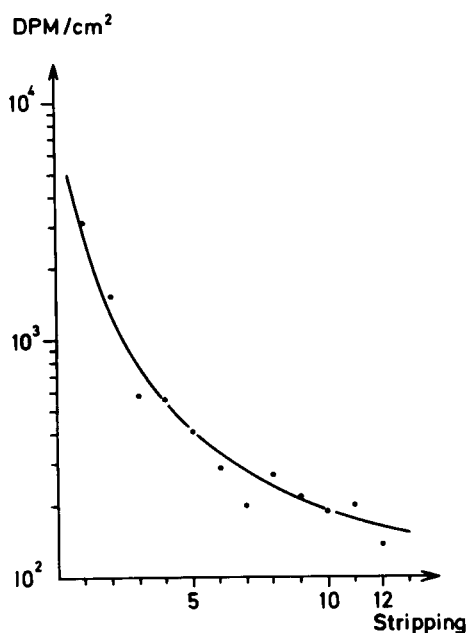


Fig. 4. Distribution of radioactivity in horny material of psoriatic skin after topical application of a labeled steroid (triamcinolone-acetonide) *in vivo*.

Reservoir Function. As a direct consequence of the barrier function in healthy (and, in part, in diseased) skin, the greater part of the applied drug remains on the skin surface and in the horny layer, even when the applied quantity is 1 mg ointment/cm² or less. This surplus represents a reservoir of the drug, permitting a steady flow into deeper layers. The reservoir function of the horny layer is the reverse of its barrier function. This reservoir can be regarded as the consequence of the adherence of the preparation to the skin surface and its presence in wrinkles and folds, as well as the result of the amount which has been taken up by the corneocytes of the horny layer by swelling.

Distribution in the Epidermis and Dermis

In the steady state of diffusion (mostly after 100-300 minutes), the distribution of the drug in the epidermis and upper dermis is represented by a rather steep concentration gradient, which in a half logarithmic plot rarely appears linear (Fig. 5); equal distribution in all layers has never been achieved. This is a consequence of the fact that the underlying dermal tissue is 10 times thicker than the epidermis, representing a relatively large distribution volume with little

restriction to diffusion in and out of the tissue, while diffusion into the epidermis is limited by the barrier function of the horny layer. Thus a three-step flux gradient pertains: The flux through the horny layer is slowest with highest concentrations in this layer, in the epidermis flux is faster establishing medium grade concentrations whereas in the dermis flux is almost as fast as free diffusion in water, with respectively low concentrations because of the resorption of the substances by the capillary and lymph systems, and/or further diffusion into the subcutaneous fat.

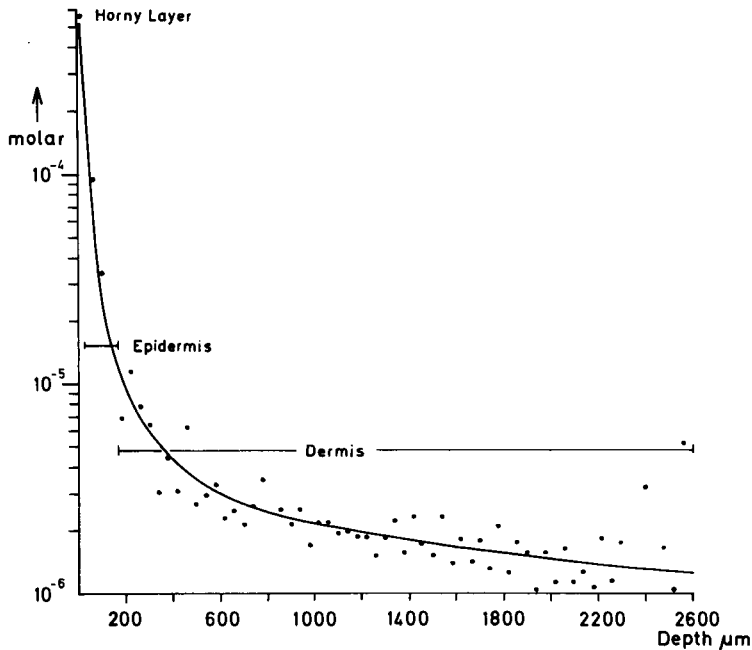


Fig. 5. Distribution of desoximetasone in human skin after topical application for 1000 min in vitro.

These two processes are illustrated in Fig. 6. After 100 minutes penetration time, high molar concentrations are achieved in the skin; however, 24 hours later these concentrations have largely disappeared and in addition, a small increase in concentration is observed in the transition region from dermis to the subcutaneous fat. In terms of pharmacological action, significant amounts are reached in most cases as early as 30 minutes after application. In both epidermis and dermis, the concentration changes slowly with most substances, since a steady flow from the reservoir maintains it nearly constant. This type of topical skin pharmacokinetics is distinctly different from that of systemically applied drugs. The drug depot sometimes desired in oral therapy, with the resulting even bioavailability, is nearly always automatically achieved in topical therapy and remains so for periods that can often extend up to 16 hours or longer.

As long as the reservoir on and in the horny layer is still present, penetration is a one way process and back diffusion can be neglected, even under steady state

conditions. In consequence, the total amount of substance which overcomes the horny layer barrier and enters into the epidermis and dermis will be, sooner or later, resorbed by the vessels. In rare cases only, small quantities can be so tightly bound to epidermal structures that in the course of epidermal proliferation they will be transported back to the surface.

As shown by Blank and Scheuplein (6), rapidly penetrating substances diffuse mainly through the cells of the stratum corneum (corneocytes). Slowly penetrating substances (i.e. electrolytes) may initially diffuse through the shunts (hair follicles and sweat glands) but later on the direct diffusion through the horny layer will become predominant, since the area of the shunts represents only 0.1% of the surface of human skin.

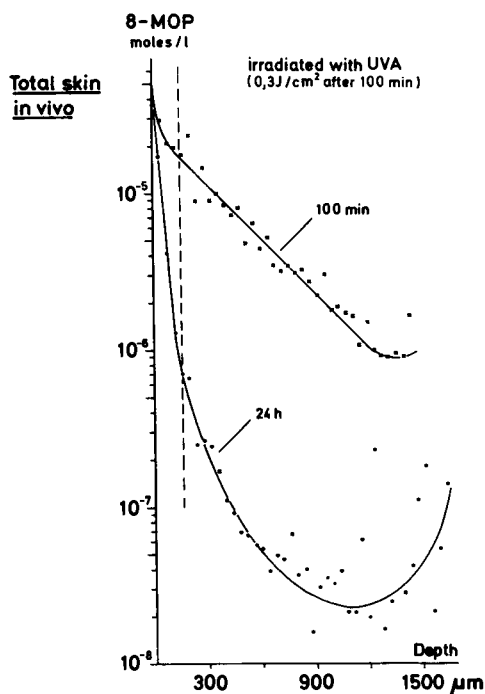


Fig. 6. Distribution of 8-methoxypsoralen in human skin 100 min and 24 hrs after topical application *in vivo*.

Penetration in Diseased Skin

In most skin diseases, the integrity of the horny layer is disturbed and its barrier function weakened (though not abolished), so that larger amounts of drugs (or foreign material in general) can migrate into the living layers of the skin (Table 1). This provides topical therapy with a considerable advantage relative to oral therapy of skin diseases: with appropriate dosage and a suitable application technique, the drug will enter almost exclusively into damaged areas in relevant

amounts. In oral therapy, on the other hand, the drug is distributed in the whole skin and in the whole body as well, though only defined skin area needs to be treated. Furthermore, small amounts of a topically applied drug may represent high concentrations in the skin because of its thinness, as mentioned in the introduction, but when percutaneously resorbed, these amounts are diluted by the whole body volume.

TABLE 1 Concentrations in the skin after topical application of 0.1% triamcinolone acetonide ointment in vivo to healthy skin and to skin of psoriatics in percent of the applied amount (upper value) and as molarity μ moles/l in the tissue (lower value)

Patient	Period of penetration, min	Healthy skin epidermis	dermis	Psoriatic skin epidermis	dermis
A	30	5.6	2.6	7.4	5.2
		16.5	1.1	21.6	2.2
B	30	0.7	0.4	7.8	2.8
		2.4	0.2	31.6	1.65
C	100	2.8	1.7	4.9	3.6
		7.6	0.7	18.1	1.95

If, however, the total skin surface is treated, i.e. about 20,000 cm², percutaneous penetration can become very significant (Table 2). If, for example, an erythrodermic psoriasis of the entire body is treated by steroid ointment, the percutaneous resorption rate can equal or surpass the rate which would be achieved by oral treatment. It has to be remembered, however, that percutaneous resorption proceeds very slowly and that in comparison to oral or intravenous therapy, the serum concentrations after this resorption remain very low, if renal function is normal (Fig. 7).

TABLE 2 Total excretion of 8-methoxypsoralen in the urine within 72 hrs after its topical application, 2 pts, upper thigh, 1000 min penetration time

A	Percent of the applied quantity: lipophilic w/o-ointment 9.60	hydrophilic o/w-ointment 4.84
B	Excreted 8-methoxypsoralen (mg) calculated on the basis of 1 m ² body surface, treated with <u>ca.</u> 30 g ointment: lipophilic w-o-ointment 28.1	lipophilic w/o-ointment 12.7
C	Oral 8-methoxypsoralen dosage administered in psoriasis therapy <u>ca.</u> 40 mg/treatment	

Besides the status of the horny layer, the localization of contact to drugs or other foreign material is of major importance for the penetration rates. Up to hundredfold higher penetration rates were observed in scrotum skin in comparison to back skin (7).

Influence of Vehicle on Penetration

The form of preparation can drastically affect the pharmacokinetic behavior of the drug. As a carrier, the physical and chemical properties of the vehicle will determine to what extent the drug can migrate into a different phase like the skin. The composition of the vehicle may affect the barrier function and thereby greatly influence the diffusion pattern. Thus the antipsoriatic agent dithranol shows excellent penetration into the skin in a vaseline ointment preparation, whereas from polyethyleneglycol as vehicle, it penetrates to a far lesser degree (8-10).

An ideal vehicle should contain a low concentration of the substance and penetration of nearly the total amount into the diseased skin. The vehicle should adhere tightly to the skin surface and the drug should be largely or totally dissolved in the base. The base should be saturated with the drug whenever possible, or should reach a saturated status on the skin by evaporation of a volatile constituent. In this way, the likelihood of preferential migration of the drug from the vehicle into the skin is increased. Since solubility is a typical property of each substance, a tailored vehicle for each drug is a practical necessity when optimal penetration is desired.

By the same reason toxic material which can dissolve in skin surface fat, i.e. lipophilic material is more likely to enter into the body via percutaneous absorption than hydrophilic electrolytes. It should be kept in mind that many pesticides exhibit appropriate physico-chemical properties for high percutaneous absorption rates.

Release of the Drug from the Vehicle

An important question is whether slow release of the drug from the vehicle is the rate limiting step in penetration. We have incorporated several drugs into some standard ointments and measured their release from these preparations through a membrane into saline as acceptor fluid. This diffusion process was found to be always faster than the penetration rates into the skin. Is the observed low penetration caused by the barrier function of the horny layer, or is nearly total retention of the drug by the vehicle the reason for the low penetration rates? A natural model to probe this question is the lower side of the dermis, which can be regarded as a physiological acceptor phase (it is, incidentally, identical with a severely traumatized skin).

When we applied Desoximetasone ointment to the lower surface of the dermis and let the drug penetrate from there into the skin, we observed a penetration of 75 to 90% of the applied quantity, whereas only 2.5 to 20% of the applied quantity permeated the normal horny layer (Table 3). These data demonstrate the usefulness of this model; animal skin can be probably used as well.

Concentration in the Vehicle

At low concentrations the penetration rates are proportional to the concentration in the vehicle. This proportionality is, however, not 1 to 1 but a doubling of the concentration leads only to 30-50% increase in the penetrating amount. At high concentration, this relation may even decrease. Solubility problems may be involved, i.e. if drugs are not dissolved in the vehicle but are incorporated in solid form, the dissolution velocity may become rather limiting.

TABLE 3 'Inverse penetration' = penetration from the dermis under-surface to the epidermis and dermis. Concentrations of desoximetasone and hydrocortisone after 1,000 minutes period of penetration as percentages of amount applied in comparison to 'normal' penetration through the intact horny layer

<u>Desoximetasone 0.05%¹</u>		
Inverse penetration (%)		Normal penetration (%)
Epidermis	3.8	14.5
Dermis	88.3	4.4
<u>Desoximetasone 0.25%¹</u>		
Epidermis	2.7	2.03
Dermis	71.0	0.41
<u>Hydrocortisone 1%²</u>		
Epidermis	3.7	0.83
Dermis	50.5	1.9

¹ In commercial ointment base

² In Eucerin cum aqua

Topical Versus Systemic Application

Essential differences in skin pharmacokinetics are to be expected between these two routes. 1) Topical treatment will achieve far higher concentrations in the skin than systemic treatment even if less drug is applied, since it enters the target organ before, not after systemic distribution. 2) As mentioned, there is a principal difference in the time course. Figure 7 shows human serum concentrations of 8-Methoxypsoralen after oral (open circles) and those after topical (closed circles) application determined by gaschromatography (11-13). The first curve achieves a distinct peak, the excretion is terminated after 7½ hours. After topical application a very flat serum concentration curve is observed and the excretion takes up to 26 hours. 3) The distribution pattern will be different in the skin. Though nobody was able up to now to demonstrate the distribution of a drug in the skin after systemic application, it will most probably resemble that after inverse penetration, (see Fig. 2), because within the dermis the diffusion velocity equals that in water, i.e. soon after general distribution an equal distribution in the dermis, or at least the lower epidermis, will be achieved. This pattern is, however, distinctly different from that after topical application of the same drug (see Fig. 5), where the epidermal concentrations are by far higher than those in the dermis. The consequences of these observations for the local versus systemic treatments of dermatoses have neither been evaluated nor even discussed.

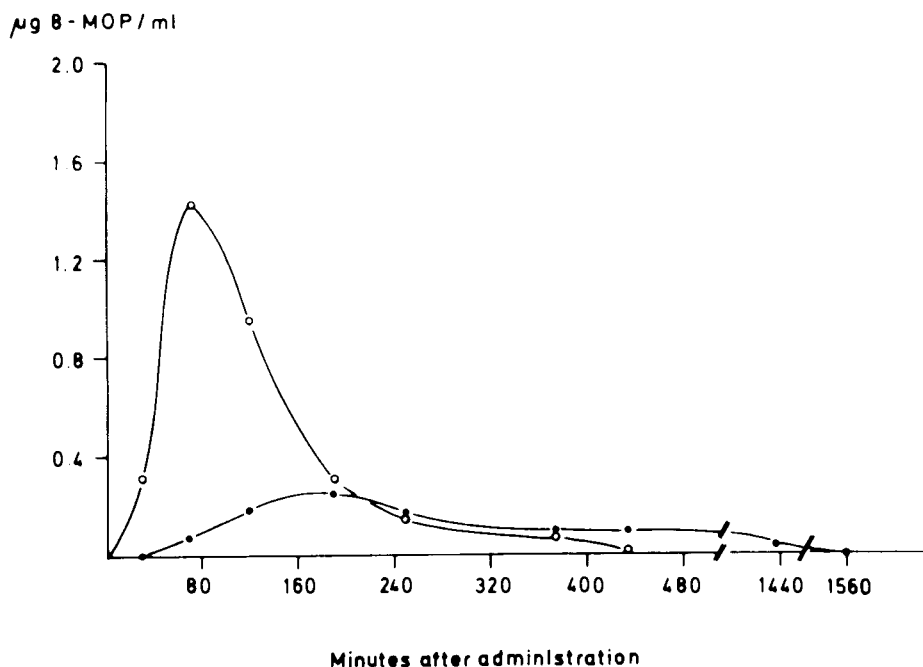


Fig. 7. Serum levels of 8-methoxypsoralen after oral application of 40 mg compared to topical application of a 0.15% emulsion to the back skin, determined by gaschromatography (13)

Percutaneous Treatment of Systemic Disorders

With growing knowledge of the principles of percutaneous absorption the possibility of treatment of systemic disorders via topical application becomes increasingly attractive. Two facts are in favor of this concept: 1. From the view of the blood system percutaneous absorption resembles a slow intravenous infusion with prolonged steady state features. Serum levels remain low, however. In consequence this mode of application is suitable for drugs which are accumulated to a certain extent at the target site. 2. The first pass effect of the liver can be bypassed. Cutaneous metabolism plays hardly any role since the metabolizing tissue (mainly the epidermis) is thin and even a high metabolic capacity per volume unit can be easily overloaded by most substances.

