MOLECULAR INTERACTIONS IN THE ASSESSMENT OF
THE GENOTOXICITY OF ALKYLATING AGENTS

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SUMMARY

The recognition and subsequent demonstration, in vitro, of the alkylating potential of dichlorvos has led to a suspicion that this triester of phosphoric acid may be a mutagen and a carcinogen. Despite consistently negative results obtained in mammalian mutagenicity and carcinogenicity tests it was never-the-less important to investigate the alkylating reactivity in vivo.

In the experiments described in this thesis, male CFE rats were exposed to atmospheres containing 0.064μg l⁻¹ of [Me⁻¹⁴C]dichlorvos (113Ci.mol⁻¹) for 12 hours. Analysis of the DNA from the total soft tissues from 20 rats revealed no methylation at the N-7 atom of guanine moieties. A comparative study with [methyl⁻¹⁴C]methanesulphonate gave rise to a readily detectable extent of methylation of the N-7 atom of the guanine moieties in DNA.

The limit of detection of methylation of the DNA in the dichlorvos study was one methyl group per 5.7 x 10¹¹ nucleotide units. The proportion of the administered dose that would be consumed in this hypothetical reaction would be 0.000001%. The exposure period employed in this study (12h) constituted a significant fraction of the half-life of 7-methylguanine moieties in DNA (3 days) and the current findings therefore indicate that dichlorvos would not methylate the DNA of mammalian tissues even when it is inhaled continuously for protracted periods of time.
The administration of radiolabelled adenine, guanine, methionine and formate to otherwise untreated rats gave rise to the excretion of radiolabelled methylated purines in urine. This finding indicates that the detection of radiolabelled methylated purines, per se, in the urine of animals exposed to methyl-labelled methylating agents does not constitute evidence for the spontaneous methylation of the purine moieties of nucleosides and nucleic acids by methylating agents, in vivo.
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CHAPTER 1

Introduction

1.1 In vivo reactions of alkylating agents with DNA

The biologically active alkylating agents are a chemically diverse group of synthetic and naturally occurring compounds whose only common feature appears to be their electrophilic reactivity. In some molecules this reactivity is inherent e.g. MMS and DMS (Fig. 1) but in many other molecules this reactivity is only manifested after metabolic activation e.g. DMN and MAM (Fig. 1). Several excellent reviews dealing with the subject of their metabolic activation have been published (e.g. Miller and Miller, 1969a; 1969b; 1971). Two mechanisms are generally accepted for in vivo alkylation reactions. By definition, alkylation reactions involve nucleophilic substitution at the electrophilic carbon atom of the alkylating agent. Two reaction mechanisms are normally available for such nucleophilic substitutions (S_N). One of them contains only one stage and proceeds via an electron transfer from the substituting agent to the seat of substitution, and from the latter to the displaced group (Equation 1). In this mechanism, two molecules simultaneously undergo covalency change and the mechanism is therefore bimolecular (S_N^2)

\[
\text{Equation 1}
\]
Fig 1  Some biologically active alkylating agents
The second mechanism involves two stages: a slow heterolysis of the alkylating agent to yield a carbonium ion followed by a rapid co-ordination of the carbonium ion and the nucleophile (Equation 2). Because only one molecule is undergoing a covalency change during the rate-determining stage, i.e. the generation of the carbonium ion, the mechanism is unimolecular (SN1).

\[
\begin{align*}
\text{Alk} & \overset{\text{slow}}{\rightarrow} \text{Alk}^+ + \text{X}^- \\
\text{N}^+ \overset{\text{fast}}{\rightarrow} \text{N} - \text{Alk}
\end{align*}
\]

Equation II

This division on the basis of mechanism is reflected in the nature of the in vivo reaction products with nucleic acids. With agents reacting by the SN2 mechanism the reaction tends to be directed towards the purine and pyrimidine ring nitrogen atoms, while agents reacting by the SN1 mechanism tend to be less selective, reacting with both oxygen and nitrogen atoms.

It is becoming evident that all of the bases in DNA and in some instances the deoxyribose phosphodiester backbone are in vivo targets for alkylating agents. However, it is clear that in vivo certain segments in DNA are more susceptible to alkylation than are others; i.e. the pattern of alkylation is not homogeneous. Thus, it has been shown that those areas of chromatin that are accessible to nucleases, i.e. regions of DNA that are not protected by histones, are the most prone
to alkylation (Cooper et al, 1975; Cooper and Itzaki, 1975; Itzaki et al, 1976; Ramanathan et al, 1976). These reactions have been the subject of many excellent reviews (Lawley, 1966; 1972a,b; 1974; Sarma et al, 1975; Singer, 1975) and therefore discussion of them here will not be extensive. Suffice it to say that with a variety of methylating agents atoms at the following nucleophilic centres have been shown to be reactive towards a variety of methylating agents:– the N-1, N-3 and N-7 atoms of adenine, the N-3, O6 and N-7 atoms of guanine and the N-3 atom of cytosine. In addition, in vitro studies have shown that the oxygen atoms of DNA bases are reactive towards nitroso compounds in vitro (Lawley et al, 1973; Singer, 1976a,b). The order of reactivity in double stranded DNA is O2 of T = O6 of G > O6 of T >> O2 of C (Singer, 1976b).

Figure 2 shows the location of these sites in their respective bases together with an indication of the agents that react with them. Quantitatively the most reactive site is the N-7 atom of the guanine ring, accounting for more than 60% of the total base alkylation. This reactivity has been attributed to its peripheral position in the wide groove of the Watson-Crick model of the double helix (Reiner and Zamenhof, 1957; Luck and Zimmer, 1972). In addition it has been proposed that the high nucleophilicity of the N-7 is related to the role of guanine as a hydrogen bond donor. In the Watson-Crick model of the DNA helix, guanine donates two hydrogen bonds but accepts only one. This, it is suggested, results in an increased electron density over the guanine ring which may be reflected by the
Fig 2 Sites of interaction of methylating agents with nucleic acids in vivo and in vitro
increased nucleophilicity of the N-7 atom (Sarma et al., 1975).

Mutagenesis and carcinogenesis, the biological effects of most concern in relation to the alkylating agents, have both been attributed to changes in base structure with subsequent misrepair or miscoding. Collectively these effects have been grouped under the general heading of genotoxicity. Thus a genotoxic compound is defined as "any agent which, by virtue of its physical or chemical properties, can induce or produce heritable changes in those parts of the genetic apparatus of somatic cells, responsible for the homeostatic control, and thereby determining them to malignant transformation" (Druckrey, 1973; 1975). Largely on the basis of its quantitative importance, alkylation at the N-7 atom of guanine was presumed to be causally related to these effects. However, in vitro studies with synthetic polymers containing varying proportions of 7-methylguanine have demonstrated that the presence of this base is not "mutagenic" per se at the level of transcription (Ludlum, 1970) or at the level of translation (Wilhelm and Ludlum, 1966). Furthermore, Hendler et al. (1970) have shown that in an in vitro DNA polymerase system, 7-methyl-2'-deoxyguanosine triphosphate can substitute, to a limited degree, for 2'-deoxyguanosine triphosphate. Further doubt has been cast on the relevance of this base to mutagenesis by studies on bacteriophage. In this system both MMS and EMS readily form 7-alkylguanine, but EMS is more potent as a mutagen than MMS (Loveless, 1959). This apparent discrepancy cannot be explained on the basis of the ethyl group being unique.
as MN\textsuperscript{U} proved to be an effective mutagen. Mechanistically EMS bridges the gap between \(S\text{\textsubscript{N}}^2\) agents (e.g., MMS) and \(S\text{\textsubscript{N}}^1\) agents (e.g., MNU) giving a measurable extent of \(\text{\textsuperscript{6}}\text{o}\text{-alkylation}\). As the biological data was amassed it became evident that there was only a poor correlation between the levels of 7-methylguanine and biological effect (in most cases tumour production).

