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Free-Radical Mechanisms in Tissue Injury
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Alkylation Intermediates in Nitrosamine Metabolism
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Many N-nitroso compounds are toxic (Magee & Swann, 1969), carcinogenic (Magee & Barnes, 1967; Druckrey et al., 1967; Magee et al., 1975), mutagenic (Magee & Barnes, 1967; Zimmermann, 1971) and teratogenic (Druckrey, 1973). The nitrosamines are chemically stable under physiological conditions, whereas some nitrosamides (e.g. N-methylnitrosurea) decompose to yield alkylation agents. Most of the available evidence indicates that the biological actions of both classes of compound are effected by decomposition products rather than the intact parent molecules. Dimethylnitrosamine damages the liver selectively (Barnes & Magee, 1954) even though it is distributed widely and fairly uniformly throughout the body after administration, probably because the liver is the main site of its metabolism (Magee, 1956). Micro-organisms that are readily mutated after exposure to nitrosamides, such as N-methylnitrosourea (Neale, 1972), are unaffected by nitrosamines. The latter agents, e.g. N-nitrosomorpholine, can, however, be activated to potent mutagens in the host-mediated assay procedure (Zeiger & Legator, 1971) and by incubation with microsomal preparations from rodent liver in vitro (Malling, 1971; Gomez et al., 1974) or with an hydroxylation system in vitro (Mayer, 1971).
Although several nitroso compounds have been clearly shown to give rise to alkylating intermediates in the organism, it has not been firmly established that their biological effects are necessarily caused by alkylation reactions. The evidence for alkylation in vivo has, until recently, been mainly derived from studies with dimethylnitrosamine and N-methylnitrosourea. Methylation of proteins (Magee & Hultin, 1962; Turberville & Craddock, 1971) and of nucleic acids (Magee & Farber, 1962; Craddock & Magee, 1963; Swann & Magee, 1968) of rat liver in vivo by dimethylnitrosamine and methylation of nucleic acids of a number of organs of rats treated with N-methylnitrosourea (Swann & Magee, 1968) have been reported. The metabolic pathways for the two compounds shown in Scheme 1 are based on these observations. There is no direct evidence for the postulated intermediates, but it is unlikely that diazomethane is formed in vivo since experiments with fully deuterated dimethylnitrosamine (Lijinsky et al., 1968) or N-methyl-N'-nitro-N-nitrosoguanidine (Süssmuth et al., 1972) indicated that the methyl group is transferred as $-\text{C}_2\text{H}_5$ rather than $-\text{C}_2\text{H}_3\text{H}$. Since the methylating metabolite is presumably formed from dimethylnitrosamine in the endoplasmic reticulum it must be of sufficient stability to reach the nuclear DNA. There is also evidence of damage to the vascular tissue of the liver (McLean et al., 1965; Butler & Hard, 1971) in which metabolism probably does not occur, and of mutation of micro-organisms introduced into the peritoneal cavity (host-mediated assay), also indicating that the alkylating intermediate must be of sufficient stability to reach the respective target sites. More recently alkylation of nucleic acids in vivo by other nitroso compounds including diethylnitrosamine and N-ethylnitrosourea (Swann & Magee, 1971; Goth & Rajewsky, 1972), di-$n$-propyl and di-$n$-butynitrosamine (Kruger, 1971) and N-nitrosomorpholine (Stewart et al., 1974) has been reported. In all the above examples alkylation was found on the N-7 position of guanine, which is the most reactive site with many alkylating agents and thus the most easy to detect in the nucleic acids isolated from animals treated in vivo with labelled nitrosamines.

\[
\begin{align*}
\text{CH}_3 \quad & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{CH}_3 \\
\text{CH}_2 \quad & \quad \text{CH}_2 \\
\text{NNO} & \quad \text{NNO} \\
\text{NH}_2 - \text{CO} & \quad \text{H}_2\text{O} \\
\text{Enzyme} \quad & \quad \text{H}_2\text{O} \\
\text{NADPH} \quad & \quad \text{H}_2\text{O} \\
\text{O}_2 & \quad \text{H}_2\text{O} \\
[\text{CH}_3 \quad & \quad [\text{CH}_3 \\
\text{NNO} & \quad \text{NNO} \\
\text{H} & \quad \text{H} \\
\text{+HCHO} & \quad \text{+HCHO} \\
\text{[CH}_3\text{N}_2\text{+OH}^-] & \quad \text{[CH}_3\text{N}_2\text{+OH}^-] \\
\text{[CH}_3^+ + \text{N}_2] & \quad \text{[CH}_3^+ + \text{N}_2] \\
\text{CH}_3\text{R} & \quad \text{CH}_3\text{R}
\end{align*}
\]

Scheme 1. \textit{Suggested metabolic pathways of dimethylnitrosamine and N-methylnitrosourea} \\
R = nucleic acid, protein, water etc.
Quantitative studies on the extent of methylation in different organs of the rat by dimethylnitrosamine or N-methylnitrosourea showed quite good correlation with the sites where tumours arise with the notable exception of the liver (Swann & Magee, 1968). Similar findings by Schoental (1969), using N-methylnitrosourethane, led her to question the significance of methylation on the N-7 position of guanine in carcinogenesis. Although tumours of the liver do not follow single doses of methyl nitroso compounds in the adult rat, they may be so induced if the animals have undergone prior partial heptectomy (Craddock, 1973). It seems therefore that cell division may be necessary within an appropriate period after the initial carcinogenic stimulus if cancer is to result. In some tissues therefore repair of the initial change induced by the carcinogen may have time to occur before a sufficient number of cells have divided to ensure tumour production.