Among others, those preconditions have to be fulfilled for topical application of drugs for systemic diseases: 1. The drug has to be innocuous to the skin since every adverse skin reaction changes the pharmacokinetic behavior dramatically. 2. It should have a high specific pharmacologic action (response intensity/drug quantity) because normal skin permits only limited quantities to permeate. Permeation rates in the range of 100 mg/day are difficult to achieve in healthy skin, even when treating the entire body surface. 3. The substance should be lipophilic,

but not nonpolar. Electrolytes - and parafins - hardly permeate the skin since they are insoluble in one of the two (hydrophilic and lipophilic) phases of the horny layer.

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Epidermal Response to Antihyperkeratotic Agents

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Mammalian epidermis is a constantly renewing tissue in which cell production is precisely balanced by cell loss from the surface. The tissue is constructed into three main compartments which differ functionally as well as morphologically (Fig. 1).

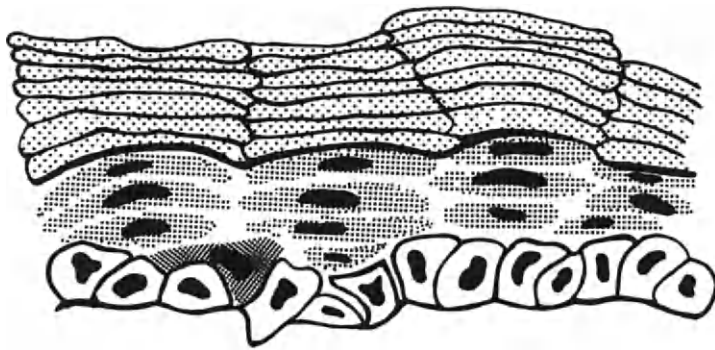


Fig. 1. Camera lucida - drawing of thin epidermis (mouse)

Cells located at the dermoepidermal junction are potential mitotic cells (basal cells). Nucleated cells located above the basal layer keratinize as they move toward the periphery (Str. spinosum + Str. granulosum). The outer layers consist of highly flattened strongly coherent anuclear horny cells (Str. corneum). This outer cell compartment functions as a protective, highly impermeable barrier against the loss of body fluids and physical and chemical damage from the environment.

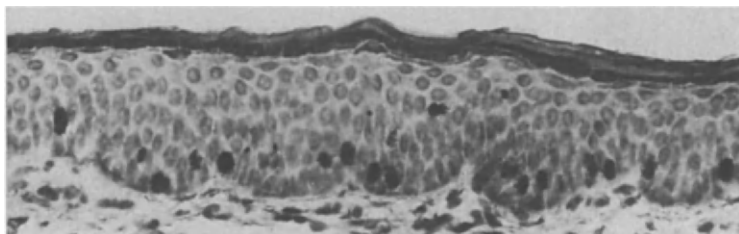


Fig. 2.a. Thicker epidermis (guinea pig ear) with ^3H -TdR-labelled nuclei 40 min. after injection of the tracer. H+E stain.

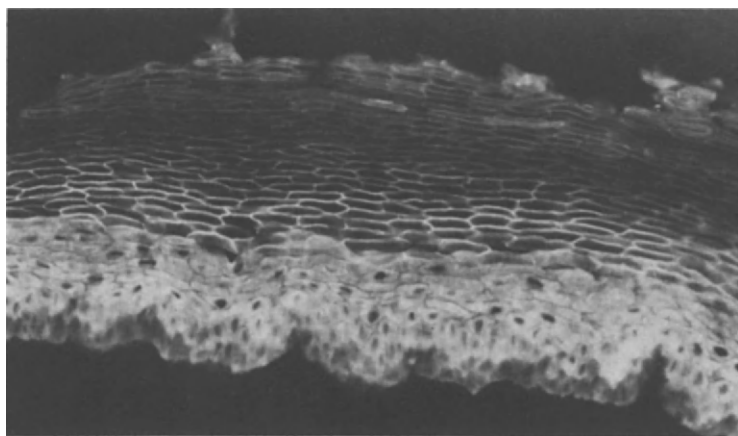


Fig. 2.b. Same specimen, frozen sectioned and stained with FITC for visualization of the horny layer.

TABLE 1 Principal changes in epidermis in response to topical agents

1. mitotic stimulation
(e.g. phorbol esters, retinoic acid, hexadecane, vaseline, low dose irritants, superficial physical trauma, UV-light)
2. mitotic inhibition
(e.g. 5 - fluorouracil, N - mustard, dithranol, podophyllin, corticosteroids of high potency)
3. altered keratinisation
(retinoic acid)
4. reduction of horny layer cellularity
(e.g. salicylic acid, resorcinol, alkaline)

Topically applied drugs may be able to affect this system in different ways (Table 1).

1. They may change the cellularity by increasing the mitotic rate in the basal layer. This is followed by thickening of the epidermis including the str. corneum. As a consequence of the increased mitotic rate, cell transition to the skin surface is accelerated and more cells will be sloughed.
2. A number of compounds inhibits the proliferative activity whereby the epidermis will become thinner and cell sloughing decreases.
3. A third mode of action consists in altering the complex process of keratin synthesis. In this way the chemical composition and the physical characteristics of the str. corneum will be changed. As in the forementioned modalities this latter aspect is of considerable importance in diseased skin.
4. A further group of substances, widely used in scaling disorders, acts on the horny layer by decreasing the cellularity.

In all of the four classes of substances a distinct, precisely focussed mode of action in epidermis would be ideal. For most topical compounds this is not the case.

In this brief report two drugs will be discussed which are extensively used in the treatment of excess scales. Although their therapeutical effectiveness is similar, they profoundly differ in the way they react with skin.

I. Salicylic acid

Since nearly hundred years salicylic acid (SA) is widely used as a "keratolytic" agent in the treatment of hyperkeratotic skin affections. Despite this, little is known concerning its mode of action in skin. Therapeutical concentrations range from 1 to 10 %, occasionally (e.g. in the treatment of callosities and warts) 40 % concentrations of SA are applied. At low concentrations SA has important bactericidal activities which are used in treating superficial bacterial infections (impetigo). Rapid penetration of the epidermis is a further feature of this compound. Significant systemic toxicity can result from long term topical application, especially in infants. Interestingly, concentrations of 2 - 12 % in aqueous cream or white soft paraffin cause no structural changes in the living epidermis as revealed by light microscopy (4). Similarly, autoradiography of SA-treated epidermis incubated with radioactive precursors (thymidin, cytidine, histidine) revealed no differences as compared to controls (10).

From these studies it appears that this drug is not significantly altering the metabolic activities of the viable epidermis. If the effectiveness of SA then solely resides in the fully keratinized layers a particular mode of action must be sought for. The way by which SA affects skin can best be studied by special horny layer stains. Incubation of guinea pig hind limbs, in which penetration from cut edges was prevented showed that the horny layer desintegrates and cells become separated (5). The experiments revealed that the loss of intercellular cohesiveness is taking place throughout the entire str. corneum. On the other hand, the viable cell layers of the epidermis appeared not to be affected. Furthermore, the morphology of the corneocytes remained unaltered with clearly stained cellular outlines. Thus the cells come apart from each other via a loss of intercellular stickiness. It should be noted that the entire str. corneum is affected, so that attempts to quantitate cell loss and establish dose-relationship were without success. Possibly, this is related to the fast rate of skin permeation of SA. One other prominent feature is the lack of topical toxicity. Also, contact allergy is extremely rare. Thereby, SA represents a suitable substance with a defined mode of action in epidermis.

II. Retinoids

The term "retinoids" includes Vitamin A and its natural and synthetic analogues. In recent years these have attracted attention because of a variety of effects in different normal tissues as well as cancer and precancerous states (3, 9). Among the derivatives, Vitamin A-acid (Retinoic acid, RA) plays an important role in skin. This compound not only proved to be of biologic significance but also became widely accepted in the treatment of hyperkeratotic disorders. The mode of action may be related to the basic processes of cell proliferation and differentiation in keratinizing epithelia. The substance furthermore elicits toxic irritant reactions upon topical application. This inflammatory reaction of the dermis is dependent upon the dose of applied RA, so that therapeutical regimens are necessary to limit this undesired side - effect.

a) DNA - synthesis

Several years ago it was shown, that topical application of RA caused a steep rise in the number of DNA-synthesizing epidermal cells in a matter of 24 hrs. (2). Continued application of the drug was followed by an increased thickening of the epidermis, the skin showed papillomatosis with greatly elongated rete ridges and numerous ^3H -TdR labelled nuclei and mitotic figures. In the treated epidermis hyperplastic changes similar to psoriatic tissue changes were present. To exclude that the enhanced proliferative activity of epidermis was not due to the inflammatory reaction of the dermis, epidermal cells were grown in vitro under the influence of RA (Table 2).

TABLE 2 Percent ^3H - TdR labelled epidermal cultured in the presence of RA

Concentration	day 1	day 2	day 3
1 ug/ml	23,2 \pm 0,61	25,9 \pm 0,58	18,4 \pm 1,52
10 ug/ml	23,8 \pm 0,62	27,7 \pm 1,51	15,7 \pm 0,75
control	2,6 \pm 1,65	5,7 \pm 1,35	13,2 \pm 3,25

Comparison of different concentrations of RA in vitro showed, that concentrations ranging from 0,1 to 50 ug/ml were effective (Table 3).

TABLE 3 ^3H - TdR incorporation after in vitro treatment of epidermal cells with various concentrations of RA for 2 days

Concentration ($\times 10^{-6}$ M/ml)	cpm
control	1081 \pm 26
0,33	3131 \pm 46
3,3	5012 \pm 54
33,0	5311 \pm 37
165,0	1057 \pm 18

In contrast to this marked effect upon epidermal proliferative activity, no stimulation of DNA synthesis was seen in dermal fibroblasts and lymphocytes treated in the same way. The amount of radioactivity incorporated into TCA precipitates showed a decrease indicating inhibitory effects of the drug in these cell types (Table 4).

TABLE 4 ^3H - TdR incorporation (cpm/culture) after in vitro treatment with RA (10 $\mu\text{g/ml}$, 2 days)

	treated	control
epidermal cells	690 \pm 105	102 \pm 13
fibroblasts	654 \pm 163	1470 \pm 895
lymphocytes	25 \pm 0,8	22900 \pm 548 ⁺

⁺ PHA stimulated

Support of the tissue specificity also comes from other work on retinoids (7).

b) Keratinization

Marked inhibition of keratin biosynthesis is a commonly noted feature of RA treated epidermis. Whereas embryonic epithelium from chick or mammals produces mucous and ciliated cells, this effect is reduced in postembryonic epidermis. When topical RA is applied to human skin or adult guinea pig skin, the differentiating epidermal cells become hypertrophic and show reduced numbers of intercellular bridges. The widened intercellular spaces contain increased amounts of

PAS-positive substances. Together with the absence of keratohyalin the str. corneum becomes parakeratotic (2). These changes last for several days of continuous treatment. With increased thickening however the granular layer eventually re-appears and the str. corneum becomes orthokeratotic. It is of interest that the cellularity of the horny layer remains low under the influence of RA. Whereas epidermal hyperplasia generally shows concomitant thickening of the horny layer, this effect is absent in RA induced hyperplasia. Instead, only a few keratinized layers remain located on the surface of the skin. Thus, several effects appear to occur simultaneously and seem to be linked to each other. These are the decrease in keratin content, decrease in cellular cohesiveness, increase of organelles (11) and increased PAS-secretory activity. The changes take place with time (days after treatment) and are dose-dependent.

Generally the loss of intercellular stickiness appears to be one of the main features of the clinical effectiveness of RA. In contrast to keratolytic agents it results from an altered differentiative biosynthesis of keratin precursors. The treatment of acne comedones or retention hyperkeratosis, both of which are due to the stickiness of keratinized cells, is based upon the effect of RA on epidermal differentiation. In addition, disorders of keratinization like Darier's disease or ichthyosis variants are likewise affected (8).

The clinical spectrum of hyperkeratotic, excessively scaling diseases constantly asks for effective treatment. The two topicals reviewed here are used since a long time. Closer look at their mode of action in skin reveals that although the target sites may differ fundamentally the clinical effects may be similar.

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Toxicological and Pharmacological Aspects of Topical Agents in Relation to the Dermis

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ABSTRACT

A new generation of topical anti-inflammatory drugs for use in the skin in which anti-inflammatory and toxic actions have been disassociated is badly needed. Studies of exudates from inflamed human skin due to a variety of injurious stimuli point to a central role for prostaglandin formation in the earlier stages of the reaction of skin to injury. However, the later stages of delayed inflammation in skin appear to be independent of prostaglandin activity. Both non-steroid and steroid anti-inflammatory drugs inhibit prostaglandin formation and the action may play a part in their anti-inflammatory activity in skin. A search for anti-inflammatory drugs with a highly specific action on the prostaglandin-forming pathway is badly needed. However, lack of data on the role of prostaglandins in the homeostasis of other cellular and non-cellular elements in the skin makes predictions regarding toxicity for putative selective inhibitors of prostaglandin formation difficult to predict.

The discovery about 20 years ago that topical hydrocortisone was a highly effective anti-inflammatory and antimitotic agent in skin revolutionised the treatment of many common inflammatory and proliferative disorders of the skin including eczema, psoriasis and occupational dermatitis. From that time to this, topical steroids have been the mainstay of routine treatment for these dermatoses. The enthusiasm with which they have been prescribed has only been matched by the enthusiasm of pharmaceutical manufacturers for synthesising variants of the steroid molecule with more or less justifiable claims to increased potency. However, like most potent drugs, the steroids possess a wide spectrum of activity in the skin including local toxicity. Especially troublesome, unwanted side effects include the production of local atrophy and telangiectasia due to the reduction of skin collagen which follows topical application of these drugs. At a more subtle but potentially more dangerous level, percutaneous absorption of potent steroids, especially when applied under polythene occlusive wrapping, and when applied to diseased skin the normal barrier function of which is weakened may occur. In such instances pituitary adrenal suppression can frequently be demonstrated and development of overt signs of systemic steroid toxicity is a well recognised complication. Despite assertions to the contrary, steroid analogues in which therapeutic efficacy has been successfully dissociated from unwanted toxicity, have yet to be devised. Industry now faces a dilemma: should further efforts be made to modify the steroid molecule in the hope of producing preparations in which the ratio of efficacy to toxicity is greatly increased, or should attempts be made to develop non-steroid anti-inflammatory drugs for use in the skin? Progress towards the latter goal has been greatly impeded by lack of formation of the molecular mechanisms involved in inflammation due to different types of skin injury. Certain non-steroid drugs have long been known to suppress inflammation in skin (1, 2, 3). It is clearly important to understand the mode of action of the drugs in order to gain clues to

their rational use in inflammatory skin disorders.