This has been demonstrated with many different alkylating agents, DMN (Craddock, 1973), MN\textsuperscript{U}, DMN, DMS and MMS (Swann and Magee, 1968), DEN, ENU and EMS (Swann and Magee, 1971), ENU and MN\textsuperscript{U} (Lijinsky \textit{et al.}, 1972), MN\textsuperscript{U} and MMS (Kleihues and Magee, 1973) and DEN (Ross \textit{et al.}, 1971). Attention was therefore focused on the minor bases, and in particular \(\text{\textsuperscript{6}}\text{o-methylguanine}\), the site of interaction suggested by Loveless (1969) as being responsible for mutagenesis in the bacteriophage. \textit{In vitro} studies with copolymers of cytidylic and \(\text{\textsuperscript{6}}\text{o-methylguanylic acids}\) used as templates for RNA polymerase demonstrate mis-incorporation of UMP or AMP into the product copolymer (Gerchman and Ludlum, 1973). The studies with poly \(\text{\textsuperscript{6}}\text{o-methylguanylic acid}\) and poly \(\text{\textsuperscript{6}}\text{o-ethylguanylic acid}\) homopolymers (Mehta and Ludlum, 1976) have shown that they do not form helices with poly (C) nor for that matter with poly (U). Thus \(\text{\textsuperscript{6}}\text{o-methylguanine}\) disrupts base pairing and affects the secondary structure and must be regarded "pro-mutagenic". \textit{In vivo} studies, (Kleihues and Magee, 1973) demonstrated a relatively high level of methylation at the \(\text{\textsuperscript{6}}\text{o-position of guanine in the DNA of rat brain following administration of MN\textsuperscript{U} (tumourigenic in brain). The level of \textsuperscript{6}}\text{o-guanine methylation was 12% of that found at the N-7
position and was not observed with MMS. A similar situation was found for $\text{O}^6$-guanine alkylation in liver DNA following exposure to DMN (approx. 12% of that at N-7; Craddock, 1973). Minor bases were found in both studies, but their generation did not correlate with tumour production, e.g. Kleihues and Magee (1973) found that the amounts of 3-methyladenine in brain DNA were higher by a factor of two following treatment with MMS. Thus it seemed, on the basis of these studies that the $\text{O}^6$-alkyl-guanine produced in the target organs was causally related to tumour production. However, it was later shown (Goth and Rajewsky, 1974a) that the initial extent of alkylation produced at the $\text{O}^6$ position of guanine by ENU was the same for both brain (target organ) and liver (non-target organ). However, the persistence of $\text{O}^6$-ethylguanine in the brain was much greater (half life approx. 220h) than in liver (half life approx. 30h). The results of a second study (Goth and Rajewsky, 1974b) showed that while the molar fractions of N-7 ethylguanine, $\text{O}^6$-ethylguanine, N-3 ethyladenine and N-7 ethyladenine were initially similar in both target (foetal or 10 day old rat brain) and non-target (liver) tissues, the rate of removal of $\text{O}^6$-ethylguanine was slower in target organs. The persistence of the lesion in target tissue was later confirmed using MNU and interestingly, the persistence in the DNA of kidney (an occasional target organ) was intermediate between that in liver and in brain (Kleihues and Margison, 1974). Repeated application of MNU to rats results in a selective accumulation of $\text{O}^6$-methylguanine in brain as compared with kidney, spleen and
liver. For comparison the terminal levels of $\theta^6$-methylguanine in liver represented less than 1% of that in brain (Margison and Kleihues, 1975). In at least one case however the rate of removal of $\theta^6$-methylguanine moieties and tumourigenesis were anti-parallel (Den Engelse, 1974).

The removal of alkylated (damaged) bases from DNA is not restricted to $\theta^6$-methylguanine. Most alkylated purines have been shown to be depurinated from DNA at a rate greater than that measured for normal bases (Lindhal and Nyberg, 1972). In some cases the rate of depurination in vivo is significantly greater than that found in vitro (Table 1), indicating the possible involvement of a "repair enzyme". Kirtikar and Goldthwait (1974) and Kirtikar et al. (1976) have described such an enzyme, endonuclease II, in E. coli. The enzyme has both phosphodiesterase and N-glycosidase activity, the latter being directed towards a limited number of purines. Thus it will release $\theta^6$-methylguanine, 3-methyladenine and small amounts of 1- and 7-methyladenines from methylated DNA. 7-Methylguanine is not released by this enzyme, and it is likely that the release of most of this base in vivo is via chemical hydrolysis of the carbon-nitrogen bond linking the N-9 of the purine to the C-1 of the deoxyribose moiety (Fig. 3). The probable steps in this excision process, derived from studies in bacteria, are shown in Fig. 4. Damaged areas are recognised probably on the basis of distortion of the DNA by the endonuclease (van Lanker and Tomura, 1972). Areas of "spontaneous" depurination, can also be recognised and Verly et al. 1973 and Verly and Paquette (1973) have described an enzyme from rat liver which acts on
<table>
<thead>
<tr>
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<th>System</th>
<th>pH</th>
<th>Temp.</th>
<th>$t_\frac{1}{2}$ (h)</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>7-Methylguanine</td>
<td>In vitro</td>
<td>5.0</td>
<td>37°</td>
<td>79.3</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>In vitro</td>
<td>7.0</td>
<td>37°</td>
<td>144</td>
<td>(1)</td>
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<td></td>
<td>In vitro</td>
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<td>37°</td>
<td>105</td>
<td>(2)</td>
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<td></td>
<td>In vivo</td>
<td>-</td>
<td>-</td>
<td>72</td>
<td>(1)</td>
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<td>7-Ethylguanine</td>
<td>In vivo (brain)</td>
<td>-</td>
<td>-</td>
<td>~89</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>In vivo (liver)</td>
<td>-</td>
<td>-</td>
<td>64</td>
<td>(3)</td>
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<td>3-Methyladenine</td>
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<td>5.0</td>
<td>37°</td>
<td>11.5</td>
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<td>37°</td>
<td>24</td>
<td>(4)</td>
</tr>
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<td></td>
<td>In vitro</td>
<td>7.2</td>
<td>37°</td>
<td>38</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>In vivo</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>(4)</td>
</tr>
<tr>
<td>3-Ethyladenine</td>
<td>In vitro</td>
<td>-</td>
<td>-</td>
<td>33</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>In vivo (brain)</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>In vivo (liver)</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>(3)</td>
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<td>37°</td>
<td>2.8</td>
<td>(2)</td>
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<td></td>
<td>In vivo</td>
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<td>-</td>
<td>2.6</td>
<td>(2)</td>
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<td>7.2</td>
<td>37°</td>
<td>&gt;105</td>
<td>(2)</td>
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<td></td>
<td>In vivo</td>
<td>-</td>
<td>-</td>
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<td>(5)</td>
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<td>-</td>
<td>~229</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>In vivo (liver)</td>
<td>-</td>
<td>-</td>
<td>~36</td>
<td>(3)</td>
</tr>
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<td>Normal purines</td>
<td>In vivo</td>
<td>7.4</td>
<td>37°</td>
<td>6.4 x 10^6</td>
<td>(6)</td>
</tr>
</tbody>
</table>

(1) Margison et al, 1973
(2) Lawley and Warren, 1976
(3) Rajewsky and Goth, 1976
(4) Margison and O'Connor, 1973
(5) O'Connor et al, 1973
(6) Lindahl and Nyberg, 1972
Fig 3  Spontaneous depurination of 7-Methylguanine in DNA
Fig 4 Probable steps in the removal of DNA lesion and repair of the damage
depurinated rather than alkylated DNA. The relationship of these two enzyme systems to each other is not known. Action of the endonuclease produces a transient break in the DNA chain. These breaks, produced either partly or entirely by enzyme activity, have been measured \textit{in vivo}, largely in liver DNA, following the application of several alkylating agents (Table 2), (Cox et al., 1973a, b; Sarma et al., 1973a; Damjanov et al., 1973; Stewart et al., 1973; Stewart and Farber, 1973; Rajalakshmi and Sarma, 1973; Goodman and Potter, 1972).

Removal of the damaged region occurs through the action of an endonuclease (Fig. 4) and is followed by repair synthesis (unscheduled DNA synthesis) presumably using the undamaged strand as the template. Incorporation of nucleotides, predominantly thymidine, into the DNA of cultured cells has been demonstrated following treatment with carcinogens (Seltow and Regan, 1972; Stich et al., 1972; Leiberman and Forbes, 1973). The gap is finally sealed by a polynucleotide ligase. The fidelity of so-called excision repair is good, as judged from studies in bacteria.

