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Genotoxicology of N-Nitroso Compounds

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Foreword

Topics in Chemical Mutagenesis is a new series dedicated to studies in the areas of environmental chemical mutagenesis and genetic toxicology. In this series we will explore some of many topics that are emerging in these rapidly developing fields.

The purpose of the present volume is to attempt to organize and compare the genotoxic properties of the N-nitroso compounds. This is a particularly interesting class of compounds because of the problems encountered with the *Salmonella* assay of Ames in generating both false positive and false negative results. The battery approach using a number of assay systems seems more appropriate to evaluate chemicals in this class.

Topics to be discussed in other volumes in this series include single-cell mutation monitoring systems, the detection of genetic damage in mammalian germ cells, the mutagenicity of pesticides, problems in monitoring human populations in genetic toxicology, and a glossary of terms in genetic toxicology. All of these books are in various stages of development and should appear within the next few years.

Frederick J. de Serres
Series Editor

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Preface

During the past ten years there has been an explosive development in the number of short-term tests to predict the biological risks, especially risks of cancer, in exposure to xenobiotic chemicals. The number of published articles in this area has reached many thousands a year and there are several new journals devoted almost entirely to the presentation of the results obtained in these tests. The developers and large-scale users of these tests often rival one another in their claims of validity as predictors of carcinogenicity. Many of the test systems have mutagenesis as the measured end point, and it is frequently forgotten that mutagenesis itself is a biological hazard and that the measurement of a mutagenic risk to man is of equal importance with the estimation of carcinogenic risk. A number of books and review articles have been written about the development and application of short-term assays for mutagenesis–carcinogenesis, but none has focused on the applications of these assays to a single group of well-tested carcinogens sufficiently large and important to be a guide to understanding the complex processes of mutagenesis and carcinogenesis and the relation between them.

Almost three years ago, Dr. F. J. de Serres organized a meeting at the National Institutes of Health that was attended by a number of scientists who had worked, sometimes cooperatively, sometimes alone, in examining the behavior of a single group of compounds, the N-nitroso compounds, in the assay with which they were most familiar. From this group of scientists, all of whom presented interesting data, several volunteered to pool their results in the compilation of a single volume that would be devoted to the comparison of the results obtained with N-nitroso compounds from mainly a single source of consistent chemical quality, and an evaluation of those results as a predictor of one assay system by another, and that would be a means of illuminating the complexity of the mechanisms of biological action of these toxicants. It is this

volume that we present as an effort to understand the meaning of all of the results, often disparate, that appear in the literature describing the application of all of these assay systems. The conclusions represent a consensus view of these combined efforts and are a beginning for the continuing process of unraveling the mechanisms of genetic toxicology through comparative studies of biological assays, based on a firm foundation of chemical structural relations among N-nitroso compounds.

T. K. Rao
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Formation of N-Nitroso Compounds and Their Significance

WILLIAM LIJINSKY

Nitrosamines were first made more than 100 years ago by the simplest means, reaction of secondary amines with nitrous acid. They were not known to have adverse biological effects until 1937, when a case of poisoning by N-nitrosodimethylamine was reported but largely ignored.[1] A detailed investigation of the toxicity of nitrosodimethylamine began in England in the 1950s as a result of another accidental poisoning, the first results being reported by Barnes and Magee in 1954.[2]

The result of acute poisoning of rats by nitrosodimethylamine was extensive liver necrosis, which led rapidly to their death; liver necrosis was also the consequence of exposure of humans to large doses of this compound. With chronic administration to rats, a natural supplement to the acute study that produced such significant results, Magee and Barnes[3] began an increasingly intense examination of the toxicology of N-nitroso compounds, which is still in full flow.