Studies of pharmacological mediators in inflamed human skin in the presence and absence of anti-inflammatory drugs are clearly of importance in this respect. We have used a suction bulla technique to obtain exudate from inflamed human skin (4).

Irradiation of abdominal skin with three times the minimum erythema dose of 290 - 320 nm ultra violet (UV-B) results in an erythema which appears at 2 h, is of moderate degree at 6 h and is maximal at 24 and 48 h. Exudate obtained at 6 and 24 h showed increased prostaglandin-like activity as identified by bioassay (5). Although the activity was maximum at 24 h, it had fallen to baseline levels at 48 h, when the erythema was still maximally intense. Radioimmunoassay for Prostaglandin $F_{2\alpha}$ showed changes in concentration which closely followed the bioassay results. Further analysis of the prostaglandin-like material was carried out by gel partition chromatography after first converting the extracted prostaglandins and related compounds to C^{14} methyl esters. This showed the presence of arachidonic acid at 6 h, reaching a maximum at 24 h and falling to control levels at 48 h. Identical changes were seen in the levels of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ at these times. Thus, although prostaglandins which were measured correlated well with the erythematous changes in the earlier stages after irradiation at 48 h, when the erythema is still intense, no prostaglandin or precursor activity is detectable. There were some differences as well as many similarities in the findings from human skin erythema due to irradiation with short wavelength ultra violet (200 - 290 nm, UV-C) (6). Abdominal skin was irradiated with six times the minimum erythema dose of UV-C. (This dosage produces a degree of erythema approximately equal to 3 times the minimum erythema dose of UV-B). Erythema was evident at 3 h, of moderate degree by 6 h and maximal at 12-24 h. By 72 h it had completely faded. Estimation in exudate samples of levels of arachidonic acid, PGE_2 and $PGF_{2\alpha}$ was carried out by combined gas-liquid chromatography-mass spectrometry using a deuterium isotope dilution technique based on preparative thin layer chromatography. Arachidonic acid PGE_2 and $PGF_{2\alpha}$ followed the same trend. Elevated levels of these agents was detectable at 6 h, 18 h and 24 h but by 48 h the concentrations approached those present in exudate from unirradiated skin. At the latter time erythema though still detectable, had greatly diminished.

The effects of the non-steroid drug indomethacin on the pharmacological and vascular responses to UV-B and UV-C are of great interest (7).

Orally administered indomethacin reduced the erythema caused by UV-B and UV-C. Topically applied indomethacin reduced UV-B erythema, but its vehicle (ethanol: propylene glycol: dimethyl acetamide 19:19:2 by vol.) had no effect on UV-B erythema. Indomethacin given orally significantly reduced the expected increase in levels of prostaglandin E_2 and prostaglandin $F_{2\alpha}$ 24 h after UV-B and UV-C irradiation. Topically applied indomethacin also significantly decreased the rise in prostaglandin E_2 and $F_{2\alpha}$. Unexpectedly the vehicle caused similar changes.

The results for arachidonic acid were of especial interest. Arachidonic acid levels were raised in both UV-B and UV-C reactions at 24 h. Oral indomethacin caused little or no rise in arachidonic acid concentrations in irradiated skin although there was a considerable increase in normal unirradiated skin. Topical indomethacin produced similar findings. However, the vehicle alone when applied topically, suppressed arachidonic acid concentration in UV-B irradiated skin below those found in untreated irradiated skin although the concentrations were still higher than those in normal, non-treated, non-irradiated skin.

In general, these findings agree well with the concept of Vane (8) that non-steroid anti-inflammatory drugs of the aspirin-indomethacin type owe their activity to their ability to inhibit prostaglandin synthetase. However, the precise relationship between UV erythema and prostaglandin activity remains uncertain. The correlation between the earlier stage of UV-B and UV-C-induced erythema and prostaglandin activity is good but in the later stages of the reaction vascular changes in the

irradiated skin remain intense despite levels of prostaglandins and arachidonic acid having returned to control values. That this later phase involves other mediators or mechanisms is also suggested by the finding that indomethacin only partially suppresses the erythema whilst at the same time reducing prostaglandin to below normal levels and by the observation that the vehicle for topical indomethacin suppresses prostaglandin formation in response to UV without influencing the overlying erythema. The possibility that prostaglandin released in response to UV have in addition to the proposed inflammatory role, a homeostatic regulatory role on epidermopoiesis also merits consideration (9).

It is instructive to compare these findings with current views on the mode of action of corticosteroids in the treatment of acute inflammation. Corticosteroids can be considered as acting at at least 3 levels: (i) inflammatory cells; (ii) blood vessels; (iii) release or formation of mediators. Neutrophilia occurs 24 h after a systemic dose of prednisolone and this appears to be at least partly due to diminution of the marginal pool of neutrophils which in turn is related to granulocyte or possibly endothelial stickiness (10). There is also substantial evidence that steroids in therapeutic concentrations can inhibit neutrophil chemotaxis (11). Corticosteroids also profoundly influence lymphocytes. Their mode of action is complex and has been reviewed by Claman (12). Steroids cause lymphopenia which does not appear to be due to lymphocytolysis. Whether or not a redistribution effect is involved remains unclear.

Although corticosteroids readily produce vasoconstriction when applied to the skin, this action is probably unrelated to their anti-inflammatory activity. Guanethidine abolishes steroid vasoconstriction which is thus probably due to sensitisation of vessels to catecholamines (13).

Are the lysosomal membrane stabilising properties of steroids described by Weissman (14) relevant to their anti-inflammatory activity? Steroids clearly enhance the integrity of lysosomal and mitochondrial membranes, probably by formation of protein-steroid-phospholipid complexes (15). That steroids interfere with prostaglandin formation, originally suggested by Greaves and McDonald Gibson (16) is now well established (17, 18). Prostaglandin biosynthesis results from release of phospholipase A₂ which in turn liberates the prostaglandin-forming substrate arachidonic acid from membrane phospholipid and the prevailing view is that corticosteroids interrupt this sequence by inhibiting release of phospholipase from lysosomes (19). Thus Weissman's original proposals take on a new significance.

One of the most troublesome toxic actions of steroids in skin results from their ability to cause atrophy and even ulceration of skin following prolonged topical application. The ability of steroids as well as non-steroid anti-inflammatory drugs to inhibit biosynthesis of skin collagen is well recognised (20, 21). Prostaglandins are known to be stimulatory on collagen biosynthesis (21). Whether or not inhibition of prostaglandin formation in skin contributes to the atrophogenic actions of corticosteroids in skin remains speculative.

Steroids like non-steroid drugs almost certainly owe their therapeutic effects to actions at multiple sites, the role of each depending upon the type of injury, cellular and biochemical components involved in an inflammatory skin lesion. However, detailed pharmacological studies of inflammatory responses, and the influences of anti-inflammatory agents on pharmacological mechanisms should do much to point the way towards new generations of topical anti-inflammatory agents with a much wider ratio between therapeutic and toxic concentrations.

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Toxicological and Pharmacological Aspects of Topical Agents in Relation to Specific Cutaneous Structures: The Melanocytes

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Irrespective of their genetic skin colour many people desire to change the function of their pigment-producing melanocytes. Some with a dark skin want to be pale and many fair-skinned long for a brown sun-tolerant complexion. In dermatology hyper- and hypopigmentation of diseased skin can result in serious cosmetic distress. In practice it is difficult to influence the melanocytes by topical applications, and there are few ways of decreasing or increasing the function of the pigment-producing cells. Nevertheless, the market abounds with products that are claimed to alter pigmentation.

When discussing the substances that can influence melanin production it is helpful to remember the key reactions involved in melanogenesis.

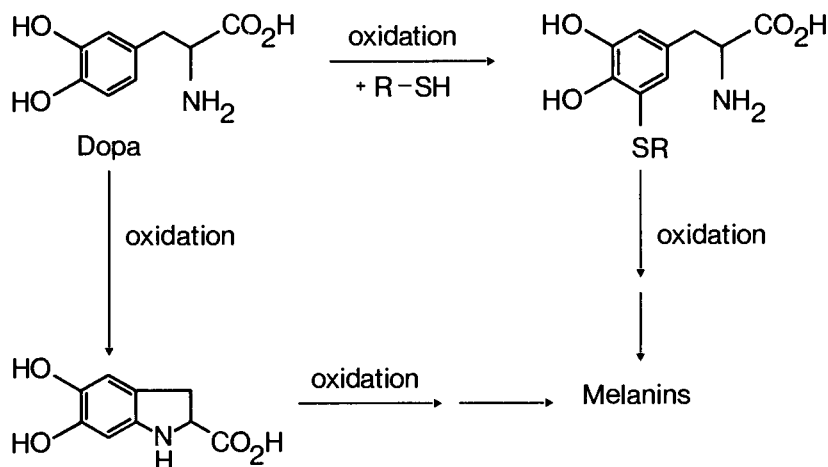


Fig. 1. Key steps in melanogenesis

The aminoacid tyrosin, which is the basic compound in the formation of melanin, is a phenol derivative. Dopa, formed by oxidation of tyrosine, is a catechol derivative. The dopaquinone, formed by the SH-containing enzyme tyrosinase, reacts with SH-groups in cysteine, glutathione or proteins.

Depigmenting Agents

The efficacy of hydroquinone and hydroquinone ethers is well documented, but not their safety. The same applies to catechols and some mercaptoethylamines. Other compounds such as ammoniated mercury, ascorbic acid, and peroxides that are currently used in skin bleaching creams on sale to the public have been scantily investigated with regard to their claimed depigmenting properties. Mercury compounds are probably tried more than other substances for inducing depigmentation, and the dogged use of such 'freckle creams' and 'bleaching creams' persists despite their doubtful results.

I shall briefly review the depigmenting effect of hydroquinone and other phenols, of catechols and of mercapto compounds.

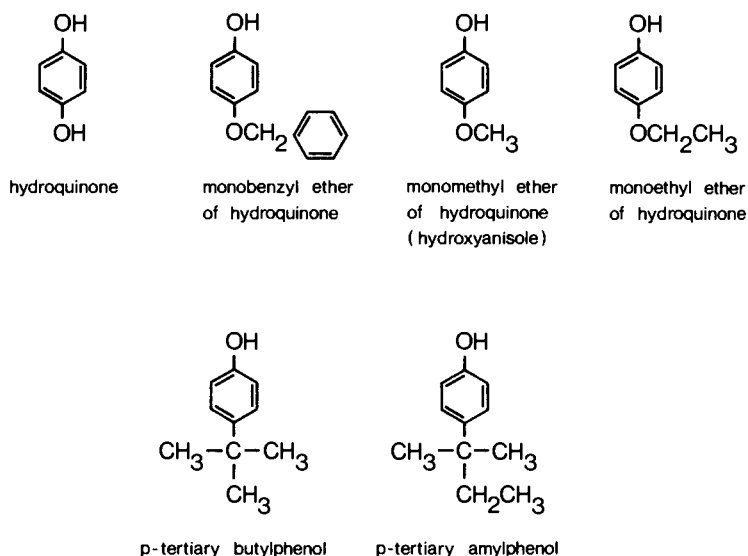


Fig. 2. Phenols with depigmenting effect

Phenols. More than 40 years ago it was found that black cats fed with hydroquinone developed grey hairs (Ref. 1). The monobenzyl ether of hydroquinone has been reported to cause occupational dermatitis in the form of vitiligo-like hypopigmentation of the hands of persons wearing rubber gloves containing this anti-oxidizing substance (Ref. 2). Monobenzyl ether of hydroquinone was also found to be an effective inhibitor of melanin-formation clinically (Ref. 3). In an experimental study on a large number of similar compounds Brun found that the monomethyl ether of hydroquinone had a good depigmenting effect. He also observed that the monoethyl ether had a more

constant effect than the methyl ether (Ref. 4). A cytotoxic effect of hydroquinone ethers on the melanocytes seems to explain their depigmenting activities. Hydroquinone itself is apparently less melanocytocidal than its ethers (Ref. 5). Of the explanations that have been put forward for the effects of the agents I have mentioned Riley's theory of the formation of free radicals from the monomethyl ether of hydroquinone is the one I find most attractive (Ref. 6).

In addition to systematically investigated compounds there are reports on depigmentation induced by occupational contact with phenolic antiseptics, p-tertiary butylphenol and p-tertiary amylphenol (Ref. 7, 8).

Catechols. Bleehen et al (9) studied a large number of catechols for their depigmenting effect, and found that 4-isopropylcatechol was a potent depigmenting agent and more active than thiols and phenols. Gellin et al (10) reported 4-tertiary butyl catechol to be an effective depigmenting agent.

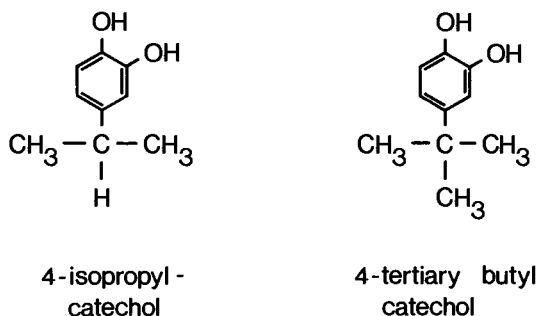


Fig. 3. Catechols with depigmenting effect

Thiols. Frenk et al (11) found that 2-mercaptoethylamine and N(2-mercaptoethyl)-dimethylamine, originally observed to have depigmenting activity in fish (Ref. 12), were both potent depigmenting agents in black guinea-pigs, apparently with a selective action on the melanocytes causing degeneration of these cells.

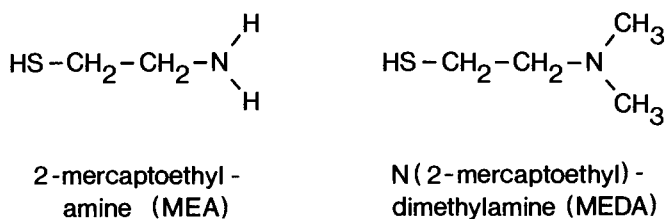


Fig. 4. Thiols with depigmenting effect

Clinical results. The clinical effects of depigmenting agents leave much to be desired.

Certain oxidizing and reducing agents alter preformed pigment in the hair, whereas the effect on the epidermal melanin is slight (Ref. 5).

Mercury products probably acting by inhibiting tyrosinase have never been subjected to systematic investigation with regard to their depigmenting effect but are widely sold over the counter. Adverse side effects are common and include kidney damage. A plea for prohibition of this 'dangerous, unproven therapy' has been made (Ref. 13).

The depigmenting mercaptoamines have a most unpleasant smell and therefore cannot be used clinically in man.

The monobenzyl ether of hydroquinone does not produce controllable depigmentation, since the leukoderma may extend beyond the area of application (Ref. 14, 15). The compound is also a sensitizer. In an editorial (Ref. 16) it was concluded that 'The therapeutic use of monobenzyl ether of hydroquinone has been responsible for a number of cosmetic disasters'. In discussing the other phenols and catechols described to have depigmenting effects, the editorial stated 'Further work on compounds that have a specific lethal effect on melanocytes is needed in order to develop a completely safe and highly effective substance for clinical use'.

Bleehen (17) recently published his 6 years' experience with the potent depigmenting substance 4-isopropyl catechol. He found it effective in selected patients with hypermelanosis, but concluded that it should be used with caution because of its irritant effect.