Failure or inability to excise damage and regions from the DNA does not necessarily block DNA synthesis. Most, if not all, cells have the ability to "bypass" such lesions by a process described as 'post-replication repair'. The operation of such a system has been reported in rat liver DNA \textit{in vivo} following exposure to DMN (Rajalakshmi and Farber, 1973). This system appears similar to that described in repair deficient \textit{E. coli} (Rupp and Howard-Flanders, 1968) and mouse L5178Y cells
Table 2 - Alkylating Agents Causing DNA Strand Breaks in Liver DNA in vivo

<table>
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<tr>
<th>Single strand breaks (rapidly repaired ≤72h)</th>
<th>Single strand breaks (slowly repaired &gt;72h)</th>
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</thead>
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<tr>
<td>Methyl methanesulphonate</td>
<td>Methylazoxymethanol</td>
</tr>
<tr>
<td>N-methyl-N'-nitro-N-nitrosoguanidine</td>
<td>Dimethylnitrosamine</td>
</tr>
<tr>
<td>N-nitroso-N-methylurethane</td>
<td>Ethyl methanesulphonate</td>
</tr>
<tr>
<td>Hycanthone methanesulphonate</td>
<td>N-nitrosomethylurea</td>
</tr>
</tbody>
</table>
Findings that micro-organisms deficient in post-replication repair are relatively resistant towards the mutagenic action of a number of alkylating agents has suggested that this process is "error-prone" (Strauss, 1974).

Replication of DNA containing damaged regions may be deleterious. Pro-mutagenic lesions such as 3-methyladenine, 3-methylguanine, \( O^6 \)-methylguanine or \( O^4 \)-methylthymine in the parent strand may introduce GC \( \rightarrow \) AT or AT \( \rightarrow \) GC transitions. Suggested anomalous pairings are shown in Fig. 5. Other lesions such as 3-methylcytosine, 3-methylthymine and 1-methyladenine are primarily inactivating (Lawley, 1974).

It would appear therefore that the genotoxic effects of the alkylating agents can be explained by a deficiency in the excision repair process. This hypothesis is supported in part by the experiments on the persistence of \( O^6 \)-alkylguanine in target organ DNA following exposure to nitroureas (Kleihues and Margison, 1974; Margison and Kleihues, 1975; Goth and Rajewsky, 1974a,b) and from the studies on xeroderma pigmentosum cells which lack the ability to excise pyrimidine dimers. In this context measurement of alkylation reaction at the \( N-7 \) atom of guanine would appear to have little relevance to the genotoxic events. However, the \( N-7 \) atom remains the most available and reactive site in DNA and therefore the most sensitive measure of alkylation reactions.
Fig 5  Suggested anomalous paring of alkylated bases

(Lawley 1974)
1.2 Naturally occurring methylated bases

In vivo studies with $^{14}\text{C}$-labelled alkylating agents have shown, that in addition to the pattern of alkylation predicted from the in vitro experiments, a general labelling of the bases occurs. Although the radiolabel is derived from the alkylating agents, its presence in the bases (e.g. adenine and guanine which have no attached methyl groups) is clearly not the result of a direct alkylation reaction. Such labelling has for example, been shown to occur in rat liver r-RNA following exposure to $[^{14}\text{C}]\text{MMS}$ (McElhone et al, 1971), in rat spleen RNA (Lee et al, 1964) and rat liver DNA (Lawley et al, 1968; Craddock 1969; Capps et al, 1973) following exposure to $[^{14}\text{C}]\text{DMN}$; in the nucleic acids of mouse colon (Hawks et al, 1972) and the DNA of mouse colon (Hawks and Magee, 1974) following exposure to $[^{14}\text{C}]\text{1,2-dimethylhydrazine}$. The RNA and DNA of rapidly dividing tissues (e.g. intestinal epithelium) are those most effectively labelled under this condition. The observed pattern of labelling closely resembled that obtained with $[^{14}\text{C}-\text{methyl}]\text{methionine}$ (Farber et al, 1967; Craddock et al, 1968; Craddock, 1972) and with sodium $[^{14}\text{C}]\text{formate}$ (Sibatoni, 1975; Smellie et al, 1958; Perretta, 1967, Craddock, 1969/70). It is assumed therefore that 1-C fragments, released during the metabolic degradation of the alkylating agents, enter the 1-C pools and are thus incorporated during de novo base synthesis.

Because biomethylation is a fundamental natural process it is important to be able to distinguish between methylated bases produced by natural methylase activity and those produced
by direct, non-enzyme mediated alkylation. In view of the contribution made by the alkylating agents to the 1-C pools, a detailed knowledge of the normal occurrence of methylated bases is essential and many comprehensive reviews have been written on the subject of nucleic acid methylation (Borek and Srinevasan, 1966; Srinevasan and Borek, 1964, 1967; Starr and Sells, 1969). In addition to methylated ribose, methylated derivatives of all four bases have been described in t-RNA and r-RNA. These are (Fig. 6) 1-methyladenine, 2-methyladenine, N^6-methyladenine, N^6,N^6-dimethyladenine, 1-methylguanine, N^2-methylguanine, N^2,N^2-dimethylguanine, 7-methylguanine, 3-methylcytosine, N^4-methylcytosine, 5-methylcytosine, 3-methyluracil and 5-methyluracil (Hall, 1965; Munns et al, 1974). A recent paper by Albani et al (1976) described the presence of 5-methoxyuracil (Fig. 6) in t-RNA of B. subtilis, but as yet no evidence exists for its occurrence in mammalian cell t-RNA. These modifications have been shown to occur post-synthetically i.e. at the polynucleotide level (Mandel and Borek, 1961; Borek, 1963; Fleissner and Borek, 1963) by the action of specific methylases with S-adenosyl methionine acting as the methyl donor (Mandel and Borek, 1961, 1963).

Until comparatively recently the m-RNA molecule was believed to be unmethylated. However, with the application of affinity chromatography highly purified m-RNA was produced. It was then shown quite clearly that m-RNA from mouse L-cells (Perry and Kelley, 1974) and from Novakoff hepatoma cells (Desrosiers et al, 1974) contained methylated bases. The suggested location
Fig 6  Methylated bases present in RNA molecules
(at the 5' terminus) was subsequently confirmed and these methyl groups were shown to be contained in a structure containing 7-methylguanine in a 5'-5'pyrophosphate linked with a 2'-0'-methylated nucleotide. Two such structures have been described in RNA viruses, cytoplasmic polyhedrosis virus (Furuichi, 1974; Furuichi and Muira, 1975) and reovirus (Furuichi et al, 1975a) as 7mGpppAmp and 7mGpppGmp. These 'cap' structures have a wide distribution and have been described in m-RNA derived from Hela cells (Nuss et al, 1975; Groner and Hurwitz, 1975; Enzinger and Moss, 1976; Wei et al, 1975,1976), mouse myeloma cells (Cory and Adams, 1975a, 1975b; Adams and Cory, 1976), Ehrlichs Ascites cells (Bajszar et al, 1976), in globin m-RNA from duck (Perry and Scherrer, 1975) and from rabbit (Mulhukrishan et al, 1975; Hunt and Oakes, 1976). These structures are also found in the m-RNA from several mammalian viruses e.g. reovirus (Shatkin, 1974; Furuichi et al, 1975), vaccinia virus (Wei and Moss, 1974), SV40 virus (Aloni, 1975a) and Rous sarcoma virus (Keith and Frankel-Conrat, 1975).

In addition to the capped ends, m-RNA contains 6-methylaminopurine located in the specific sequences Gpm ApC and Apm ApC and not in the poly A segment at the 3' end (Desrosiers et al, 1974; Perry et al, 1975; Desrosiers et al, 1975).

The situation with regard to mammalian DNA is less complex than that of RNA. 5-Methylcytosine, first identified in the DNA from calf thymus, beef spleen and ram sperm (Wyatt, 1950, 1951a, 1951b) is the only methylated base found in mammalian DNA, although 6-methylaminopurine is found in the DNA of bacterial
cells. Subsequent studies in rat liver and spleen (Sheid et al., 1968), chick embryo (Kappler 1971), cultured mouse fibroblasts (Burdon and Adams, 1969), Hela cells (Geraci et al., 1974) and various organs of the rat (Vanyushin et al., 1973) have all confirmed the presence of 5-methylcytosine in mammalian cell DNA and have demonstrated both an inter- and intra-species variation in its distribution. The methyl groups of these residues are introduced into the DNA at the polymer level. The methyl group is donated by S-adenosylmethionine and its transfer to cytosine is effected by a specific methyl transferase. The fact that 5-methylcytosine is synthesised at the polynucleotide level clearly distinguishes it from thymine (5-methyluracil), the methyl group of which is incorporated at the monomer level with the methyl carbon originating from $N^5,N^{10}$-methylene-tetrahydrofolate (FH$_4$).