Although it cannot be said that the mechanism of toxicity and carcinogenesis by nitrosodimethylamine is completely understood, the biochemical studies carried out by Magee and his collaborators have been of immense importance, following the discovery that chronic feeding of this compound to rats gives rise essentially to a 100% incidence of liver tumors.[3] A number of analogs and homologs of nitrosodimethylamine were tested within a short time after the reports of the carcinogenicity of this prototype, mainly for the purpose of establishing types of nitrosamine structure that were associated with carcinogenic activity. Information obtained by this approach, standard in toxicological

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studies, is important to an understanding of the mechanisms of carcinogenesis by this group of compounds. Some of this work was done by Magee and his collaborators in England, but the bulk of the important studies of the relationship between chemical structure and carcinogenic activity was carried out in Freiburg. Associates of H. Druckrey, and especially R. Preussmann, were the principal investigators, with the extensive studies of nitrosodiethylamine carcinogenesis in rats by D. Schmähl being the starting point. The dose–response study of nitrosodiethylamine in rats conducted by Schmähl, Preussmann, and Druckrey provided sufficient information for an attempt at quantification of carcinogenic response and risk extrapolation. The enormous amount of work carried out in Freiburg was summarized in a monumental paper in 1967.[4]

Among the N-nitroso compounds studied by the German group (many being tested in species other than the rat) were several nitrosoalkylamides, almost all of which were potent carcinogens. These were of special interest because several of them, including nitrosomethylurea and nitroso-N-methylurethane, had been commonly used in laboratories for decades in the preparation of the methylating agent diazomethane. It was tempting to believe that their action as carcinogens might be due to this ability to give rise to diazomethane, although this is not now thought to be true.[5] Another nitrosoalkylamide tested by Druckrey's group was nitroso-N-methyl-N′-nitroguanidine, originally prepared for use as a detonator, but now used as a standard mutagen, being a powerful mutagenic agent for bacteria and many higher organisms. Interest in the nitrosoalkylamides grew when it was found that they were not only mutagenic and carcinogenic by direct action (they produced tumors where they were applied, as distinct from nitrosamines, which are systemic carcinogens and are believed to require metabolic activation), but were directly acting alkylating agents for cellular macromolecules.

In the beginning it was not believed that nitrosamines represented a carcinogenic risk outside the laboratory, even though they are so easily formed from amines and nitrosating agents. It was not easy to determine nitrosamines analytically, and the sensitivity of methods available in the late 1960s was no greater than 1 to 10 parts per million, at which levels attempts to find them in the environment were not seriously made. An experiment of Druckrey's in the early 1960s was designed to test the obvious possibility that nitrosation of amines might occur in the acid milieu of the mammalian stomach and thereby result in exposure to this group of carcinogens. The amine chosen was diethylamine, which was fed to rats as the hydrochloride together with sodium nitrite for 2 years. It did not lead to the induction of tumors typical of treatment of rats with nitrosodiethylamine. It was concluded that formation of nitrosamines *in vivo* was unlikely.[6] It was later realized that nitrosation of diethylamine (and dimethylamine) is very slow because of the strong basicity of these amines, and therefore it was improbable that sufficient nitrosodiethylamine was formed to

give rise to tumors within the lifetime of the rats. The nitrosation of secondary amines (formation of nitrosamines from secondary amines and nitrous acid) had been studied very thoroughly by Ridd,[7] and kinetic experiments showed that under most conditions the rate of reaction is proportional to the square of the nitrite concentration.

The reaction of tertiary amines with nitrous acid had been largely ignored because of the commonly held belief (based on inadequate experiments) that such a reaction did not take place. There had been sporadic reports in the literature of formation of nitrosamines by nitrosation of tertiary amines, but these findings could have been due to contamination of the tertiary amine with secondary amine. However, in 1967 Smith and Loeppky reported their studies of the reaction of tribenzylamine with nitrous acid, which showed that the reaction did take place,[8] but only at a weakly acid pH (above pH 3). Starting in the 1970s, this finding encouraged others, including this author, to undertake a more comprehensive study of the nitrosation of tertiary amines.[9] Among the tertiary amines of interest were the large number of drugs and agricultural chemicals that belong to this class.[10,11] There is, of course, a comparably large number of secondary amines that are used as drugs and agricultural chemicals. Although there have been some kinetic studies of nitrosation of tertiary amines,[12,13] the mechanisms are not well esfablished and the stoichiometry of the reaction is not clear, although it is known that one or more nitrosamines and carbonyl compounds are the most prominent products.[14]