It has been observed that hydroquinone itself does not have some of the negative effects of its monobenzyl ether (Ref. 18), but the efficacy of hydroquinone also is less than that of the ethers. Kligman and Willis (5) have combined hydroquinone with two other types of compounds clinically observed to diminish pigmentation - tretinoin, used in acne treatment, and glucocorticoids (Ref. 19, 20). Extensive clinical trials led to the following formula,

tretinoin 0.1%
hydroquinone 5%
dexamethazone 0.1%
in hydrophilic ointment or in equal parts of ethanol and propylene glycol

Kligman and Willis noted that the depigmenting preparation did not induce disappearance of melanocytes. In contrast, the number of dopa positive melanocytes increased. The effects of this treatment are illustrated in Table 1.

TABLE 1 Therapeutic Effectiveness of Kligman and Willis' Formula

Disorder	No. of Patients Treated	No. of Patients With Satisfactory Response
Melasma	16	14
Ephelides	11	9
Postinflammatory pigmentation	18	12
Senile lentigines (hands)	7	0

The mechanism of the effect of the combined preparation is not known, but the inventors put forward interesting hypotheses. Bleeher (21) has also recently reported good results in hyperpigmentations with a similar formula.

Pigmenting Agents

The commonest stimulation of melanocytes is by ultraviolet light. Sun-screening agents of course decrease UV-exposure and stimulation of melanocytes, but can actually increase pigmentation by preventing desquamation due to an overdose of sunlight. Topical application of irritant compounds may induce some stimulation of melanocytes, but the most efficient melanocyte stimulation is produced by phototoxic substances, which need light to become reactive, phlogistic and hyperpigmenting. Many phototoxic compounds active after topical application have been identified (Ref. 22). If a phototoxic reaction, due to small amounts of the chemical or of the light, is very weak it may pass unnoticed, and may later only manifest itself as hyperpigmentation (Ref. 23).

Phototoxic reactions to creams and ointments leading to ugly hyperpigmentation are now uncommon, because the cosmetic and pharmaceutical industries are awake to the risks and perform tests to avoid the phototoxic products.

The best investigated photosensitizing compounds are psoralens (Ref. 24, 25), of which 8-methoxypsoralen (methoxsalen) and 4,5', 8-trimethylpsoralen (trioxsalen) have been more widely used clinically than the other compounds.

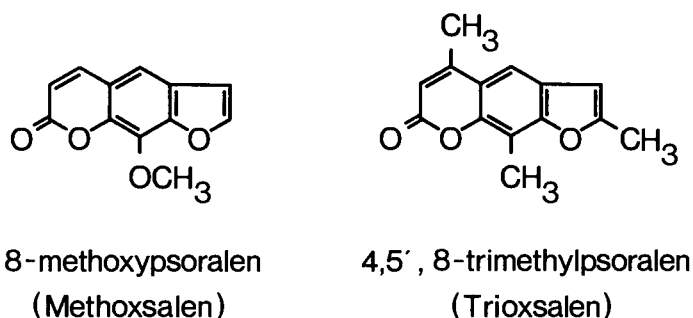


Fig. 5. Commonly used photopigmenting psoralens

The most important condition for which pigment induction would be helpful is vitiligo. El Mofty (26) at the University of Cairo introduced 8-methoxypsoralen in the treatment of vitiligo in 1947. Methoxsalen has strong photosensitizing properties after topical and oral administration, whereas trioxsalen seems to produce definite phototoxicity after topical application only (Ref. 27). There has been a general tendency to standardize hyperpigmenting treatment with psoralens for systemic administration, since the pigment response after topical applications is less predictable (Ref. 28).

By using low concentrations (0.1%) of methoxsalen topically and by carefully standardizing the light exposure, the pigment response can be kept under acceptable control (Ref. 29), and there are several reports on the efficacy of topical psoralens (Ref. 30, 31). Very low concentrations of trioxsalen, 0.5 mg/l, have been used for baths in the treatment of psoriasis (Ref. 32), but this form of therapy, reported to give an even pigmentation as end result, is of course not suitable for the treatment of facial vitiligo lesions.

Whereas psoralens in combination with longwave ultraviolet light have rapidly proved to be highly efficient for psoriasis, this treatment seems to be less useful in vitiligo. Unfortunately, we have nothing better at the moment.

It seems reasonable to hope that the intense research on melanocytes and on the effect of psoralens as photopigmenting compounds that is going on may lead to better ways of stimulating the melanin production in the skin.

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The Pilosebaceous Unit: Toxicological and Pharmacological Aspects of Topical Agents

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INTRODUCTION

The pilosebaceous unit consists of the follicular canal, the hair, the sebaceous gland, and the apocrine gland. Since there is a separate presentation on sweat glands by another author, the apocrine glands will not be discussed herein. Furthermore, since there is no information on the effect of topical agents on the follicular canal, only the sebaceous gland and the hair will be discussed.

The literature abounds with reports of the effects of systemically administered agents on both hair and sebaceous glands. On the other hand, there is more limited knowledge of the effect of topical agents on the pilosebaceous unit. The paucity of information may be related to the relative lack of experimental animal models. The hair and sebaceous glands are slow to respond to topical agents, and therefore ingestion could easily occur during prolonged applications. It is necessary, therefore, to use a model in which it is possible to check for oral ingestion. Another factor that may have served to limit the accumulation of data, as will be discussed, is the fact that the reproductive cells of these appendages are deep in the dermis, and unless histological techniques are used, indirect measurements must be made.

For practical purposes, in the human, only hormonal agents have been shown to alter hair growth or sebaceous gland function, and therefore this presentation will be limited to a discussion of their influence on these appendages. Furthermore, with hormonal agents it is impossible to define what is a pharmacological and what is a toxicological action. Hence no differentiation will be made.

METHODOLOGY FOR MEASURING HAIR GROWTH AND SEBACEOUS GLAND FUNCTION

It is necessary to understand the methodology for evaluating both hair growth and sebaceous gland function in order to place results in the correct

prospective. The available measurement parameters have some drawbacks, as detailed below.

Measurement of Hair Growth

Under certain circumstances, gross changes of hair growth may be readily apparent. For instance, if testosterone is applied to the axilla of a prepuberal child, hair growth will be induced. The change from no hair to definitely visible hair is easily seen. However, in most instances where pharmacological or toxicological products are applied to the skin, the changes will be in existing hair and, therefore, more accurate estimation of hair growth is required.

Human hair growth has been measured mainly by weighing or by measuring the length of hairs removed from a study area. The site is prepared by shaving or clipping and then the hair is removed for measurement after a finite interval. Where hair growth presents as a cosmetic defect, such methods have limited acceptance and usefulness. Furthermore, it is often impossible to accurately define the exact site or dimensions of area.

When hair length measurements are made with clipped hairs, the investigator must make certain that the skin is not depressed when the hair is cut. The same is true with shaved hair samples and, in addition, with shaved samples, one must be certain that partial clippings are not obtained. In order to overcome these drawbacks, a technique has been developed whereby after the hair is initially cut, subsequent growth is measured by passing a calibrated capillary tube over the hair in vivo (Ref. 1). In this method the measured length is dependent upon the pressure with which the capillary tube is applied to the skin. With all of these measurement techniques, the error will be proportionally greater if the hair is short. The incremental difference will be less if the hair is longer.

It has been shown that parenterally injected S^{35} cystine is rapidly incorporated into the hair shaft of animals and can serve as a pulse label for the hair (Ref. 2,3). Munro (4) has adapted this technique to human hair, using intradermally injected labeled cystine. He injected $0.05 \mu\text{c}$ of S^{35} L-cystine in 0.05 ml of isotonic saline, using 9 injections to cover a $2 \times 2 \text{ cm}$ square. Pulse-labeling was done twice at three to four week intervals. Two weeks later the hairs were clipped and processed for radioautography. By measuring the distance between the fronts of the two pulse labels, he was able to obtain a very accurate assessment of hair growth. Since the biological half-life of the isotope is very short and the use of intradermal injections limits the amount of radioactive isotope that is required, the procedure can be repeated as often as required. This probably represents the most accurate measurement procedure for hair-length measurement.

It is important to realize that the cross section of hair may change as a result of inhibition or stimulation of the hair, so that a measurement of longitudinal growth alone may not be sufficient. Furthermore, since hairs are not fully circular in their cross section, a micrometer measurement of hair thickness is not necessarily a reflection of hair diameter. The hair should be sectioned with a fiber microtome, an instrument used quite widely in the wool industry. By combining the cross sectional diameter with a measurement of length, a true measure of hair growth can be obtained and this is probably the best measurement of hair growth presently available.

Human hair growth, as in most species, is cyclic in nature with the hair progressing through a growth phase (anagen) to a resting stage (telogen). There is an intermediate stage of hair growth in between anagen and telogen known as catagen, but since this is a transient stage of hair growth, it is rare to find catagen hairs in any sample. The duration of anagen and telogen varies from area to area, and has not been fully determined in the human. As can be readily appreciated, the longer the ultimate length of the hair, the longer the period of growth. Obviously scalp hair has a longer anagen phase than axillary hair. If a large percentage of the hairs in a given area are in telogen, as is the case over most of the body, then very little hair growth will be observed over a unit period of time. The reverse will be true if most of the hairs are in anagen. Therefore, as part of any study of the influence of a topical agent on hair growth, an anagen/telogen count should be done. This can easily be accomplished by forcibly extracting a group of hairs, cutting off the roots, and floating them on water in a culture dish for examination with a dissecting microscope (Ref. 3). The roots of anagen hairs are surrounded by root sheaths and the bottom border is irregular where the hair shaft breaks off in the follicle. Telogen hairs, which have lost their attachment in the follicle, have a smooth round bulb ("club hairs") and no attached root sheaths.

Another factor that must be considered in all hair growth studies is the fact that there may be differences in the measurement parameters within an area. This has been demonstrated on the scalp, particularly in reference to hair length growth by Pecoraro *et al* (6).

Measurement of Sebaceous Gland Function

The sebaceous glands of most animals are uniform in size and estimation of gland size can be accomplished by planimetric measurements. However, in the human, the glands are not uniform in size, and planimetry would require the examination of a large number of samples. Another drawback to planimetry is that it requires repeat biopsies. It also has been impossible to measure sebum formation directly at the gland level.

Therefore, over the years many different methods of collecting and measuring sebum at the skin surface have been devised. The lipid which is collected from the skin surface is a mixture of lipid from the sebaceous glands and lipid derived from the epidermis. Therefore, not only is sampling occurring at a level distant from the gland, but the lipid is not purely of sebaceous gland origin. Because most samples have been taken from the face, an area where less than 5% of the lipid is of non-sebaceous origin, epidermal lipid is a minor component of the collected sample unless the glands are small.

There are two major factors that must be considered in the analysis of surface lipid samples. These are the collection technic and the method of analysis. Since the material that is being collected is a lipid, a lipid-absorbing material must be used for collecting the sample. Procedures that involve collection of the lipid on non-absorbing material such as ground glass are subject to suspicion since the lipid can not be collected quantitatively from the skin surface, even if short collection times are used. Since the lipid will flow away from the collection site, the area must be protected so that all of the lipid remains in place. The size of the collection area should be as large as possible in order to minimize any source of error related to the variability in follicular size. Finally, collections made from the unprotected skin ("casual samples") are subject to error due to the fact that the lipid may be inadvertently wiped away between the time of the preparation

of the collection site and when the sample for analysis is collected.

Many different methods of analysis have been used. Procedures such as monolayer film spreads and nephelometry are influenced by the composition of the sample. The same can be said for methods involving the staining of the lipids with osmium tetroxide, since only unsaturated fatty acids are stained with osmium.

Our method of collection and analysis (Ref. 7) has been in use for over 20 years and has been adapted with minor variations by other laboratories. In the procedure the forehead is first wiped clean with dry gauze sponges. Solvents are not used as we have found that solvent-extraction removes variable amounts of the surface and follicular lipids, and reproducible results are not readily obtained. The measurement area on the forehead (usually 2.54 cm x 1.91 cm--1 inch x 0.75 inch) is delineated with overlapping strips of non-perforated cloth adhesive tape. Absorbent papers (we use cigarette papers) are placed over the collection site and held in place with folded gauze squares and an elastic bandage encircling the head. After two preliminary 15 minute collection periods, which are used to reach a stable base-line, ether-washed papers are placed over the collection site and held in place for 3 hours. The collection papers are then removed and the central lipid-bearing portion is cut out so that any lipid outside the collection area is eliminated from the extraction procedure. The lipid is subsequently extracted from the collection papers with diethyl ether into tared aluminum weighing cups, taken to dryness and weighed after temperature equilibrium has been reached.

The method is time-consuming and definitely is inconvenient for the subject. However, it takes into account all of the factors outlined above: 1) the lipid is collected quantitatively from a measured area; 2) the lipid is trapped and not allowed to flow out of the collection area; and 3) all of the lipid that is collected is measured, regardless of the composition of the sample. For these reasons we consider it to be the standard method, and replacement methods should be calibrated against it.

PRINCIPLES OF ACTION OF TOPICAL AGENTS ON THE PILOSEBACEOUS UNIT

Before discussing the effect of topical agents on the hair and sebaceous glands, a few general principles should be understood. All mitotic activity in hair is localized to the hair bulb. Similarly in the sebaceous gland, new cells are generated from the basal cells at the periphery of the glands. Therefore, when topical agents are applied to the skin they must penetrate to the hair root or to the level of the basal cells of the sebaceous glands.

In the two subsequent sections the effect of topical steroids will be discussed. Steroids are absorbed through the skin but the rate-limiting area is in the stratum corneum of the epidermis, and there is no scientific evidence to support the often quoted transfollicular route of absorption. Once the steroid has passed through the stratum corneum, it is rapidly absorbed into the circulation. It is, therefore, difficult to restrict the actions of the steroid to the area of application, and when potent agents are used, as will be illustrated, a systemic effect may be observed from topically applied agents. Therefore, it is essential that control areas of the skin be examined in order to rule out a systemic effect.

Another factor that must be considered is the state of responsiveness of the specific end-organ. Since the pilosebaceous unit is very androgen-sensitive, under normal circumstances it is impossible to further stimulate either the hair

or the sebaceous gland in an adult. Thus it is absolutely necessary to use the correct subject in any study. Topical androgens will not have any stimulation in the adult male; similarly estrogens will not inhibit the sebaceous glands in an unstimulated pilosebaceous unit.

THE RESPONSE OF THE HAIR TO SPECIFIC TOPICAL AGENTS

Hair growth can be stimulated by the application of testosterone to a site where secondary sexual hair would normally occur (Ref. 8-10). For instance, hair growth can be stimulated in the axilla of prepuberal children with topical testosterone (Ref. 9), but the concentration of applied material is critical, since if too high a concentration of the androgen is applied, or the applications are continued for too long, enough material may be absorbed to induce hair development in the opposite axilla. Maquire (10) applied a 1% testosterone ointment to the arm, nasal vestibule and auditory vestibule for 9 months to 4 post-menopausal women. Coarsening of the hair occurred only on the arm.

Another susceptible area for studying the effects of hormones on hair growth is the axilla of the aged individual, and indeed, Papa and Kligman (11) have shown that hair growth can be stimulated in the axilla of aged individuals. Thirteen of 14 individuals studied by them noted an increase in hair growth when 1% testosterone propionate in hydrophilic ointment was applied to the axilla. The hairs were reported to be thicker, longer and to contain more pigment after 4 months. These authors reported lesser, but definite stimulation of hair growth with 1% progesterone in hydrophilic ointment and 0.5% pregnenolone acetate in a water and oil emulsion base. A 0.5% ethinyl estradiol ointment caused decreased hair growth in 3 of 4 individuals, and 0.5% triamcinolone acetonide resulted in marked diminution of hair growth of the axilla in 3 of 4 aged subjects.