In cultured mouse fibroblasts, the methylation of cytosine occurs within 1 h of DNA synthesis, during the S-phase of the cell cycle (Burdon and Adams, 1969); the same is true for DNA synthesis by Hela cell nuclei in vitro (Geraci et al., 1974). The distribution of 5-methylcytosine in the DNA molecule appears to be non-random (Shapiro and Chargaff, 1960; Smith and Markham, 1952). In Novikoff hepatoma cells the 5-methylcytosine is associated with pyrimidine isostichs (Sneider, 1971) and virtually all is associated with the doublet 5MeCpG (Sinsheimer, 1955). Use of antiserum to 5-methylcytosine has shown that it is associated with the C-band regions of centromeric heterochromatin, and that mouse satellite DNA contains more 5-methylcytosine than main band DNA (Miller et al., 1974).
Probably the most significant finding, in the present context, is that the methylated bases produced by direct chemical alkylation are normally absent from mammalian DNA. However, reports of the presence of minor methylated bases other than 5-methylcytosine have been made, for example Culp et al (1970) claimed the presence of \(N\)-3 methylcytosine in human embryonic lung cells and \(N\)-3 methylcytosine, \(N\)-1 methylguanine, \(N\)-7 methylguanine, \(N^2\)-methylguanine and \(N^2,N^2\)-dimethylguanine in Hela cell DNA. These experiments in Hela cells have since been repeated by Lawley et al (1972) who found that no methylated base other than 5-methylcytosine was present in such DNA. **In vivo** studies in the rat (Craddock et al, 1968) failed to detect 7-methylguanine in liver DNA following \(^{14}\text{C}-\text{methyl}\) methionine whilst its presence was clearly demonstrable in the RNA fraction. These findings were later confirmed in a study using selenomethionine, which as selenoadenosylselenomethionine (active selenomethionine) can apparently act as a methyl donor in place of \(\text{S}\)-adenosyl methionine (Craddock, 1972).

Evidence for the urinary excretion of methylated bases in the human dates back to 1898 when the presence of 7-methylguanine was documented by Kruger and Saloman (1898a; 1898b). However, it was not until the work of Weissmann et al (1957a; 1957b) that the full range of methylated base excretion became apparent. Chheda (1975) has reviewed the current situation which is summarised in Table 3. The origin of these bases would appear to be the methylated bases present in the various RNA-species, released from the RNA during its metabolic degradation. As they
Table 3 - Purine and Pyrimidine Derivatives Excreted in Normal Human Urine (after Chheda, 1975)

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>1.4</td>
</tr>
<tr>
<td>1-methyladenine</td>
<td>0.3-0.4</td>
</tr>
<tr>
<td>N6-methyladenine</td>
<td>0.37</td>
</tr>
<tr>
<td>Adenosine</td>
<td>Trace</td>
</tr>
<tr>
<td>1-methyladenosine</td>
<td>Not quantified</td>
</tr>
<tr>
<td>N6-methyladenosine</td>
<td>10-14</td>
</tr>
<tr>
<td>S-adenosylmethionine</td>
<td>23.8</td>
</tr>
<tr>
<td>Cytosine</td>
<td>0.5-0.6</td>
</tr>
<tr>
<td>3-methylcytosine</td>
<td>1.1</td>
</tr>
<tr>
<td>Cytidine</td>
<td>Trace</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.3-1.3</td>
</tr>
<tr>
<td>1-methylguanine</td>
<td>0.6</td>
</tr>
<tr>
<td>N2-methylguanine</td>
<td>0.3-0.6</td>
</tr>
<tr>
<td>N2N2-dimethylguanine</td>
<td>Trace</td>
</tr>
<tr>
<td>7-methylguanine</td>
<td>2.7-7.8</td>
</tr>
<tr>
<td>8-hydroxy-7-methylguanine</td>
<td>1.6</td>
</tr>
<tr>
<td>Guanosine</td>
<td>Trace</td>
</tr>
<tr>
<td>N2N2-dimethylguanosine</td>
<td>1.1-2.2</td>
</tr>
<tr>
<td>N2-methylguanosine</td>
<td>0.2-0.3</td>
</tr>
<tr>
<td>1-methylguanosine</td>
<td>0.4-0.6</td>
</tr>
<tr>
<td>Uracil</td>
<td>3.7-13.7</td>
</tr>
<tr>
<td>5-methyluracil</td>
<td>3.5</td>
</tr>
<tr>
<td>5-hydroxymethyluracil</td>
<td>Trace</td>
</tr>
<tr>
<td>Xanthine</td>
<td>2.8-8.7</td>
</tr>
<tr>
<td>1-methylxanthine (1)</td>
<td>Not quantified</td>
</tr>
<tr>
<td>3-methylxanthine (2)</td>
<td>3.0</td>
</tr>
<tr>
<td>7-methylxanthine</td>
<td>0.7-7.0</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>6.8</td>
</tr>
<tr>
<td>1-methylhypoxanthine</td>
<td>0.4-0.9</td>
</tr>
<tr>
<td>Inosine</td>
<td>0.18</td>
</tr>
<tr>
<td>1-methylinosine</td>
<td>2.1-2.9</td>
</tr>
<tr>
<td>1-methylnicotinamide</td>
<td>2.8</td>
</tr>
</tbody>
</table>

(1) Young et al, 1971
(2) Butts et al, 1971
represent post-synthetic modifications to the RNA they would not be re-utilised, and there would therefore seem little advantage to be gained by their salvage. Consequently they are excreted unchanged in the urine. This conclusion may be supported by the fact that no methylated nucleotide or nucleoside precursor pools have been detected in tissue extracts or bacterial cells (Borek, 1963). Also, if chemically prepared methylated bases are offered to the intact organism they are not incorporated and in the case of 7-methylguanine, are excreted largely unchanged in the urine (Borek, 1963; Craddock et al, 1968b). With the exception of xanthine, 1-methylxanthine, 3-methylxanthine and 7-methylxanthine (Young et al, 1971; Butts et al, 1971; van Gennip et al, 1973) the concentrations of methylated bases in urine are independent of diet. The fact that administration of $[^{14}\text{C}}$-methyl)methionine to the intact animal results in the labelling of the urinary purines (Mandel et al, 1966; Ahonen et al, 1972) argues very strongly for their endogenous origin.

The daily excretion of 7-methylguanine has been determined in the rat. Craddock and Magee (1967) reported a daily excretion of 65 µg., Chu and Lawley (1975a) have reported an excretion rate of 90 µg/day. Administration of DMN (30 mg.kg$^{-1}$) caused a 2-fold increase in the urinary excretion of 7-methylguanine (Chu and Lawley, 1975a). That at least part of this was derived from DNA was demonstrated using rats with DNA pre-labelled with sodium $[^{14}\text{C}}$formate (Magee et al, 1967; Craddock and Magee, 1967). However, after treatment with MMS (100mg.kg$^{-1}$) urinary levels of 7-methylguanine remained at the control level. Whilst
it can be clearly demonstrated that radioactivity derived from
the methyl group of $^{14}$C]MMS can be transferred to urinary 7-
methylguanine (Chu and Lawley, 1974), the position of this label
in the 7-methylguanine molecule is unknown.

Although the urinary excretion of methylated xanthines
probably reflects dietary intake it has been suggested that
3-methylxanthine may be derived from 3-methyladenine. This latter
base, the most abundant of the minor products generated by
the interaction of methylating agents and DNA, is not normally
recognised as being present in RNA. However, it has been reported
as a product in E. coli and yeast t-RNA following hypermethylation
with t-RNA methylase derived from various hamster tissues
(McFarlane and Lee, 1970; McFarlane, 1972). These findings were
not confirmed by Jackson and Pegg (1975). However, this issue
must remain open in view of the description (Axelrod and Daly,
1962) of a methylase that is capable of methylating adenine
at the $N$-3 position.

It is therefore apparent that the measurement of radioactivity
contained in urinary purines cannot be utilised as a method
for quantitatively measuring in vivo alkylating reactivity
and its utility in the qualitative sense is also severely limited.
Demonstration of alkylating reactivity in vivo must therefore
rest on the isolation of methylated bases from cellular nucleic
acids, and in this context it would appear that DNA with its
very low background of natural methylations is the best receptor
molecule for such studies.
1.3 Alkylating activity of phosphorus esters

Esters of pentavalent phosphorus constitute the most important class of biologically active organophosphorus compounds. They are employed extensively as insecticides in both commercial and domestic situations, in many cases replacing the more environmentally and biologically persistent organochlorine compounds. With few exceptions these organophosphate pesticides are neutral esters of phosphoric acid or phosphorothionic acid (its sulphur analogue). In such esters the phosphorus atom tends to be electron deficient due to the polarised phosphoryl groups. Thus there is a net positive charge on the phosphorus atom, which as a consequence displays electrophilic characteristics. Nucleophilic attack on the organophosphates by hard nucleophiles (OH\(^-\), HPO\(_4\)^{2-}\) is centred at phosphorus, as shown in Equation III.