Comprehensive studies of amine nitrosation were very few until a decade ago. It was known that, in addition to nitrous acid, alkyl nitrites and nitrogen oxides (mixtures of NO_2 and NO) reacted with secondary amines to form nitrosamines, and several were prepared this way, e.g., nitrosoiminodiacetic acid.[15] Nitrogen oxides in flue gases are also the source of nitrosamines in beer.[16] It has been proposed that nitrogen oxides might lead to formation of nitrosamines *in vivo*, but this has been disputed. It is believed, now, that many types of oxidized nitrogen compounds can act as nitrosating agents, e.g., peroxyacyl nitrites and nitrates, which are components of the modern "smog" in cities (see review on nitrogen in AMBIO.[17]) Some N-nitroso compounds can themselves act as nitrosating agents for amines. Among them is the aromatic nitrosamine, nitrosodiphenylamine, which finds extensive use in the rubber industry and is itself a carcinogen,[18] but which can also be an effective nitrosating agent.[19] More recently it has been found that many aliphatic nitrosamines, as well as aromatic ones, are excellent nitrosating agents,[20] particularly in the presence of nucleophiles, such as thiocyanate, which act as catalysts. Thiocyanate has long been known as a good catalyst of nitrosation.[21] Nitrosamines usually considered noncarcinogenic, and therefore of little or no risk, such as nitrosoproline and nitrosohydroxyproline,[22-24] or others considered only weakly carcinogenic, such as nitroso-N-methylpiperazine,[4,25] react quite rap-

idly with easily nitrosated secondary amines, such as morpholine, at acid pH to form nitrosamines that can be carcinogenic, in this case nitrosomorpholine.[26] Nitrosoalkylureas are also good nitrosating agents, in some cases nitrosating themselves.[27]

In the course of repeating a study of the nitrosation of oxytetracycline by nitrous acid to form nitrosodimethylamine,[28] Mirvish and co-workers discovered that ascorbic acid was an effective inhibitor of nitrosation of amines[29] by competing with the amine for the nitrous acid, as demonstrated earlier by Dahn.[30] This finding, with its obvious implication for the prevention of formation of carcinogenic N-nitroso compounds, led to a large number of studies of the role of inhibitors and accelerators of nitrosation, in conjunction with studies of nitrosation itself. In addition to ascorbic acid, alpha tocopherol, glutathione, some phenols, and tannins are inhibitors of nitrosation, also through competition for nitrous acid.[31] Among accelerators of nitrosation, in addition to nucleophilic anions, are some phenols[32] and metal ions.[33] Among the more provocative discoveries was that of Keefer and Roller,[34] who showed that certain carbonyl compounds, including formaldehyde and chloral, were able to facilitate reaction of nitrites with secondary amines (but not with tertiary amines), even at alkaline pH. This could greatly broaden our awareness of the range of sources of exposure of humans to N-nitroso compounds. It goes far to explain the presence of nitrosodiethanolamine in synthetic cutting oils, which contain diethanolamine as an impurity in a main component, triethanolamine, in addition to nitrite.[35,36] Nitrosodiethanolamine is also present, in much lower concentrations, in many types of cosmetic preparation.[37] It is becoming increasingly obvious that nitrosodiisopropanolamine [nitroso-bis(2-hydroxypropyl)amine], a homolog of nitrosodiethanolamine, and seemingly a more potent carcinogen than the latter, is also quite widely distributed. The concentrations of the homolog are usually lower than those of nitrosodiethanolamine, and they are derived from nitrosation of the secondary and tertiary aromatic bases di-isopropanolamine and tri-isopropanolamine.

Another type of nitrosamine found in cutting oils, nitroso-5-methyloxazolidine,[38] illustrates the variety of N-nitroso compounds that can be formed in mixtures. It has been known for some time that alkanolamines react with aldehydes in the presence of nitrites to form nitrosooxazolidines and nitrosotetrahydrooxazines.[39] Many of these compounds are potent carcinogens, although some are not, and considerable work is in progress on their formation and occurrence.

There have been reports of nitrosamines in air of industrial areas, especially nitrosodimethylamine in the vicinity of factories in which dimethylamine is manufactured or used.[40] There have also been reports of the presence of nitrosamines in some foods in which their presence would be far from obvious, such as dried milk and vegetable oils. Some of these reports might be spurious, and the curious identification of a nitrosamine in dried milk as nitrosodi-

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