These authors assumed that the effect of testosterone was pharmacological rather than endocrinological, and therefore they applied the same 1% testosterone propionate ointment to 21 men with male-patterned alopecia (Ref. 12). Approximately 0.5 gm of the ointment was applied daily; a control group of 21 men were treated with the ointment base alone. They reported that in 15 of the 21 androgen-treated subjects there was approximately a 10-15% stimulation of hair growth. A method of ameliorating male-patterned alopecia would naturally result in marked interest. Suffice it to say that the results have not been confirmed and nothing further has been heard of this potential cure for baldness.

THE RESPONSE OF THE SEBACEOUS GLANDS TO SPECIFIC TOPICAL AGENTS

The effect of topical hormones has been well studied in human sebaceous glands. The studies are more conclusive than those related to the hair, which probably reflects the better methodology for measuring sebaceous gland function, as well as more intensive study related to the potential use of hormonal agents in the treatment of acne. Furthermore, there is an animal model which can be used in that it is possible to differentiate between a local and systemic effect.

The prime stimulus for sebaceous gland development is hormonal (Ref. 13,14). Prior to puberty the glands are small in size. Sebaceous gland hyperplasia occurs as part of the early, if not the earliest, display of puberty and the sebaceous glands remain large until old age in the male. In women, involution of the glands occur at the time of menopause. Therefore, if one is interested in studying the effect of an agent which might stimulate the glands, the study should involve the use of either prepuberal subjects or post-menopausal women.

When topical testosterone is applied to the skin of susceptible subjects, the sebaceous glands enlarge (Ref. 13,14). It can be shown that this enlargement is a local effect in that under the proper conditions, enlargement is localized to the treated site. However, as has already been pointed out, the glands are very sensitive to androgens, and therefore even when small amounts of androgens are applied, comparison examinations of the treated and untreated site are necessary in order to be certain that the observed effect is local and not due to systemic absorption.

Because sebum, the product of the sebaceous glands, is involved in the pathogenesis of acne, and because systemic administration of estrogens in pharmacological dosages results in the inhibition of sebaceous glands, it is only natural that considerable effort has been spent on studying the topical effect of estrogens. High dosages of estrogens given locally are also capable of inhibiting the sebaceous glands (Ref. 13,14). For instance, 1% ethinyl estradiol in a cream base will cause sebaceous gland inhibition. However, in all instances, there is suppression of the sebaceous glands in the contralateral untreated site. The time sequence and the level of inhibition is the same on the treated and control sites. It would thus appear that in man the effect of estrogens is due to systemic absorption and not due to a true local effect. This conclusion is confirmed by the fact that if 1/2% ethinyl estradiol in a cream base is applied to the human skin, there is adequate absorption of the hormone, as witnessed by the appearance of gynecomastia and loss of libido in the male patient, yet there is no sebaceous gland inhibition either locally or at distant sites.

The quest for a topical inhibitor of sebaceous glands has continued and has centered around the potential use of antiandrogens, as recently summarized (Ref. 15). Animal studies have been of use in investigating the effect of topical antiandrogens on the sebaceous glands. The golden hamster has a pair of flank organs (costovertebral organs or spots) which are clearly visible and hormonally responsive. The costovertebral spots are oval, pigmented areas which measure approximately 8-9 mm in the intact male and approximately half that in the female. They contain a mass of large sebaceous glands, but in addition are pigmented. Since the size of the pigmented area will change as the size of the glands change, the effects of various agents on the costovertebral spot are grossly apparent on visual inspection. Any changes can also be followed by either histological examination or weighing of the excised gland. The pairing of the glands makes it possible to determine whether there is a local or systemic effect from a topical agent.

Burdick and Hill (16) were the first investigators to make use of the costovertebral spot for studies of antiandrogens. They did show that the response of the gland was similar to that of the human in that topical testosterone caused unilateral gland enlargement whereas topical estrogens resulted in a decrease in the size of both glands, indicating that there was systemic absorption and activity. Twenty-five mg. of a 5% concentration of chlormadinone acetate, cyproterone acetate, Δ^1 -chlormadinone acetate, Δ^1 -chlormadinone, 19-nor-chlormadinone acetate or Δ^1 -chlormadinone acetonide was applied to one flank organ five days a week for varying time periods. The vehicle itself was applied to the other flank organ. Gland response was assayed either by histologic methods or functionally by incubation with labeled acetate to determine incorporation of the label into lipids. The results of these studies were far from dramatic. The glands of the male hamsters treated with cyproterone acetate showed a questionable bilateral decrease in gland size. Two of 6 animals showed a unilateral decrease in gland size with topical Δ^1 -chlormadinone acetate, whereas in 2 of 6 animals there was a bilateral decrease in gland size. There was no response in the other 2 animals.

Another receptor antagonist that has been studied in the hamster flank organ system is flutamide (α,α,α -trifluoro-2-methyl-4'-nitro-m-propionotoluidide). Lutsky *et al* (17) did a series of elegant studies involving weighing of the glands, measurements of lipogenic potential, histochemistry and electron microscopy following the topical administration of flutamide to the costovertebral spot of intact male or testosterone-treated female hamsters. They were able to show that flutamide inhibited androgen-induced sebaceous gland growth in the costovertebral spot in a dose responsive manner, but since the untreated gland unit on the opposite side of the animal was equally inhibited, they could not prove that this was a local effect.

Flutamide and the cyproterone derivatives are antiandrogens by virtue of being competitive inhibitors for dihydrotestosterone receptors in the cell cytoplasm. On the other hand, agents such as progesterone are antiandrogens by virtue of inhibiting 5α -reductase. Voigt and Hsia (18,19) have used the costovertebral spot to study another 5α -reductase inhibitor, 4-androsten-3-one-17 β carboxylic acid. Using the female hamster, they were able to show that the topical application of this antiandrogen prevented the increase in size that normally would have occurred when testosterone propionate was applied topically concomitantly. Since the application of the antiandrogen topically with dihydrotestosterone did not block the enlargement of the sebaceous glands in the costovertebral spot, it was clearly shown that this was a 5α -reductase blocking agent.

The potential use of a 5α -reductase inhibitor in humans has been demonstrated by Mauvais-Jarvis *et al* (20) who were able to show approximately 75% inhibition of 5α -reductase activity in skin specimens obtained after 100 mg of radioactive progesterone in absolute alcohol had been applied to pubic area skin in two individuals. Since more than 70% of the applied progesterone was metabolized in the skin, they felt that this might be a potential useful antiandrogen for topical applications.

Cyproterone acetate is being used in Europe for the treatment of acne and hirsutism (Ref. 21), and it has been used experimentally in topical preparations. Winkler (22) has reported that a 1% cyproterone alcohol cream reduced sebum production in approximately 50% of his subjects. However, these studies are not confirmed by three other groups of investigators using 10% cyproterone acetate applied with 50% DMSO (Ref. 23), 1% cyproterone acetate in a cream base (Ref. 24), 1% chlormadinone cream (Ref. 25), and 5% Δ^1 -chlormadinone acetate cream (Ref. 25).

The effects of several other antiandrogens on serum production in man have been summarized by Strauss and Pochi (25). Of the compounds studied, 17 α -methyl-B-nortestosterone was most extensively studied (Ref. 25,26). The topical application of a 10% cream of 17 α -methyl-B-nortestosterone resulted in a decrease in sebum production in 14 of 21 subjects. The mean percentage for the change in sebum production was 14.4%. However, there was evidence of systemic absorption, since plasma testosterone values decreased in most of the men after topical application of this antiandrogen. There was no correlation between the level of decrease of plasma testosterone and the decrease in sebum production. Furthermore, sebum production was measured at the application site and the contralateral control site in 6 subjects, and in only one was there any decrease in sebum production on the control site. Even here, the decrease was much less (33.1% vs. 6.1%). The other topically applied antiandrogens studied by Strauss and Pochi (25), none of which had any significant effect on sebum production, included 5% 2-formyl-prednisolone-21-acetate cream, 1% lotion of clomethalone (6- α -chloro-16 α -methylpregn-4-ene-3,20-dione), and 5 and 10% A-norprogesterone cream. Therefore, at present, no topical antiandrogens have proven to be of

benefit in controlling sebaceous gland activity.

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Toxicology and Pharmacology of Topically-applied Agents on the Eccrine Sweat Gland

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NORMAL STRUCTURE AND FUNCTION

About 3 million eccrine sweat glands are distributed throughout the human skin (1). Each gland weighs about 30-40 mg so that their total weight approximates one kidney or about 100 g (2). Together these glands can produce up to several liters of fluid per hour or 10 l/day which far exceeds the secretory rate of other exocrine glands. The major function of this profuse secretory capacity is to provide for evaporative heat loss and, as a result, the eccrine sweat gland plays a major role in the control of body temperature.

Anatomically, the eccrine gland consists of three distinct components - the secretory coil, the sweat duct and an intraepidermal sweat duct unit each of which has its own unique attributes and, thus, each is affected in different ways by the action of various topically-applied agents. The secretory coil consists of three distinct cell types - large pale or clear cells between which are an elaborate series of canaliculi, dark cells containing numerous mucoprotein granules and myoepithelial cells. Because of its similarity to other fluid and electrolyte secreting cells, the clear cell probably serves as the water and electrolyte-producing cell (3). The dark cell, although its exact function is unknown, appears to produce a Schiff-positive, diastase-resistant material that lines the duct. The purpose of this material is unknown but it may assist in the reabsorption of cations. Originally, myoepithelial cells were thought to aid in the expulsion of preformed sweat through their contraction (4). However, Sato (2) has shown that Ca^{++} ionophore A23187 stimulates sweat secretion without myoepithelial contraction. Acetylcholine, on the other hand, induces both secretion and contraction. Based on these observations, myoepithelial contraction does not seem to be essential for inducing or maintaining sweat secretion. The function of the myoepithelium, therefore, is probably to maintain the structural integrity of the delicate secretory cells that produce a fluid roughly isosmotic to plasma.

The sweat duct consists of two cell types - an inner luminal cell and an outer basal cell. As the secretory product flows through the duct, sodium is reabsorbed in excess of water so that sweat appearing on the skin surface is invari-

ably hyposmotic with respect to plasma. The most distal portion of the duct undergoes tight spiralling as it traverses the epidermis making it vulnerable to a variety of topically-applied agents. The intraepidermal portion of the duct together with the surrounding epidermis has been termed the epidermal sweat duct unit (5).

A considerable mass of data, often conflicting, concerning the pharmacology of eccrine sweating has accumulated during the past half-century. However, the development of in vitro techniques by Sato (6) to study isolated sweat glands has provided the basis for much of our current knowledge. Samples collected directly from the secretory coil contain Na^+ and K^+ concentrations isotonic to plasma whereas specimens from the duct show Na^+ concentrations varying from 20-80 mM and K^+ concentrations from 5-25 mM indicating reabsorption of Na^+ and probable secretion of K^+ by the duct.

Sweating can be stimulated both in vivo and in vitro by both cholinergic and adrenergic agonists. In vitro sweating induced by mecholyl is completely inhibitable by atropine, cyanide, dinitrophenol and ouabain indicating that sweat secretion is an active metabolic process that requires $\text{Na}^+ + \text{K}^+$ -activated ATPase (6). Adrenergic agents induce a sweat rate approximately half that of acetylcholine. Epinephrine and isoproterenol are about equally effective. Neither propranolol nor isoproterenol affects the rate of mecholyl-induced sweating tending to invalidate the beta-receptor inhibitory control theory of Hemels (7).

Dibutyryl cyclic AMP 10^{-3} M induces a sweat rate in vitro about one-tenth that of cholinergic sweating but its effect is markedly enhanced by the addition of theophylline which, by itself, stimulates sweating probably via inhibition of degradation of endogenous cAMP. Phenylephrine-induced secretion is enhanced by theophylline but epinephrine-induced sweating is not. The maximum secretory rates of epinephrine plus theophylline and phenylephrine plus theophylline are similar which, according to Sato (2) indicates that there is a limit to the sweat rate inducible by cAMP that is constantly below that of cholinergic agents. However, the sweat rate induced by PGE_1 is comparable to cholinergic stimulation creating a puzzling situation since PGE_1 stimulates adenylcyclase and cAMP production (8).

As in other secretory glands, Ca^{++} seems to play a major role in sweat secretion. Apparently, an increased intracellular concentration of free calcium ions initiates exocytotic processes by mechanisms as yet unknown (9). Sato's in vitro studies (2) indicate that Ca^{++} but not Mg^{++} in the incubation medium is essential for sweat secretion. Calcium ionophore A23187 alone strongly induces sweating and this is inhibitable by ouabain but not by atropine or propranolol. Thus, it appears that an influx of Ca^{++} into the secretory cell is essential to its function and Sato proposes that the major role of acetylcholine may be the introduction of Ca^{++} into the cell as has been shown in the pancreas (10).

In order to function, the secretory coils require glucose as an energy source. Endogenous glycogen is capable of fueling the active gland for only a few minutes and Dobson (11) has observed that acclimatized sweat glands, capable of more profuse sweating than unacclimatized glands, contain less glycogen than unacclimatized glands. Glucose can be metabolized via four different pathways: 1) incomplete oxidation to lactate, 2) tricarboxylic acid cycle, 3) hexose-monophosphate shunt, 4) pentose phosphate shunt. Active glycolytic metabolism undoubtedly occurs as originally suggested by Werner and Van Heyningen (12) based on the high lactate content of sweat. Wolfe et al (13) postulated this as the major energy source because of their inability to detect oxygen utilization by the isolated sweat gland. However, this is unlikely to be correct since the application of a tourniquet to an extremity increases sweat lactate production but markedly in-

hibits sweat production (14). In addition, isolated sweat glands have a high $^{14}\text{CO}_2$ production inhibited by cyanide which increases lactate production and, at the same time, lowers sweat production (15). Thus, it appears that the oxidative phosphorylation of glucose is the main source of energy providing ATP to fuel the secretory process.

Absorption of NaCl by the duct is due to the active transport of sodium since it is completely inhibitable by ouabain (15). As in the renal tubule, sodium reabsorption by the sweat duct is facilitated by aldosterone (16). However, in contrast to the kidney, antidiuretic hormone does not influence water and electrolyte secretions by the sweat gland after systemic administration (17).

Based on these observations a reasonable model of sweat gland function can be constructed. After stimulation, oxidative glucose metabolism is activated providing ATP as an energy source for sodium pumps located within the secretory cell. Sodium is thus actively transported into the intercellular canaliculi. Water moves passively across the cell to restore osmotic equilibrium in conformance with the "backward transporting system" (18). As a result, a fluid isosmotic to plasma, is secreted into the lumen of the secretory coil. As this fluid passes through the coiled, proximal portion of the duct, NaCl in excess of water is actively reabsorbed, so that surface sweat is invariably hypotonic. However, surface sweat is more than a dilute solution of NaCl since it contains a number of organic components including protein and mucopolysaccharides that may play a critical role in events occurring within the epidermal sweat duct unit as they interact with topically-applied agents.

EFFECT OF TOPICALLY APPLIED AGENTS

When the normal structure and function of the eccrine sweat gland is considered, it is apparent that topically-applied agents can affect secretion, ductal reabsorption or can interfere with the delivery of sweat by poral obstruction. A thorough review of this topic has recently been published by Reller and Luedders (19) and can be recommended to the reader interested in details beyond the scope of this paper.