![Equation III](image)

The net result of such an attack is phosphorylation of the nucleophile. It is by this reaction that the organophosphorus insecticides are thought to exert their biological effect. Thus attack of the phosphorus atom by the serine hydroxyl situated at the active esteratic site of acetylcholinesterase, activated as a nucleophile by the presence of the imidazole group of an adjacent histidine residue, results in phosphorylation
of the serine hydroxyl and inactivation of the enzyme.

Nucleophilic attack of the organophosphates by soft nucleophiles (RSH, I⁻, S₂O₃²⁻) is usually directed towards the α-carbon of the ester grouping rather than the phosphorus atom. This results, as shown in Equation IV in alkylation and not phosphorylation of the nucleophile.

\[
\begin{align*}
\text{R''-X-H} & \quad \rightarrow \quad \text{R''XCH₃} + \\
\text{CH₃-O-P} & \quad \text{OR'} \quad \rightarrow \quad \text{CH₃-O-P} \quad \text{OR'}
\end{align*}
\]

Equation IV

The alkylating reactivity of the non-insecticidally active phosphorus esters has been exploited for many years in synthetic chemistry. Trialkyl phosphates, for example, have been used to alkylate amines (Billman et al, 1942; Thomas et al, 1946), aliphatic alcohols (Toy, 1944), thiourea (Parker and Smith, 1961) arylamines (Jones et al, 1966) and imidazole (Yamanchi and Kiroshita, 1973a). The alkylation of molecules of greater biological significance, have been recently reported to occur under non-physiological conditions. Yamanchi and Kiroshita (1973b) demonstrated the alkylation of the pyrimidines thymine and uracil with trimethyl phosphate. In subsequent studies the same authors demonstrated the alkylation of a range of purines, namely xanthine, theophylline, theobromine and adenine by trialkyl phosphates (Yamanchi and Kiroshita, 1975).
Although such experimentation has demonstrated that organophosphates can act as alkylating agents, the conditions employed are too vigorous for any meaningful extrapolation to in vivo. This was recognised by Yamanchi et al. (1976) who have studied the alkylating reactivity of trimethylphosphate towards the free bases cytosine, thymine, uracil, adenine and guanine under much milder conditions (aqueous solution, pH 9-12 at 35-60°C). Employing at least a 3-fold molar excess of trimethylphosphate methylation of all bases was demonstrated as follows: cytosine N-1 > N-3, thymine N-1 = N-3, uracil N-1 = N-3, adenine N-9 = N-3 > N-7 > N-1 and guanine N-1 > N-7 > N-3 > N-9 > O6.

Two interesting points emerged from their study, firstly the N-7 atom of guanine was not the major reaction site and secondly that reaction at the O6 atom of guanine was detected, not alone but in a trimethylated derivative (O6, 3, 7-trimethylguanine).

Although poorly documented alkylation of these nucleophilic centres has been demonstrated in DNA, Kononova and Gumarou (1971) demonstrated the in vitro alkylation by trimethylphosphate of the DNA of bacteriophage T4B and were able to identify 7-methylguanine and 1-methyladenine as the reaction products. Rosenkranz and Rosenkranz (1972) also demonstrated an interaction between DNA and trimethylphosphate in vitro. Whilst these workers did not identify the products of the reaction with DNA, they found a reduction in the sedimentation coefficient of DNA following treatment with organophosphates.

Information on the alkylating reactivity of the insecticidal organophosphorus esters is sparse in comparison with their
phosphorylating reactivity. The preference of soft nucleophiles for the α-carbon of the ester has provided a convenient route to the preparation of des-alkyl derivatives (Hilgetag and Teichmann, 1965). In addition many unwanted modifications that occur during the manufacture and storage of organophosphates are attributable to alkylating reactions. A good example is provided by demeton-S-methyl which, during storage, is converted into a sulphonium compound of much greater toxicity. This was shown by Heath and Vandekar (1957) to be due to one molecule alkylating the sulphide group of another (Equation V).

\[
2(CH_3O)_2PSCH_2CH_2SC_2H_5 \rightarrow (CH_3O)_2PSCH_2CH_2S-C_2H_5 + (CH_3O)_2PSCH_2CH_2SC_2H_5
\]

Equation V

Eto and his co-workers have studied the alkylating reactivity of the saligenin cyclic phosphorus esters and have shown that they will alkylate mercaptans such as glutathione (Eto et al., 1968) and cysteine (Ohkawa and Eto, 1969). They have also shown that these compounds will react with and inhibit the 'SH enzymes' such as papain and yeast alcohol dehydrogenase (Ohkawa and Eto, 1969). Despite the possible implications of these findings to the toxicity of the organophosphorus pesticides only one such compound 2,2-dichloroethenyl dimethyl phosphate (Fig. 7a, dichlorvos) has been studied in any detail. Låforoth et al. (1969) reported the in vitro alkylating reactivity of this compound towards a number of nucleophiles including water, chloride ions, several mercaptans and 4-(4-nitrobenzyl)pyridine. This list of nucleophiles
Fig. 7 Organophosphorus pesticides examined for \textit{in vivo} alkylation reactivity
has since been extended to include guanosine (Löfroth, 1970; Rosenkranz and Rosenkranz, 1972), isolated DNA (Löfroth, 1970; Rosenkranz and Rosenkranz, 1972; Wennerberg, 1973; Wennerberg and Löfroth, 1974; Lawley et al., 1974). A detailed comparative study of the reaction of organophosphates including dichlorvos and established alkylating agents with 4(4-nitrobenzyl)pyridine by Bedford and Robinson (1972) showed dichlorvos to be less reactive than both MMS and DMS, but more reactive than trimethylphosphate, which gave only a very weak positive reaction. Studies with cells in culture have also shown that dichlorvos is capable of alkylating the nucleic acids of both bacterial cells (Wennerberg, 1973; Lawley et al., 1974; Wennerberg and Löfroth, 1974) and Hela cells (Lawley et al., 1974).

These essentially in vitro studies demonstrate that phosphoric acid triesters such as dichlorvos possess alkylating properties. Dichlorvos methylates DNA via an $S_N^2$ mechanism. In this respect dichlorvos resembles MMS but of the two, the organophosphate is much the weaker alkylating agent. However, the results obtained in these model systems cannot be expected to provide a reliable evaluation of in vivo reactivity and are therefore of little relevance to the assessment of in vivo alkylating reactivity in the mammal.

Several attempts have been made, with varying degrees of success, to measure the in vivo alkylating reactivity of organophosphorus pesticides. Wennerberg (1973) examined the DNA and RNA fractions derived from the lungs and livers of rats and mice exposed to [Me-$^{14}$C]dichlorvos by inhalation or i.p. injections. Whilst labelling of the purine ring systems via
the 1-C pool was readily detected, no 7-methylguanine was found in either DNA or RNA fractions. However, examination of the urine from these and other experiments (Wennerberg and Löfroth, 1974) revealed the presence of radiolabelled N'-methylnicotinamide, 7-methylguanine and 3-methyladenine. The detection of these radiolabelled methylated bases, which with the possible exception of 3-methyladenine are normal excretion products, was taken as evidence for in vivo alkylating reactivity. The biological half-lives for radiolabelled 7-methylguanine and 3-methyladenine were approximately 50 hours and 20 hours respectively.

Subsequently, a similar approach has been adopted by Dedek et al (1976) to study the in vivo alkylating reactivity of trichlorophone and butonate (Fig. 7 b & c) administered intraperitoneally to mice at high dose levels. These workers were able to detect the presence of radiolabelled 7-methylguanine in a combined nucleic acid (DNA and RNA) fraction derived from a number of organs. However, the radioactivity had a short life in the nucleic acid fraction, being undetectable in animals killed 24 hours or later after administration of the compound. The overall half-life of 7-methylguanine, as measured by urinary excretion, was \( <24 \) h following trichlorphon and 2.0 h following butonate. Thus, although there is evidence which indicates a degree of methylating activity, there is no evidence to implicate DNA as the target, furthermore such an experimental approach would not discriminate between spontaneous and enzymatic methylation.
By analogy with the powerful alkylating agents, the demonstration of the, albeit weak, alkylating properties of dichlorvos led to a suspicion that this compound might be a mutagen and a carcinogen (i.e., genotoxic). However, although very high concentrations of dichlorvos, relative to those encountered in practical use situations, can cause mutations in bacterial cells there is no evidence that this compound produces genotoxic effects in mammals (Table 4). Thus, dichlorvos has been thoroughly evaluated for mutagenicity and carcinogenicity in mammals at dose levels of up to at least 100 times those achieved in practical use conditions and the results of these tests have been entirely negative (Voogd et al., 1972; Buselmaier et al., 1972; Dean, 1972b; Epstein et al., 1972; Dean and Thorpe, 1972a, 1972b; Witherup et al., 1971).