The suggestion by Loveless (1969) that alkylation on the O-6 position of guanine may be important for mutagenesis and carcinogenesis has led to further study of this reaction in several laboratories. DNA alkylated on this position is unstable under the acid conditions of hydrolysis of alkylated nucleic acids used in much of the earlier work and O6 alkylation was therefore often overlooked. Recently a comparison of the stability of O6-methylguanine with that of N7-methylguanine in the brains and livers of 10-day-old rats treated with [1-14C]ethylnitrosourea has been made by Goth & Rajewsky (1974). N-Ethylnitrosourea induces tumours of the brain in rats under these conditions with virtually no tumours elsewhere. Elimination of O6-ethylguanine from the brain was strikingly slower than from the liver and also much slower than the elimination rates from brain DNA of N7-ethylguanine and N3-ethyladenine. Since the presence of O6-ethylguanine in DNA is likely to lead to anomalous base-pairing during DNA replication (Gerchman & Ludlum, 1973) the persistence of this ethylated base in the brain may be related to the highly selective carcinogenic action of N-ethylnitrosourea on this organ.

Recent findings at the Courtauld Institute (J. W. Nicoll & A. E. Pegg, unpublished work) are consistent with those of Goth & Rajewsky (1974). Their finding that the relative proportion of O6 to N7 alkylation with N-ethylnitrosourea was considerably higher than the known proportion with N-methyl nitroso compounds has been confirmed and found also to apply to nucleic acid alkylation in vivo by diethylnitrosamine. There is thus a much closer agreement with the degree of formation of the O6-alkylguanine product than the N7 product by doses of the ethyl and methyl derivatives that induce an approximately equal incidence of tumours (Swann & Magee, 1968, 1971). Also in agreement with Goth & Rajewsky (1974) a marked organ-specific difference in the rate of loss of N7 and O6 alkylguanines from DNA in vivo was found in rats treated with N-ethyl nitrosourea or diethylnitrosamine. O6-Ethylguanine was lost rapidly from rat liver DNA, at a much faster rate than N7-ethylguanine. In contrast, O6-ethylguanine was removed from brain DNA of rats treated with N-ethyl nitrosourea much more slowly than in liver. In the kidney, after administration of N-ethyl nitrosourea or diethylnitrosamine, O6-ethylguanine was lost from DNA faster than in the brain but slower than in the liver. As already stated, tumours are not produced in the liver of adult rats by single doses of these agents, but tumours of the kidney and brain are so produced. At present nothing is known of the mechanism by which the alkylated purines are removed. It seems probable than an enzymic mechanism is involved in the removal of O6-alkylguanine as this product is chemically stable in DNA at neutral pH and the rate of loss varied with the tissue and was different from that of N7-alkylguanine, indicating that loss was unlikely to be due solely to cell death.

The loss of alkylated guanines from DNA of liver and kidney after treatment of rats with dimethylnitrosamine at two dose concentrations, 2.5 mg/kg body wt. and 20 mg/kg body wt respectively, has been compared recently (J. W. Nicoll & A. E. Pegg, unpublished work). At the lower dose (2.5 mg/kg body wt.) N7-methylguanine was lost from the liver DNA with a half-life of 2-3 days, but O6-methylguanine was lost more rapidly with a half-life substantially less than 1 day. These results are in agreement with those of O’Connor et al. (1973). There was a somewhat slower rate of loss of O6-methylguanine from kidney DNA so that the ratio of O6- to N7-alkylguanine fell in both organs but more
rapidly in the liver. At the higher dose (20mg/kg body wt.), however, a strikingly different result was obtained. The alkylated guanines were lost from liver DNA at rates similar to those seen after the smaller dose, but in the kidney O\textsuperscript{6}-alkyguanine was lost much more slowly and the ratio of O\textsuperscript{6}- to N\textsuperscript{7}-alkyguanine in the kidney DNA increased, doubling in 72h, whereas after the low dose of dimethylnitrosamine this ratio fell by 70\% in the same period. A possible explanation of these findings is that the high dose of dimethylnitrosamine inhibits in some way the removal process in the kidney but not in the liver. If there is a correlation between the ability to remove O\textsuperscript{6}-methylguanine from DNA and a protective mechanism against carcinogenesis by these agents such an inhibition of the removal process might be of considerable importance. In this respect it is of interest that liver tumours are induced in the rat by repeated exposure to dimethylnitrosamine, but large single doses induce tumours of the kidney but not of the liver in the adult rat.

It is concluded that recent evidence is consistent with the hypothesis that carcinogenesis and other biological actions of N-nitroso compounds are dependent on the generation of alkylating metabolites within the organism.

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Inactivation and Mutation Induction in Bacteria by Microsomal Metabolites

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The phenomenon of mutation induction by mustard gas was demonstrated over 20 years ago (Auerbach & Robson, 1946). Since this time numerous other chemical and physical agents have been found to have mutagenic activity. Many of these, such as the