Few agents applied topically have been found to stimulate sweating probably because of the inability of standard cholinergic and adrenergic drugs to cross the epidermal barrier. 2,4-hexadienol induces sweating for as long as 12 hours due to a muscarinic-like stimulation of the secretory cells that atropine inhibits (20). Other alcohols such as 3-hexen-1-ol, 1,5-pentanediol and 1,6 hexanediol increase the rate of thermally-induced sweating (19). Organophosphorus insecticides penetrate the skin readily and induce sweating because of their anticholinesterase activity.

Since the ability to control or abolish excessive sweating has great medical and cosmetic implications, a great deal of effort has been expended in an attempt to identify agents that inhibit sweating. The major classes of compounds studied include anticholinergics, antiadrenergics, aldehydes, tanning agents and metal salts.

Anticholinergic drugs have been extensively studied. Herman and Sulzberger (21) first reported inhibition of sweating after the application of atropine and scopolamine and their observations have been repeatedly confirmed. As anticholinergic drugs became more widely available for the treatment of gastrointestinal disorders, most were tested for topical activity. Compounds such as dephemanil methylsulfate (22), propantheline bromide (23) and hexapyrronium bromide (24) have antiperspirant properties but these have proven to be of limited clinical

value. Goodall (25) and MacMillan et al (26) have studied numerous anticholinergic agents and have ranked them in order of efficacy. Of the commercially available drugs, scopolamine methylbromide, aminopentamide and dibutoline sulfate appear to be the most active. O-esters of scopolamine HBr are even more active probably due to their high skin penetrability rather than enhanced anticholinergic activity. Adrenergic antagonists have been less well studied. Of these, dibenzylamine has the greatest activity (19).

Formaldehyde and glutaraldehyde are effective in reducing the sweat rate and enjoy some popularity in the treatment of palmar and plantar hyperhidrosis (27,28). Both these chemicals produce an obstruction of the distal pore and this blockage can be abolished by cellophane stripping of the stratum corneum (27). Sato and Dobson (28) have shown that after stripping sweat gland function returns to normal indicating the superficial location of the obstruction.

Most attention, by far, has been paid to metal salts mainly because of the enormous commercial interest in axillary antiperspirants. The inorganic salts of aluminum, particularly, have been extensively studied since they are the most widely used. Several theories have been proposed to explain their mechanism of action and, until recently, this has been a subject of considerable controversy. Sulzberger et al (30) postulated that metal salts neutralized the negative charge at the distal end of the pore thus impeding sweat delivery. Others have suggested denaturation of the keratinous proteins surrounding the pore (31,32). Since the antiperspirant activity of aluminum salts cannot be removed by stripping, Papa and Kligman (33) concluded reabsorption of sweat occurred within the upper dermis.

The most widely used antiperspirant is 5/6 basic (neutralized) AlCl_3 generally written as $\text{Al}_2(\text{OH})_5\text{Cl} \cdot 2\text{H}_2\text{O}$ and referred to as aluminum chlorhydrate (ACH) or aluminum hydroxychloride. Reller and Lueders (20) have extensively studied its mechanism of action and conclude that ACH produces blockage of the distal pore by precipitation of an hydroxide gel. Their results have been confirmed (34).

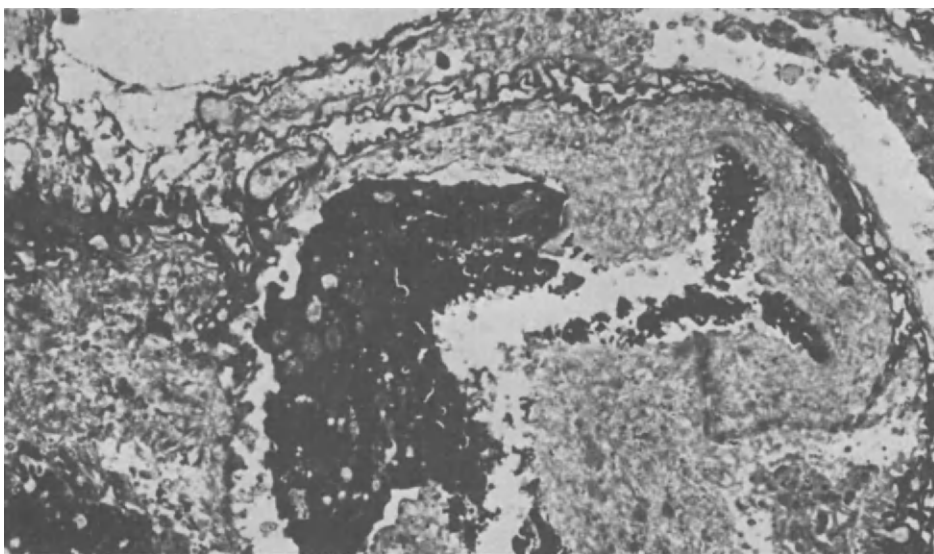


Fig. 1. Electron micrograph of sweat pore showing $\text{Al}(\text{OH})_3$ plug within lumen. (Courtesy of Gillette Research Institute).

The more acidic salt, AlCl_3 , has a similar mechanism but, in addition, appears to produce anatomic alterations within and around the pore as well as a non-specific inflammatory response which may also interfere with sweat delivery (20). After aluminum salt antiperspirants have been used for 10 weeks and then discontinued, the half-life of their effect is about 19 days. This repair process corresponds well to the time required for normal epidermal regeneration.

Although the basic mechanism of action of aluminum salt antiperspirancy seems well established, there are a number of unanswered questions. Despite the ability to produce virtually complete inhibition of sweating on the back, the same ACH preparations applied to the axilla usually produces a reduction of less than 50%. The presence or absence of sweat within the pore as well as the rate of sweating at the time of application seem to be critical factors in determining whether hydroxide gelation will occur within the pore. In addition to flow rates, the numerous components within sweat which facilitate or inhibit gelation are poorly understood. Perhaps major individual differences exist in sweat composition which determine, to a great extent, the degree of responsiveness to aluminum antiperspirants. Anatomic factors may also be significant since Sato (35) has recently shown that axillary "apocrine" glands respond to acetylcholine and produce an abundant aqueous secretion and, thus, may be responsible for a larger component of the axillary sweat output than previously believed. Since the duct of these glands empties into the pilary canal rather than onto the skin surface, it may be inaccessible to aluminum salts because of its distance from the skin surface.

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Carcinogenicity of Cosmetic Materials

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I INTRODUCTION

Definition of Cosmetic Materials

Cosmetic materials are by definition intended only to promote attractiveness ; they are not intended to affect any bodily functions (15). It is clear that : "to promote attractiveness" is one element of the human social and sexual behaviour, a point which should not be underestimated. The second part of the sentence is also clear : "Cosmetic materials are not intended to affect any bodily functions". It means that cosmetic materials must remain at the surface of the body and, must not interfere with the activity of any organ. Since the skin is also an organ, to say that cosmetic materials are not intended to affect skin functions means that these materials should be totally inert. But, inertness depends upon the parameters used for its evaluation and a compound which appears inert to the clinician may well be active at the cellular and molecular level. This is precisely the problem with carcinogens which may seem to be inactive clinically at the very time when they are inducing profound changes in cell molecules.

The Initiation-Promotion Theory of Cancer

The general idea is that initiation corresponds to an alteration of cellular DNA. Alteration of cellular DNA by chemical as well as physical agents elicits complex mechanisms which aim at repairing the altered portion of the DNA chain. An important point is that among DNA repair mechanisms some are error free such as the excision dark repair of ultraviolet radiation induced DNA damage and some are error prone such as the removal and repair of depurinated (or depyrimidinated) sites which is

triggered by most alkylating agents (*). Once its DNA has been altered the cell will have to wait for a chance to divide and develop into a clone. In normal adult tissues, these chances are rare because the rate of mitotic activity is low. In human epidermis for example no more than 4 to 5 per cent of the basal cells replicate DNA. But if promoting substances such as croton oil are applied, or if promoting factors such as chronic inflammation are at work (3) mitotic activity of epidermis increases and chances that an initiated cell divides and produces a clone increase in proportion. An example of how the initiation-promotion scheme works is furnished by recent experiments in gravid mice (29). When a potent initiating substance like Dimethyl benzantracene (DMBA) was given orally to gravid mice it was possible to induce tumors in the skin of the baby mice after their birth in using doses of promoting substances such as 12-O-Tetra decanoyl Phorbol-13-Acetate (TPA) which alone were not sufficient to induce tumors in non initiated controls. This example shows that the initiating substance does not induce clinically visible damage but increases the susceptibility of the target tissue to the promoting chemical. For this reason the initiating potential of cosmetic materials will be considered first and the promoting potential of cosmetics will be dealt with independently.

II INITIATING POTENTIAL OF COSMETIC MATERIALS

Basics in Chemical Carcinogenesis (12, 17)

According to the theory schematized above, a chemical with initiating potential is a substance which increases the susceptibility of the target tissue. This is best achieved by interacting with the hereditary cell system, that is cellular DNA. This interaction is the result of covalent binding with DNA or RNA or proteins. However, the majority of chemicals which exhibit carcinogenic potential are not able to bind directly with nucleic acids and/or proteins. They must be chemically or enzymatically transformed into reactive intermediates in order to interact with cellular macromolecules. It has been shown that enzymatic transformation of Polynuclear Aromatic Hydrocarbons (PAH) which are the best known initiating substances is largely due to the microsomal bound enzymes monooxygenases. These monooxygenases are linked to and depend on cytochrome P-450. They usually produce inactive or inert metabolites and, as such they can be considered as detoxifying agents. But it has been found recently that the monooxygenases can also behave as activating agents, responsible for the production of epoxides which are highly electrophilic metabolites susceptible to react with cellular macromolecules. These epoxides represent "ultimate carcinogens". These ultimate carcinogens can be detoxified by other cellular enzymes among which are the epoxide hydratase and the glutathion transferase. Thus, the intracellular level of ultimate carcinogens finally depends not only on the activity of the monooxygenase system but also on that of the other detoxifying enzymes. In general, the capacity of a given tissue to produce high amounts of intracellular ultimate carcinogens rests on complex enzymatic interactions. The point of interest is that these interactions vary not only from tissue to tissue but also from species to species and in a given species from individuals to individuals (14).

As regards the problem of topical applications the main question is to know whether skin cells, both epidermal and dermal are able to metabolise potential carcinogens.

(*) Miller, J.A. Course on Chemical Carcinogenesis given at the International Agency for Research on Cancer, Lyon (France) in Nov. 1977.

Metabolising Capacity of the Skin

Recent studies (19) on microsomal enzymes of dermis and epidermis have revealed that skin does contain Aryl Hydrocarbon Hydroxylase (AHH) a cytochrome P-450 dependent monooxygenase which is inducible by PAH. A dose response relationship exists between the application of PAH and the formation of papillomas and carcinomas and a correlation has been found between the tumor-initiating ability of several hydrocarbons and their ability to bind covalently to epidermal DNA *in vitro* (16, 31). The capacity of epidermal cells to metabolise potential carcinogens is not surprising since the first chemically induced cancer to be recognized in man was that caused by chimney sweeps two hundred years ago and since the first experimental skin cancer was produced sixty years ago by repeated applications of coal tar to rabbit ears. Coal tar contains numerous polycyclic hydrocarbons including benzo(a)-pyrene. In a recent study by Dr R. Bickers (personal communication) it was shown that 24 hours after topical application of a coal tar solution there was a 2-5 fold increase of AHH activity in human skin. As pointed out by Dr Bickers, quote : "this suggests that cutaneous tissue contains enzyme activity that could produce reactive metabolites capable of inducing cancer in the skin or, following percutaneous absorption, in other tissues as well".

The metabolising capacity of epidermal cells is different from that of fibroblasts. With benzo(a)pyrene human epidermal cells do not produce the same metabolites as fibroblasts. It is possible that this difference explains why carcinomas in man are more frequent than sarcomas (10).

Examples of Initiating Substances in Cosmetic Materials

The question is to know whether cosmetic materials contain substances which directly or after metabolism can react with cell macromolecules. Most of our knowledge in this field comes from the use of biological systems. There exists many such biological systems (17) but two are of especially wide use at the present time. One is the bacterial test of Ames (see ref. 23). The principle of this test is that most carcinogens are mutagens even if not all mutagens are carcinogens. Practically mutagenicity is evaluated in counting revertants in a system which uses bacteria as target cells. This is a good choice because our knowledge of bacterial genetics is relatively advanced which allows a variety of experimental situations to be developed. The other system is the induction of tumors in laboratory animals. In addition, occupational exposure to chemicals corresponding to unwilling experiments in man, epidemiology of cancer may be of help in revealing unexpected links between the incidence of certain forms of cancer and chemical compounds.

Hair dyes have been the subject of recent research since the reports by Umeda in 1955 (33) and Ito et al., in 1969 (18) of the production of tumors in the rat with M-Toluylenediamine. This was followed by Ames who reported in 1975 (1) that nine of the ingredients used in hair-dye manufacture were mutagenic to *Salmonella typhimurium* Strain 1538, the most mutagenic being 2,4 diaminoanisole. In the last few years, a study sponsored by the American National Cancer Institute (NCI) has been started for the testing of hair dye chemical ingredients in laboratory animals. Since this bioassay programme is still underway final results are not known. However, preliminary data indicate that so far nine ingredients have been found carcinogenic when administered in the feed to rats and mice. A typical example of how carcinogenicity is established in the NCI sponsored study is given by the testing of 2,4 diaminoanisole (24). The time-weighted average dietary concentration was of 0.5 per cent high-dose and 0.12 per cent low-dose for the rats and 0.24 per cent high-dose and 0.12 per cent low-dose for the mice. With high doses, and after 78 weeks of administration, in rats there was increased incidence of thyroid tumors in each sex, malignant tumors of the ear in each sex, malignant skin and preputial tumors in males ; in mice thyroid tumors were induced in each sex and malignant lymphomas in females. With low doses only was the incidence of malignant lymphomas

increased among female mice.

As regards topical application, most studies have yielded negative results (see ref. 5). However, two semi-permanent hair dyes have been shown to be carcinogenic for mice when applied topically (30). Interestingly, two strains of mice were tested and only one was susceptible to tumor induction. This suggests that highly sensitive animals must be selected for topical testing. Positive tumor inductions in animals are listed in TABLE I.

TABLE I
CARCINOGENICITY OF HAIR-DYE INGREDIENTS IN ANIMAL TESTS

ORIGIN	INGREDIENTS	TUMORS	METHOD
NCI 1978 (24)	0-nitro-p-aminophenol 2,4-diaminoanisole direct black 38 * direct blue 6 * 2-nitro-p-phenylenediamine m-toluenediamine 2-methoxyaniline 4-chloro-o-phenylenediamine 0-phenylenediamine	thyroid, ear, skin, lymphomas	Feeding MTD to rats and mice
UMEDA 1955 (33)	m-toluenediamine	sarcoma	injection-rat
ITO et al., 1969 (18)	m-toluenediamine	carcinoma (liver)	feeding-rat
SEARLE and JONES 1977 (30)	GS : 2-nitro-p-phenylenediamine 4-nitro-o-phenylenediamine Acid black 107 RB : 4-aminol-2-nitrophenol	ovary uterus skin lymphomas	Topical Applica- tion in DBAf mice.

(*) no proper hair-dye.