Tests for mutagenic effects in mammals exposed to dichlorvos have included dominant lethal assays in male and female mice (Epstein et al., 1972; Dean and Thorpe, 1972a; Dean and Blair, 1976) investigations of the chromosomes of bone marrow cells and spermatogonia in mice and Chinese hamsters (Dean and Thorpe, 1972b) and host-mediated assays (Voogd et al., 1972; Buselmaier, 1972; Dean et al., 1972). None of these studies has given any indication that dichlorvos by any route of exposure exerts mutagenic effects.

In vitro tests employing mammalian cells have also failed to show any compound-related chromosome aberrations even at concentrations which gave rise to cytotoxicity (Dean, 1972b).
Table 4 - Mutagenicity Tests with Dichlorvos

### a. Bacterial systems

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli WP2</td>
<td>Negative</td>
<td>(1)</td>
</tr>
<tr>
<td>Escherichia coli WP2</td>
<td>Positive</td>
<td>(2,3,4)</td>
</tr>
<tr>
<td>Escherichia coli W3110</td>
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<td>(6)</td>
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<tr>
<td>Escherichia coli W3478</td>
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<tr>
<td>Escherichia coli WP67</td>
<td>Positive</td>
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</tr>
<tr>
<td>Escherichia coli CM561</td>
<td>Negative</td>
<td>(5)</td>
</tr>
<tr>
<td>Escherichia coli WP12</td>
<td>Negative</td>
<td>(5)</td>
</tr>
<tr>
<td>Escherichia coli CM57</td>
<td>Negative</td>
<td>(5)</td>
</tr>
<tr>
<td>Escherichia coli CM611</td>
<td>Negative</td>
<td>(5)</td>
</tr>
<tr>
<td>Escherichia coli K12</td>
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<td>(7,9)</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Negative</td>
<td>(5)</td>
</tr>
<tr>
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<td>(5)</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>(3)</td>
</tr>
<tr>
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<td>(3)</td>
</tr>
<tr>
<td>Salmonella typhimurium C 117</td>
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</tr>
</tbody>
</table>

(1) Dean, 1972a  
(2) Bridges et al, 1973  
(3) Shirasu et al, 1976  
(4) Ashwood-Smith et al, 1972  
(5) Hanna and Dyer, 1975  
(6) Rosenkranz, 1973  
(7) Mohn, 1973  
(8) Dyer and Hanna, 1973  
(9) Voogd et al, 1972

### b. Mammalian systems

<table>
<thead>
<tr>
<th>Species</th>
<th>Test system</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human lymphocytes</td>
<td>Chromosome analysis</td>
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<td>(1)</td>
</tr>
<tr>
<td>Chinese hamsters</td>
<td>Azaguanine resistance</td>
<td>Negative</td>
<td>(2)</td>
</tr>
<tr>
<td>Mice (male)</td>
<td>Dominant lethal assay</td>
<td>Negative</td>
<td>(3)</td>
</tr>
<tr>
<td>Mice (male and female)</td>
<td>Dominant lethal assay</td>
<td>Negative</td>
<td>(4)</td>
</tr>
<tr>
<td>Mice (male)</td>
<td>Dominant lethal assay</td>
<td>Negative</td>
<td>(5)</td>
</tr>
<tr>
<td>Mice</td>
<td>Host-mediated assay</td>
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<td>(6)</td>
</tr>
<tr>
<td>Mice and hamsters</td>
<td>Host-mediated assay</td>
<td>Negative</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>Cytogenetic studies</td>
<td>Negative</td>
<td>(8)</td>
</tr>
</tbody>
</table>

(1) Dean, 1972b  
(2) Green et al, 1974  
(3) Epstein et al, 1972  
(4) Dean and Blair, 1976  
(5) Dean and Thorpe, 1972a  
(6) Dean et al, 1972  
(7) Buselmaier et al, 1972  
(8) Dean and Thorpe, 1972b
The consistently negative results with respect to mutational events in mammalian germ cells and the absence of genetic effects in suspensions of mammalian somatic cells, is complemented by negative results in mammalian carcinogenicity studies. Rats were fed diets containing dichlorvos (up to nominal concentrations of 500 ppm) for 2 years and no compound-related tissue damage or carcinogenic responses were observed (Witherup et al., 1971). A more recent 2 year study (Blair et al., 1976) where rats were exposed for 23 hours daily to atmospheres containing 0.05, 0.5 and 5.0ug dichlorvos per litre, failed to establish any carcinogenic effects attributable to exposure to dichlorvos.

It has been suggested (Lawley et al., 1974) that the failure of dichlorvos to induce genotoxic effects in mammals is due to the limiting effect of the known rapid metabolic degradation of this compound on the extent of methylation of DNA in vivo. Such metabolism (Hutson and Hoadley, 1972a,b) leads to the loss of methylating potential as evidenced by the negative results obtained in host-mediated assays (Voogd et al., 1972; Buselmaier, 1972; Dean et al., 1972) and the non-reactivity of the metabolic products, des-methyldichlorvos and dimethylphosphate in the 4-(4-nitrobenzyl)pyridine colour reaction (Bedford and Robinson, 1972).
1.4 Aims of the present study

The experimental work described in this thesis was specifically designed to investigate, at the molecular level, the genotoxic potential of dichlorvos \textit{in vivo}. In comparative studies with MMS interactions with DNA were assessed in both chemical and biological terms, i.e. examining the nature of the reaction products with DNA and the effect on DNA integrity respectively.

In addition, a comparative examination of the urinary excretion of methylated bases following exposure to alkylating agents and 1-C pool donors was performed. These latter experiments were designed to assess the utility of methylated urinary purine measurements in the determination of \textit{in vivo} alkylating reactivity.
CHAPTER 2

Materials and syntheses

2.1 Materials

Ion-exchange resin, Dowex AG 50 W x 8 (\(^+\)H form; 100-200 mesh) was purchased from Bio-Rad Laboratories, Bromley, Kent. Blue dextran, Sephadex G-10, 2-amino-6-chloropurine, glutathione (reduced), S-methylcysteine, S-methylglutathione, protease (type VI from \textit{Streptomyces griseus}), ribonuclease A (type III from bovine pancreas) and DNA (type I, calf thymus) were purchased from the Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey. The reference bases adenine sulphate, 2-methyladenine hemisulphate, 6-methylaminopurine, guanine, 7-methylguanine, thymine, uracil, cytosine, 5-methylcytosine, xanthine and hypoxanthine were also obtained from Sigma Chemical Co. Ltd. The methylated bases, 1-methyladenine, 3-methyladenine, 7-methyladenine, and 9-methyladenine were purchased from Cyclo Chemicals Inc., Los Angeles, California, USA. Methyl methanesulphonate, dimethylnitrosamine and diethyl pyrocarbonate were obtained from Ralph Emanuel Ltd., Wembley, Middlesex. Tri-isopropynaphthalene sulphonate was purchased from Kodak Ltd., Kirkby, Liverpool. m-Cresol (GPR grade, redistilled before use), phenol (Analar), 8-hydroxyquinoline (Analar), sucrose (Aristar), sodium dodecyl sulphate ('specially pure grade'), hydrochloric acid (Analar), perchloric acid (72% w/v, Aristar), ammonium formate (Analar), sodium 4-aminosalicylate, \(t\)-butanol and methyl ethyl ketone (butanone, specially pure for chromatography) were purchased from British Drug Houses Ltd., Poole, Dorset. All other chemicals and reagents were of the
purest grade available and were purchased from British Drug Houses Ltd., or Hopkin and Williams, Romford, Essex. Chromatography paper was obtained from Whatman LabSales Ltd., Maidstone, Kent. Thin-layer cellulose and silica gel plates (Merck) were obtained through Anderman and Co. Ltd., London.

\[\text{[Methyl-}^2\text{H}_2\text{]}\text{Dichlorvos}:\] This compound, synthesised from deuteromethanol and 2,2-dichlorovinyl phosphodichloridate was a gift from Dr. D. H. Hutson.

\[\text{Dimethyl napthylphosphate}:\] Synthesised from 1-napthol and dimethyl phosphoryl chloride was also a gift from Dr. D. H. Hutson.

2.2 \textbf{Radiochemicals}

\[\text{[Methyl-}^{14}\text{C]}\text{methanesulphonate (56mCi.mmol}^{-1})\]; \text{Di[Methyl-}^{14}\text{C]}\text{ sulphate (46mCi.mol}^{-1})\]; \text{L-}[\text{Methyl-}^{14}\text{C]}\text{methionine (56mCi.mmol}^{-1})\]; \text{sodium [}^{14}\text{C]}\text{formate (56mCi.mmol}^{-1})\]; \text{[6-}^{14}\text{C]}\text{orotic acid monohydrate (57mCi.mmol}^{-1})\]; \text{[8-}^{14}\text{C]}\text{adenine (54.2mCi.mmol}^{-1})\]; \text{[8-}^{14}\text{C]}\text{guanine sulphate (56mCi.mmol}^{-1})\] and \[\text{[Methyl-}^3\text{H]}\text{thymidine (20Ci.mmol}^{-1})\] were purchased from the Radiochemical Centre, Amersham. Before use the radiochemical purity of all materials with the exception of \[\text{[}^{14}\text{C]}\text{MMS}\] and \[\text{[}^{14}\text{C]}\text{DMS}\] was checked by paper or thin-layer chromatography (Table 5).

\[\text{[Me-}^{14}\text{C]}\text{Dichlorvos}:\] This compound (113Ci.mol}^{-1}) was synthesised at Shell Biosciences Laboratory, Sittingbourne Research Centre, from \[\text{[}^{14}\text{C]}\text{methanol and 2,2-dichlorovinyl phosphodichloridate.}\]

\[
2\text{ }^{14}\text{CH}_3\text{OH} + \begin{array}{c} \text{Cl} \\ \text{OCH}=\text{CCl}_2 \end{array} \xrightarrow{\text{P}} \begin{array}{c} \text{Cl} \\ \text{OCH}=\text{CCl}_2 \end{array} \rightarrow \begin{array}{c} \text{14CH}_3\text{O} \\ \text{OCH}=\text{CCl}_2 \end{array} + 2\text{ HCl}
\]

The radiochemical was stored at \(-20^\circ\) as a solution (2 mg.ml}^{-1}) in di(2-ethylhexyl)-adipate.
Table 5 - Purity of Commercially Available Radiochemicals

<table>
<thead>
<tr>
<th>Radiochemical</th>
<th>Support</th>
<th>Solvent</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>[8-(^{14})C]Adenine</td>
<td>Paper</td>
<td>1,2</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>Thin-layer silica</td>
<td>3</td>
<td>99%</td>
</tr>
<tr>
<td>[8-(^{14})C]Guanine</td>
<td>Paper</td>
<td>1,2</td>
<td>99%</td>
</tr>
<tr>
<td>L-[methyl-(^{14})C]methionine</td>
<td>TLC cellulose</td>
<td>4,5,6</td>
<td>99%*</td>
</tr>
<tr>
<td>Sodium [(^{14})C]formate</td>
<td>TLC silica</td>
<td>7</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>Cellulose</td>
<td>8</td>
<td>99%</td>
</tr>
<tr>
<td>[6-(^{14})C]orotic acid</td>
<td>Paper</td>
<td>1,5,9</td>
<td>99%</td>
</tr>
<tr>
<td>[methyl-(^{3})H]thymidine</td>
<td>TLC silica</td>
<td>3</td>
<td>99%</td>
</tr>
</tbody>
</table>

*After removal of approx. 1% of impurity, probably sulphoxide

Solvents:

System 1. isopropanol/water/hydrochloric acid (130:37:33)
System 2. t-butanol/methyl ethyl ketone/water/ammonia (4:3:2:1)
System 3. n-butanol saturated with water
System 4. n-butanol/water/acetic acid (12:5:3)
System 5. ethanol/ammonia/water (80:4:16)
System 6. n-butanol/pyridine/water (1:1:1)
System 7. ethanol/ammonia (3:1)
System 8. ethanol/2N sodium hydroxide/water (144:32:14)
Mass spectrometry

For the subsequent experimentation it was necessary to confirm the isotopic abundance with respect to $^{14}$C in the methyl groups of the [Me-$^{14}$C]dichlorvos. Mass spectrometry provided the most convenient method for measuring the isotopic distribution. However, owing to the very high specific radioactivity of the [Me-$^{14}$C] dichlorvos a full spectral analysis was not possible. The isotopic abundance in the methyl groups of the parent compound was therefore assessed by multiple ion monitoring which required a smaller quantity of compound and therefore presented less of a contamination hazard. Examinations of the GC-MS spectrum obtained with an analytically pure sample of dichlorvos (Fig. 8) showed, in common with many organophosphates, a very weak parent ion (Safe and Hulzinger, 1973). The most abundant ion occurred at M/e 109 corresponding to $[(\text{CH}_3\text{O})_2\text{P}=0]^+$ and was at least ten times as intense as the parent ion. Analysis of dichlorvos containing deuterium in the methyl groups (Fig. 9) showed a shift in the parent ion of 6 AMU. The ion present at M/e 115 was exactly that expected for the $[(\text{C}^2\text{H}_3\text{O})_2\text{P}=0]^+$ ion. Subsequent analysis of the sample of [Me-$^{14}$C]dichlorvos for ions occurring at M/e 109, M/e 111 and M/e 113 corresponding to $[(\text{C}^{12}\text{H}_3\text{O})_2\text{P}=0]^+$, $[(\text{C}^{12}\text{H}_3\text{O})\ (\text{C}^{14}\text{H}_3\text{O})\text{P}=0]^+$ and $[(\text{C}^{14}\text{H}_3\text{O})_2\text{P}=0]^+$ respectively (Fig. 10) failed to detect any non-radioactive molecules. However, approximately 15% of the total molecules had only one radiolabelled methyl group. Thus the isotopic abundance of $^{14}$C in the methyl groups approximated to 92.5% of theoretical.
Fig 8 GC–Mass spectrum of an analytically pure sample of dichlorvos
Fig 9  GC–Mass spectrum of deuterated dichlorvos
Fig 10 GC—Mass spectrum of dichlorvos obtained by multiple ion monitoring
S-[Methyl-\textsuperscript{14}C]glutathione: This compound was synthesised by reacting glutathione (16\textmu M) in 1ml 0.05M phosphate buffer pH 7.6 with di-[\textsuperscript{14}C]methyl sulphate (10.88\textmu M, 500\textmu Ci) in 1ml phosphate buffer, for 2h at 37\degree. After removal of residual dimethylsulphate in vacuo the residue was dissolved in 0.5ml water and was purified by chromatography on Whatman 3MM paper in butan-1-ol/acetic/acid water (11:4:5, v/v; Rf 0.45). The radioactive band was eluted with water and the extract was dried in a stream of dry nitrogen. The product, obtained in 50% yield (250\textmu Ci), chromatographed as a single spot coincident with unlabelled S-methylglutathione on cellulose TLC in ethanol/water/ammonia (90:5:5 v/v), and on silicic acid TLC in butan-1-ol/acetic/water (4:2:1, v/v). Following hydrolysis in 4M HCl for 2h at 100\degree, a sample of the S-[\textsuperscript{14}C]methylglutathione gave a single spot coincident with marked S-methylcysteine when chromatographed on cellulose thin-layers developed with butan-1-ol/acetic acid/water (4:2:1, v/v).

2.3 Synthesis of reference compounds

3-Methyladenine: This compound was prepared by refluxing adenine and trimethylphosphate in dimethylformamide (Yamauchi et al, 1975). The product obtained in 43% yield was recrystallised from ethanol (Found: C 48.30; H 4.73; N 46.95; Calc: For C\textsubscript{6}H\textsubscript{7}N\textsubscript{3}; C 48.32; H 4.73; N 46.95). Mass spectrometry revealed a parent ion at M/e 149 and the spectrum (Fig. 11) was identical with that obtained with the authentic compound. Paper chromatography (Whatman 3MM) in t-butanol/methyl ethyl ketone/ammonia/water (4:3:2:1, v/v) gave a single spot, Rf 0.62. Column chromatography
Fig 11 Mass spectrum of 3-methyl-6-amino purine
(8 most intense peaks are indicated)
on Sephadex G-10 eluted with 0.05M ammonium formate pH 6.8 gave a single peak eluting at fractions 24-29. This component behaved identically with the authentic compound during co-chromatography in this system.