The American National Institute for Occupational Safety and Health (NIOSH) has conducted two epidemiological studies which point to excess cancer among cosmetologists. The first suggests an excess of cancer of specific genital sites (corpus uteri, ovaries). In the second quote : "age and race adjusted proportional morbidity ratios (PMbR's) have been constructed for 24 selected occupational groups. Elevated PMbR's were found, among cosmetologists, for cancer of the digestive organs, respiratory system, trachea, bronchus and lung, breast and genital organs. Also cosmetologists had a greater number of elevated PMbR's for specific primary malignant neoplasms than any other tabulated occupational group" (25). A different conclusion has been reached in relevant epidemiologic studies which do not demonstrate clearly an association between hair dyes and human cancer. Of particular interest is the study conducted by E.C. Hammond (**) which concludes that there was not a single site of cancer from which significantly more beauticians than non-beauticians died. Results of recent epidemiological studies are listed in TABLE II. Obviously the situation is far from clear. More epidemiological studies are in progress which aim at answering the question of possible co-carcinogenic effects of various environmental factors such as hair-dyes, tobacco smoke and contraceptive practices among others.

(**) Hammond, E.C. : Some negative findings polio, small pox, tetanus and diphtheria vaccines, beauticians and evaluation of risks. American Cancer Society - News Service Apr. 6, 1977.

TABLE II

RECENT EPIDEMIOLOGICAL STUDIES ON HAIR DYES
AND CANCER INCIDENCE AMONG COSMETOLOGISTS

ORIGIN	RESULTS
NIOSH (CIB 19) 1978	Excess of cancer (corpus uteri, ovaries, lymphomas). High PMBR's in females (digestive, respiratory, breast, genitals).
Danish Report 1977 (*)	Excess of cancer (most types) in females Higher rates bladder and lung
Hammond 1977 (see footnote **)	No excess of cancer at any site
Connecticut Cancer Epidemiology Programme 1978 (*)	No excess of cancer at any site (except acute leukemia)
Garfinkel et al. 1977 JNCI, 58 : 141-43	6 X increased risk of lung cancer in females
Menck et al. 1977 JNCI, 59 : 1423-25	2 X increased risk of lung cancer in females
Kinlen et al. 1977 Br. Med. J., 2 : 366-68	No excess of breast cancer

(*) Personal communication

In the list of chemicals commonly applied to skin as established by Maibach and Marzulli in 1977 (21), light mineral oil as cosmetic ingredient ranks fourth in frequency in the United States. In Europe the consumption of mineral oils in 1973 was over 100,000 tons of which 9,410 went to cosmetic industries (communicated by INRS, Nancy, France). Various regulations have been enforced concerning the tolerable amounts of carcinogenic polycyclic hydrocarbons in mineral oils. As a result the content of polycyclic hydrocarbons is usually low (between 0.1 to 500 μg per kg as compared to 1 to 10 g in coal tar). However, Thony et al., have reported in 1975 that the prohibition in France in 1947 of coal derived anthracenic oils as cutting oils did not result in the expected disappearance of occupational skin cancers in relation to mineral oils (32). The same situation appears to exist in the United Kingdom according to a recent survey of J.D. Everall (8). In fact the question is posed of the possible carcinogenic potential of oils containing polycyclic hydrocarbon amounts of the order of as little as 100 micrograms of benzo(a)-pyrene per kg. In addition, as pointed out by Lijinsky et al., it may be misleading to draw negative conclusions about the carcinogenic effect of materials extracted from sources such as petroleum simply from chemical analysis (20). Recent studies at the Pasteur Institute have shown that the mutagenicity of mineral oils can be evaluated in the bacterial test of Ames (M. Hermann et al., personal communication). If confirmed it would become possible for cosmetic firms to assess the mutagenic potential of the mineral oils they use by both chemical analysis and biological testing.

Another example of initiating substances possibly being present in cosmetic materials is the N-nitroso derivatives of di- or triethanolamine. It has been shown by Fan et al., in 1977 that skin care products, cosmetics, beauty aids and hair shampoos may contain variable amounts of N-nitrosodiethanolamine, a compound which was found carcinogenic in the rat at least in one experiment (9). The need for confirmation is important and urgent.

III PROMOTING POTENTIAL OF COSMETIC MATERIALS

Basics in Epidermal Growth Regulation

As said above promotion consists in increasing the mitotic activity of the target tissue. Thus, any factor which increases the number of divisions of epidermal cells has at least a theoretical promoting potential. This promoting potential is expressed by the increase in thickness of the epidermis, i.e. acanthosis. An interesting point is that acanthosis can be the result of different mechanisms such as sun exposure, stripping of horny layers and even simple massage. It is well established that 90 to 95 per cent of epidermal cells are blocked along the cell cycle (13). Eighty to eighty five per cent are blocked in the G1 phase immediately before the S phase of DNA synthesis. Ten per cent are blocked in G2. The remaining cells are in S and Mitosis. This means that five to ten per cent of epidermal cells are in cycle. Let us neglect mitosis which represent only 0.5 per cent. Since S phase is limited in time and since there are cells in S at any given time it is necessary that five to ten per cent cells leave G1 to enter S. Consequently the population of G1 cells is composed of two parts : one which is blocked and one which is in cycle. The cells in cycle represent the cycling cell pool. As a consequence of this, increase of epidermal cell divisions will depend upon one of three mechanisms : either the duration of the cell cycle decreases, or an enlarged number of cells leave the G1 phase and augment the cycling cell pool or cells blocked in G2 are freed and enter mitosis. If we know little about the mechanisms which decrease the duration of the epidermal cell cycle, we do have some knowledge of how epidermal cells move from G1 to S and from G2 to mitosis. It has been firmly demonstrated that basal non keratinizing cells produce a G2 inhibitor and that keratinizing suprabasal cells produce a G1 inhibitor (22). The capacity of basal cells to respond to these two inhibitors is very likely based on cell surface receptors able to "read" the inhibitory signals. The reading of G2 blocking signals seems to be dependent upon various factors including catecholamine and intracellular c-AMP level. The reading of G1 blocking signals is not influenced by the above factors and would be based on cell surface structures which depend upon cell maturation. Going back to the various causes of acanthosis there are some evidences to suggest that sun exposure acts through the lift of the G2 block. Stripping would induce an increase of the cycling cell pool and massage would reduce the duration of the cell cycle (4). There are many ways by which both G2 and G1 blocks can be lifted up. For example, in the case of stripping it has been proposed that suprabasal and basal cells bearing receptors being forced up to replace the lost horny cells the pool of non bearing receptor cells increases. An other example is that of the effect of TPA. Topical application of 0.016 μM of TPA on mouse skin induces a sharp increase in DNA synthesis 18 hours later (28). This can be interpreted as an interference of TPA molecules with cell surface receptors for G1 inhibitor. It is quite probable that the promotion potential of chemicals is due to a variety of interactions with the surface of epidermal cells which finally result in the alteration of inhibitory signal reading. For example when a cream is being massaged onto the skin there is possible combination of massage and penetration through the horny layers of chemicals which in reaching the surface of basal cells interfere with regulatory signals.

Examples of Promoting Substances in Cosmetic Materials

In fact, a wide assortment of materials have been shown to induce acanthosis in Guinea pig skin most of which are commonly used in cosmetic creams, milks and other formulas (2). They include paraffin oil, petrolatum jelly, castor oil, hexyl, decyl, lauric and oleic acids, fatty acids both saturated and unsaturated, sorbitol mono-laurate, oil-in-water as well as water-in-oil emulsions. Characteristically, most of these acanthotic reactions receded in spite of the continuation of topical application. This can be interpreted in saying that only transient interference with regulatory signals occurred. As a consequence of this it is a widely accepted idea

that cosmetic topical application has only a weak (if any) promoting potential. This view may be too optimistic. The fact is that new cosmetic materials are being produced which may not have only transient promoting capacity. In addition, the cosmetic market has extended in the recent years not only to more individuals, which increases the chances of unexpected reactions due to genetic predispositions, but also to much younger subjects, since skin care products are being commonly applied to the skin of infants and babies whose reactions toward even weak promoting substances have not been investigated. This last point is of particular import in view of the fact that the stimulation of DNA synthesis increases the susceptibility of epidermal cells to low-doses of carcinogens (11). In other words it is a good precaution to test new cosmetic final preparations for their acanthogenic capacity.

IV SAFETY REGULATION AND PUBLIC HEALTH

Proposals of the American Occupational Safety and Health Administration (OSHA)

The problem of the potential carcinogenicity of cosmetic materials is part of the more general question of manufactured environmental hazards. Its importance depends upon the number of consumers, actual or potential, and that of workers involved in their production. As such it is not basically different from that of potential occupational carcinogens. In terms of public health the first thing is to decide whether such or such cosmetic material is carcinogenic or not. To achieve this it is logical to demand that the most stringent experimental conditions be used to detect carcinogenicity. This is the base of the concept of the Maximal Tolerated Dose introduced by the NCI in 1976 (26). This done and if some carcinogenic potential is found it will be the task of public health services to grade the risk and decide either to ban the product in question or help the consumer make a decision by proper diffusion of information. A basis for dealing with known or suspected occupational carcinogens has been recently provided (27) by the American Occupational Safety and Health Administration (OSHA). In this important document a potential occupational carcinogen is defined as follows : any toxic substance which (1) causes at any level of exposure, or dose, as a result of any oral, respiratory or dermal exposure, or any other exposure which results in the systemic distribution of the substance under consideration in the organism tested, an increased incidence of benign or malignant neoplasms, or a combination thereof, in (i) humans, or (ii) in one or more experimental mammalian species, or (2) in a statistically significant manner decreases the latency period between exposure and onset of neoplasm in (i) humans, or (ii) in one or more experimental mammalian species. OSHA proposals include the classification of all toxic substances in 4 categories. In category I would fall all substances that meet the definition of potential occupational carcinogen given above in (i) human, or (ii) two mammalian test species, or (iii) a single mammalian species, if those results have been replicated in the same species in another experiment or (iv) a single mammalian species if those results are supported by short-term tests. Also any substance for which the Secretary considers there is sufficient evidence to classify as category I. In category II : any substance defined as a potential occupational carcinogen in tests on animals or humans, but for which the evidence is considered only "suggestive". Also, any substance that meets such a definition in an unreplicated experiment in a single test species, and any other substance for which the Secretary considers there is sufficient evidence to classify as Category II. Category III : any toxic substance which is used in the US workplace, but which is not classified in Category I or Category II. Category IV : foreign toxic substances, i.e., those not found in the US workplace. As commented by D. Dickson, quote : "the new proposals have been widely welcomed by many members of the Scientific Community as well as trade unions and environmentalist groups as an appropriate response to the current lack of precise knowledge about the mechanisms of carcinogenicity, and to increasing evidence of the dangers of low levels of exposure which were previously considered safe" (6). There may be changes in OSHA's proposals. However, these changes are likely to remain minor and no major shift in

philosophy is being expected.

Some Questions Raised by Industry

The chemical industry raises several important scientific issues. One of them deals with the concept of the Maximal Tolerated Dose. As pointed out by L. Golberg (***) the organ damage observed at high doses may bear little or no relationship to the nature of the physiological perturbations induced by the compound at doses approximating those of human exposure and, quote : "Is the object of tests for carcinogenic potential to produce cancer under the most extreme conditions than can be devised ? Or are we seeking a rational animal model that constitutes a reasonably accurate representation of the human population"? On a general basis, industry is arguing that due to the many scientific uncertainties which exist at the present time, the only rational approach would be to firmly establish scientific knowledge including quantifiable parameters such as relative potency of different carcinogens. Specific questions are raised by the cosmetic industry. Thus, the carcinogenic effect of hair dye ingredients tested in the NCI sponsored assay programme has been detected only by feeding high doses of chemicals to rats and mice, a situation which does not correspond to reality since hair dyes are applied topically. As regards oxidation dyes, only topical application of freshly prepared model dye systems including addition of hydrogen peroxide would be appropriate (5) a condition which is not fulfilled when ingredients alone are mixed with rat or mice feed. In this regard, one must admit and regret that too little work has gone into the development of model systems to measure the carcinogenic effects of cutaneously applied substances.

Practical Regulation and, a Word to the Public

On practical grounds, statutory requirements respecting premarket clearance are currently limited primarily to pesticides, drugs, and food additives. Clearance of cosmetic products by public health services prior to marketing is not mandatory in any country. In the American Cosmetic Safety bill it is required that cosmetic products be tested before they are marketed but test results need not be submitted to the Food and Drug Administration for premarket clearance. However manufacturers are obliged to retain test data and such other information as may be necessary to substantiate the safety of their products (7).

Last but not least what should the public think of the carcinogenicity of cosmetics ? First it should understand that if some cosmetic materials include chemical ingredients with carcinogenic potential, this potential is very weak as evidenced by the fact that high doses or highly susceptible animals have to be used to reveal it. Furthermore the right move has already been taken. Thus, as regards hair dyes, the carcinogenic compound M-Toluylenediamine is no longer in use and 2,4 diaminoanisole is in the process of being replaced. Second it should realize that virtually all substances present a hazard under appropriate conditions and concentrations (15). Carcinogenic risks do not come only from chemical industry. Besides cigarette smoke, carcinogenic agents are naturally present in the environment including bacterial as well as viral agents, fungi, certain non-radioactive inorganic compounds, products arising from interactions of organic and inorganic compounds, products derived from physical decomposition of organic cellular components, not to speak of ultraviolet light and other radiations. This does not give the right to industry and in particular to cosmetologists to increase already existing risks since the combination of weak carcinogens has yet unpredictable effects, but this means that the consumer must face the problem of environmental carcinogenicity in its entirety and not in a

(***)GOLBERG, L. : Introductory remarks to the first International congress on Toxicology Symposium on Rationale for high-dose exposure held on Thursday March 31, 1977.

prejudiced and/or emotional way. Two things can be done by the public : one is to maintain pressure to assure (i) that cosmetic materials with potential carcinogenicity be replaced by nonhazardous ingredients and (ii) complete, impartial information be available regarding the carcinogenic potential of cosmetic products. The other is to learn how to interpret biological pieces of information and accept the fact that biological knowledge is always relative. In this respect, man remains in permanent quest of truth.

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Toxins Acting at the Presynaptic Part of the Neuromuscular Junction

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The great potency and specificity of the actions of toxins have always fascinated and stimulated scientific imagination. It is, however, not until recently that advances in protein chemistry have allowed the purification and isolation of the active principles from toxins and thereby laid the foundation for their use as tools in biological research. Particularly has this been the case for toxins which affect neural function and which therefore are named neurotoxins. During the last congress in Helsinki a symposium was devoted to "Toxins as tools in receptor studies". At the present congress I have been asked to review toxins which affect presynaptic functions. Presynaptically acting neurotoxins are of potential interest since they may help us to understand the mechanisms involved in the storage and release of chemical transmitters. Furthermore, they can be used as tools in studies on the nature of neurotrophic influences.

The presynaptic neurotoxins to be discussed are:

1. Notexin, taipoxin, β -bungarotoxin and crotoxin. These are neurotoxins isolated from the venoms of snakes belonging to the families Elapidae (respectively tiger snakes, taipans, sea snakes and rattle snakes).
2. Neurotoxins produced by the microorganism Clostridium botulinum.
3. Neurotoxic principles of the venom of black or brown spiders (Genus Latrodictus).

The snake venom neurotoxins and botulinum toxin act selectively on cholinergic nerves blocking the release of acetylcholine while the main effect of spider venom neurotoxins is to cause a massive release of transmitter from the nerve irrespective of the type of the chemical transmitter involved.

Before discussing these neurotoxins I will give a short schematic presentation of current views of the mechanism for transmitter release at the cholinergic neuromuscular junction (Fig. 1). Transmitter release is triggered by the entry into the axoplasm of calcium ions through voltage dependent channels in the axolemma which are activated by the depolarization caused by the nerve terminal action potential. Calcium ions by some, as yet, unknown mechanism allow the synaptic vesicles, which contain acetylcholine, to fuse with specialized active zones in the axolemma. The acetylcholine content of the synaptic vesicle is released into the synaptic cleft during vesicle fusion. The vesicle membrane is incorporated into the nerve terminal membrane and vesicles are reformed by subsequent endocytic invagination and budding off of new vesicles from the nerve axolemma. The reformed vesicles are filled with in the axoplasm synthesized acetylcholine. The free calcium inside the nerve ending is removed by being taken up by intracellular calcium binding organelles like mitochondria.

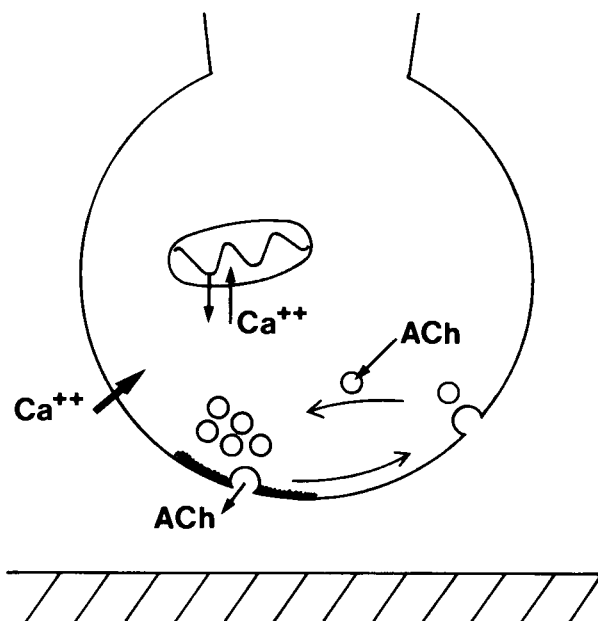


Fig. 1. Schematic presentation of events involved in transmitter release. For explanation, see text.

PRESYNAPTIC SNAKE VENOM NEUROTOXINS

The chemical composition and molecular size of these neurotoxins varies but all of them have in common, as an essential part, a very basic and catalytically active phospholipase A_2 (Ref. 1). Typically this is a single peptide chain of about 120 amino acids cross-linked by 7 disulfide bridges. The phospholipase A component may, or may not, be complexed with acidic, neutral or basic protein constituents. The neurotoxicity resides in the phospholipase part of the molecule but, when present, the other subunits greatly enhance the potency of the toxin. However, other phospholipases A_2 such as those from mammalian pancreas and bee venom lack neurotoxicity.

The lethality of the snake venom neurotoxins varies between LD_{50} values of 2-80 $\mu\text{g}/\text{kg}$ body weight. Taipoxin ($LD_{50} \approx 2 \mu\text{g}/\text{kg}$) is the most potent animal neurotoxin known. Their neurotoxicity seems to be limited to cholinergic synapses, death resulting from respiratory paralysis.

When injected into an animal or applied to a nerve-muscle preparation *in vitro* the toxins gradually block spontaneous as well as evoked release of neurotransmitter, a latency period of about 60 min preceding paralysis. Nerve activity with release of transmitter shortens the duration of the latency period while increasing the dose of the toxin has relatively little effect on the time required to block neuromuscular transmission (Ref. 2-5). Characteristically, dose-response curves for these toxins are linear on a bilogarithmic scale (Ref. 6).

During the latency period the toxins are irreversibly bound to nerve terminals,

washing the preparation with toxin-free solution fails to prevent the development of neuromuscular block and similarly the duration of the latency period is unaffected. Before a neuromuscular block develops spontaneous transmitter release, recorded as miniature endplate potentials (mepps), may be affected. The nerve terminals appear initially to become "labilized" since short bursts of moderate increases in mepp frequency and some giant mepps are observed (Ref. 2-7, 34). Evidence has also been obtained that the toxins are immediate and potent inhibitors of the high-affinity choline transport system in nerves (Ref. 8).

Apparently the toxins have a selective affinity for cholinergic nerve terminals. Ziegler (9) observed that taipoxin irreversibly blocked the effects of vagal stimulation on the isolated nerve-atrium preparation of the guinea pig heart leaving the effects of sympathetic stimulation unchanged. Abe, Alema and Miledi (10) have reported that β -bungarotoxin even at high concentrations failed to block transmission at the locust leg neuromuscular junction where glutamate is supposed to be the transmitter nor did the toxin block transmission at the giant synapse of the squid where the unknown transmitter is not acetylcholine.

Species differences exist regarding the susceptibility to the toxins. For instance, taipoxin is 3 times more potent than β -bungarotoxin and 5 times more potent than crotoxin in blocking the mouse nerve-diaphragm preparation but 30 and 100 times less potent, respectively, than crotoxin and β -bungarotoxin in the chick biventer muscle (Ref. 11).

Ultrastructural examination (Ref. 12, 4) of affected motor endplates show that in the early stages of intoxication in vivo the majority of the nerve terminals have a normal structure. Some, however, exhibit increased numbers of Ω -shaped axolemmal indentations and a slight reduction of synaptic vesicle number and some vesicles with larger than normal size. In the later stage of intoxication, i.e. at the time of paralysis, the presynaptic portion of the junction shows striking alterations. The synaptic vesicles are markedly reduced in number and the remaining vesicles show a wide variation in size. The axoplasm has a granular structure with greatly increased electron density. The mitochondria are swollen and disrupted. No increase is observed in the surface area of the axolemma which in fact appears reduced in mass. Similar observations, have been made on cholinergic terminals in the adrenal gland of affected mice, while nerve terminals from the iris of these animals, which belong to the sympathetic nervous system, showed no alterations (Ref. 13). Very high doses of the toxins (20-100 $\mu\text{g/ml}$ of β -bungarotoxin) causes in vitro disintegration of motor axon terminals and their replacement at the endplate by the Schwann cell (Ref. 14, 15).

With the exception of β -bungarotoxin these neurotoxins have a myotoxic action on mammalian skeletal muscle. A single intramuscular injection results in an acute necrotizing myopathy of surrounding muscle fibres. After the acute phase of muscle necrosis muscle regeneration is swift and complete (Ref. 16, 17).

Possible Mode of Action

From the aforementioned description it is obvious that the presynaptic snake venom neurotoxins have a complex mode of action affecting a number of processes at the cholinergic synaptic junction. Despite of that, it seems worthwhile to attempt to present, at least, a provisional hypothesis for their mode of action.

The phospholipase A activity of the molecule is essential for the toxicity but there is no correlation between catalytic activity and toxicity (Ref. 1). The other subunits of the molecule are non-toxic but enhance toxicity, probably by shielding the basic phospholipase from binding to non-specific acidic tissue sites (Ref. 18). The neurotoxins bind rapidly and with low reversibility to cholinergic nerve ter-

minals. At the same time is the high-affinity choline transport system inhibited which might indicate that this protein serves as the binding site for the neurotoxin in the axolemma. Binding to the axolemma has initially little effect on neuromuscular transmission, only a slight to moderate increase being observed in mepp frequency. This effect on spontaneous transmitter release could result from a small depolarization of the resting membrane potential of the nerve terminal. However, no major hydrolysis with rupture of the axolemma occurs at this time since massive release of transmitter is not observed (compare the effects of spider venom neurotoxin). When transmitter release gradually declines and ceases the most marked alterations are observed inside the nerve terminal. The axoplasm is electron dense and granular, the number of synaptic vesicles greatly reduced, mitochondria swollen and disrupted. This suggests that the toxins enter the axoplasm and only thereafter exert their hydrolytic action on membrane constituents. A hydrolysis which would be hastened by the entry of calcium ions during nerve activity since calcium is an activator of the enzyme (Ref. 19). Since the toxins apparently gain access to the axoplasm without rupturing the axolemma it appears that they enter the terminal by an endocytic mechanism. As pointed out endocytosis is a prominent phenomenon in nerve terminals accounting for the recycling of synaptic vesicle membrane during transmitter release. If the toxin molecule entered the terminal by the endocytosis accompanying vesicle recycling the dependence of onset of paralysis on the rate of transmitter release would be explained. The myotoxicity could be similarly explained since it has been shown that macromolecules are internalized in skeletal muscle fibres by endocytic activity of the transverse tubular system (Ref. 20).

A direct interference by the toxins with the formation of synaptic vesicles is another possibility (Ref. 4). When the pre-existing store of vesicles is depleted by neural activity a transmission block results. Under those conditions, however, one would expect an increase in axolemmal mass (compare the effects of spider venom neurotoxin) and this is not observed.

BOTULINUM TOXIN

These neurotoxins, which are components of the toxin produced by Clostridium botulinum exist in several immunologically distinct forms designated types A-F. They are proteins with a molecular weight of approximately 150 000 with no phospholipase or proteolytic activity (Ref. 21). Botulinum neurotoxin is the most poisonous poison with an LD₅₀ of the order of 5 ng/kg body weight (Ref. 22).

Botulinum toxin causes a selective paralysis of cholinergic synapses by blocking the release of acetylcholine from the nerve terminals. The action of the toxin is long-lasting, several weeks and therefore in principle irreversible. Transmission is reestablished as a result of nerve sprouting and the formation of new synapses.

Botulinum toxin blocks transmitter release without interfering with the synthesis or storage of acetylcholine nor are the ultrastructure of the nerve terminal or the number of synaptic vesicles altered (Ref. 23). As with snake venom neurotoxins paralysis is preceded by a latency period during which the toxin binds irreversibly to the nerve terminal. Nerve activity with resulting transmitter release shortens the duration of the latency period (Ref. 24, 25). The toxin has been shown to enter the nerve terminal axoplasm and to be transported by retrograde axonal flow since after the intramuscular injection of ¹²⁵I-labelled toxin a significant accumulation of radioactivity is present in the ipsilateral but not in the contralateral half-segment of the spinal cord (Ref. 26, 27).

Electrophysiological examination (Ref. 28) of an intoxicated muscle reveals that spontaneous transmitter release (miniature endplate potentials, mepps) as well as

transmitter release evoked by nerve impulses (endplate potentials, epps) are reduced to a level of less than 1% of normal. Raising the extracellular calcium concentration from 2-16 mM increases, in a dose-dependent manner, the frequency of mepps in normal muscles but not in botulinum toxin poisoned ones. Similarly procedures which are believed to moderately raise the intracellular calcium level in the nerve terminals, i.e. exposure to potassium free solution or to ouabain increase mepp frequency in normal but not in poisoned muscles. When the calcium-ionophore A 23187, which allows the passage of calcium ions through biological membranes, is added to the bathing medium a massive release of transmitter occurs from normal nerve terminals but not from botulinum toxin poisoned ones. However, if the extracellular calcium concentration, in the presence of the calcium-ionophore, is raised to 4 mM or more a high rate of transmitter release is seen also from botulinum toxin poisoned nerves. These observations indicate that in botulinum toxin poisoned nerve terminals the transmitter release mechanism has an elevated threshold to calcium ions but can release large amounts of transmitter provided that a sufficient amount of calcium is allowed to enter the axoplasm by the use of a calcium-ionophore and a high extracellular calcium concentration.

The extracellular calcium concentration which causes a certain level of transmitter release in response to nerve impulses is similarly considerably higher at botulinum toxin poisoned endplates than at normal ones. The slope value for calcium dependence of evoked transmitter release is about half that at normal endplates (Ref. 28). Drugs which affect voltage dependent calcium channels in the nerve terminals and thereby greatly increase the amount of calcium entering the axoplasm during an action potential antagonize the neuromuscular block produced by botulinum toxin (Ref. 29, 30). Tetraethylammonium and guanidine in concentrations of about 3 mM restore neuromuscular transmission to a normal level while 4-aminopyridine has a similar restorative effect but is about 20-30 times more potent. *In vivo* 4-aminopyridine is particularly effective, the intravenous injection of the drug temporarily restoring neuromuscular transmission in the rat from complete paralysis to normal as shown by the recording of normal twitch and tetanic tensions in the muscle. When rats are given a lethal amount of botulinum toxin, 4-aminopyridine is able to restore posture and general motor activity, the effect lasting 1-2 hours.

Possible Mode of Action

The results presented suggest that in botulinum toxin poisoning the mechanism for synaptic vesicle fusion with the axolemma, i.e. vesicle exocytosis with release of acetylcholine, has an increased requirement for calcium ions but is otherwise intact. Once, the intracellular calcium concentration in the nerve terminal is raised to a sufficiently high level by the use of a calcium-ionophore and high extracellular calcium or one of the presynaptically acting drugs (tetraethylammonium, guanidine or aminopyridines) transmitter release occurs in a manner similar to that in normal nerve terminals. Botulinum toxin has been shown to enter the nerve axoplasm. Maybe, the toxin after binding to the release sites for cholinergic synaptic vesicles is taken up into the axoplasm by the endocytosis responsible for vesicle membrane recycling. That would explain how this large molecule (m.w. appr. 150 000) could gain access to the interior of an axon and why nerve activity with transmitter release increases the rate at which a neuromuscular block develops in poisoned animals. However, it does not explain how the toxin, after being internalized, affects the calcium dependence of transmitter release. This is in itself not so surprising since, at present, precious little is known about the mechanism by which calcium ions initiate transmitter release.

LATRODECTUS SPIDER VENOM NEUROTOXINS

This spider venom is a complex mixture of many proteins with varying modes of

action. From the venom has a neurotoxic component, the so-called B₅ fraction with a molecular weight of approximately 130 000 been isolated (Ref. 31, 32). This component which is devoid of phospholipase or proteolytic activity, seems to account for the majority of the neurotoxic action of the crude venom. The toxicity is in the order of LD₅₀ = 0.5 mg/kg body weight.

When added to a synapse the toxin rapidly, within minutes, evokes a massive increase in the rate of spontaneous transmitter release, mepp frequency increasing up to 1000-fold in skeletal muscle (Ref. 33). The increase in rate is not antagonized by an absence of extracellular calcium or an excess of magnesium ions. The toxin also causes a massive release of transmitter from botulinum toxin poisoned nerve terminals (Ref. 28, 34). Apparently the neurotoxin changes the properties of the nerve terminal membrane in such a way as to promote explosive release of transmitter. The toxin has this mode of action on all kinds of chemical synapses irrespective of type of transmitter.

Morphological examination (Ref. 35) shows that the toxin causes synaptic vesicles to fuse with the axolemma and finally the disappearance of synaptic vesicles. The terminal now contains all of its usual components with the exception of the synaptic vesicle, the cytoplasm appearing less electron dense than normal. Concomitantly with the loss of vesicles by their fusion with the axolemma there is a great increase in the surface area of the nerve terminal membrane.

Possible Mode of Action

It has recently been shown that the neurotoxic principle enters artificial lipid bilayers and forms channels which are highly permeable to cations (Ref. 36). If this happened in the axolemma of nerve terminals it would account for the physiological and morphological effects observed (Ref. 34). The increased permeability to cations would allow sodium and calcium to enter the terminal. Depolarization and entry of calcium will enormously increase transmitter release, i.e. mepp frequency. Also, in the absence of extracellular calcium the entry of sodium ions would displace bound calcium from intracellular binding sites and thereby increase the rate of transmitter release. Since in the presence of the neurotoxin synaptic vesicles are not reformed one must assume that the toxin also blocks the mechanism responsible for endocytic retrieval of vesicles. It has been shown that the toxin in neurons interacts with cytoplasmic assemblies of microtubules and microfilaments i.e. with structures believed to be associated with endocytosis (Ref. 36). Unlike synthetic cationic ionophores, like A 23187, the neurotoxin is specific for neurons, glial cells, fibroblasts, macrophages and muscle cells are not affected (Ref. 36). The specificity of toxin action presumably resides in a differential toxin binding to lipids or proteins present only in the preterminal axolemma.

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