\( 0^6 \)-Methylguanine: This compound was synthesised by refluxing 2-amino-6-chloropurine with sodium methoxide (Balsinger and Montgomery, 1964). The product obtained in 80% yield was recrystallised from water (Found: C 43.6; H 4.25; N 42.37; 
Calc: For \( \text{C}_6\text{H}_{15}\text{N}_5\text{O}_5 \): C 43.63; H 4.25; N 42.37). Mass spectrometry (Fig. 12) showed a parent ion at M/e 165 and was identical with the of \( 0^6 \)-methylguanine obtained by the alkylation of guanine with diazomethane (Friedman et al, 1965). Paper chromatography (Whatman 3MM) in t-butanol/methylethyl ketone/ammonia/water (4:3:2:1, v/v) gave a single spot (Rf 0.69). Column chromatography on Sephadex G-10 gave a single peak eluting at fractions 95-105. This component behaved identically with the authentic compound during co-chromatography in this system.

2.4 Animals

Albino rats of the CFE strain, originally supplied by Carworth Farm and then maintained as a specific pathogen free colony in this laboratory were used in these studies. Where appropriate partial hepatectomy was performed on 10-12 week old rats by the method of Higgins and Anderson (1931), 60% of the liver being removed. Labelling of liver DNA occurred during the period of restorative hyperplasia following the partial hepatectomy (as described in subsequent sections) (Bresnick, 1971).
Fig 12 Mass spectrum of 2-Amino-6-methoxy purine
(8 most intense peaks are indicated)
CHAPTER 3

Direct Alkylation Studies

3.1 Introduction

By analogy with powerful alkylating agents, the recognition and subsequent demonstration of the alkylating properties of dichlorvos suggested that this mixed triester of phosphoric acid might be a mutagen and a carcinogen. Although the results of mammalian carcinogenicity and mutagenicity studies have been consistently negative it was, nevertheless, important to determine whether dichlorvos methylated DNA in vivo.

The experimental work described in this Chapter was specifically designed to investigate this possibility. In the practical use situation, the inhalation route is the major route of exposure to dichlorvos. Consequently the inhalation route was chosen for the experimental programme. The air concentration employed in these studies was equivalent to a typical high practical use concentration (Elgar and Steer, 1972) and in order to maximise the sensitivity of detection of DNA alkylation, it was decided that the DNA from the total soft tissues of exposed animals would be examined for the presence of 7-methylguanine. Pilot studies were conducted with positive control agents (MMS and DMS) in order to establish the validity and sensitivity of the experimental procedures employed in this study.
3.2 Methods

Alkylation of DNA in vitro

Calf thymus DNA (ca. 50mg; type I) was dissolved in 8ml of 8mM sodium phosphate buffer, pH 7.6 containing 2mM EDTA at 4°C. A solution of the alkylating agent, \([^{14}\text{C}]\text{MMS} \) (250μCi; 4.8μmoles) or \([^{14}\text{C}]\text{dichlorvos} \) (250μCi; 2.2μmoles) in 2 ml of buffer was added and the mixture incubated for 4h at 37°C. After incubation 0.5ml of 2M NaCl was added, and the DNA was precipitated by the addition of ethanol (12ml). The methylated DNA was collected on a glass-rod and washed successively with ethanol 0.25M sodium acetate solution (3:1, v/v), five times, ethanol, ethanol/diethyl ether (1:1, v/v), and, finally with diethyl ether. After drying over P₂O₅ and paraffin wax (to remove traces of ether) the methylated DNA was stored, desiccated as a felt, at -20°C.

Hydrolysis of DNA

Marker 7-methylguanine (approx. 100μg per mg DNA) was added to all DNA samples prior to hydrolysis. Two methods of hydrolysis were employed, (i) In some of the in vitro experiments, samples of DNA were hydrolysed in 0.1M HCl (250μl per mg DNA) at 70°C for 1h; (ii) In the in vivo experiments and in some of the in vitro experiments samples of DNA were hydrolysed in 72% (w/v) perchloric acid (50μl per mg DNA) for 1h at 100°C. Prior to chromatography these latter hydrolysates were diluted to approximately 1M, with respect to the perchloric acid, by the addition of distilled water.
Fractionation of DNA hydrolysates

Two methods of column chromatography were employed in these studies. Perchloric acid hydrolysates and some hydrochloric acid hydrolysates were chromatographed on Dowex 50. Dowex AG50 W x 8, (100-200 mesh) H⁺ form was washed exhaustively with 4M HCl and then to neutrality with glass distilled water prior to packing into glass columns (1.5cm x 12cm or 35cm x 3.5cm) fitted with a sintered glass disc. The DNA hydrolysates were applied to the columns in 1M perchloric acid and washed on to the resin with distilled water (10ml for the small column and 50ml for the large column). The columns were then eluted with a linear gradient of 1 to 4M HCl at a flow rate of 25 ml h⁻¹. Individual fractions of 99 drops (6.4ml) were collected using an LKB Ultrorac fraction collector (LKB, Croydon, Surrey) equipped with a drop counter. The absorbance of each fraction was measured at 254nm using a Unicam SP 800 recording spectrophotometer. Aliquots (1ml) of each fraction were removed for the determination of radioactivity. The remainder of each of the fractions constituting radioactivity peaks were pooled, dried in a stream of dry nitrogen, and redissolved in 0.1M HCl (5ml).

Chromatography of the 0.1M HCl hydrolysates and, in some experiments, rechromatography of the 7-methylguanine peaks from Dowex chromatography (described above) were performed on Sephadex G-10. The Sephadex was stirred in distilled water containing 0.1% sodium azide for 48 hours at room temperature. It was then packed into an all-glass column (95cm x 1.5cm)
fitted with a sintered glass disc, and equilibrated with 0.05M ammonium formate, pH 6.8 (generally 4 litres). After the addition of blue dextran (to mark the void volume) and any other marker bases that were required, the samples in 0.1M HCl were applied directly to the column as a solution in 0.1M HCl and were then eluted with 0.05M ammonium formate pH 6.8. Individual fractions of 99 drops (5.9ml) were collected, and the absorbance of each fraction was determined as described above.

**Measurement of radioactivity**

Radioactivity was determined by liquid scintillation spectroscopy which was performed in a Packard Tri-Carb (Model 526 or 3003) spectrometer. Samples (1ml) of nucleic acid hydrolysates and the column eluant fractions were blended with NE 260 scintillator mixture (10ml). Such samples regularly counted with an efficiency of 60-65% as determined by the channels ratio method.

**Animal experimentation**

The apparatus used, in the inhalation experiments with [Me-\(^{14}\)C]dichlorvos, consisted of five separate atmosphere generators supplying five individual rat holders. Each holder was sealed into a polythene bag to minimise the risk of radiochemical contamination. The generators consisted of paper-wick saturators contained in glass tubing, 14cm in length and 1.5cm outside diameter. A solution (1.5ml) of [Me-\(^{14}\)C]dichlorvos in di-(2-ethylhexyl)-adipate (0.116mg.ml\(^{-1}\)) was applied to each wick. The atmosphere generated from these wicks, containing 0.064µg.1\(^{-1}\)
dichlorvos (22°, flow rate, 0.46 l.min⁻¹) as measured by g.l.c.,
was brought immediately into contact with the breathing zones
of the rats at a constant flow rate (0.46 l.min⁻¹). The atmosphere
was drawn out of the head section of the rat holder, at a point
just above the head of the rat at a flow rate (0.56 l.min⁻¹)
that was marginally greater than the rate of supply. As a con­
sequence, the holders were under a slight negative pressure
which caused them to leak inwards, reducing even further the
risk of radiochemical contamination whilst ensuring efficient
removal of respired gases. This exit atmosphere was drawn suc­
cessively through a water-sealed bubbler, a silica gel drying
tube, a flow restrictor and finally an activated carbon filter
to trap the radioactive waste (Fig. 13).

Two replicate experiments were performed. In each experi­
ment, two groups of five rats were exposed to the atmosphere
containing 0.064μg.l⁻¹ [Me⁻¹⁴C]dichlorvos for a 12h period.
Based on a minute volume (Vm) of 0.13 l.min⁻¹ (Roderick, H.
personal communication) the total inhaled dose of dichlorvos
amounted to 6μg per rat. The animals were killed by decapit­
immediately at the end of the exposure period. The organs (brain,
heart and lung, liver, spleen, kidney and testes) were rapidly
dissected from the carcasses, pooled according to type, e.g.
liver, frozen in liquid nitrogen and stored at -20°. The urine,
which collected in the polythene bags during the exposure period
of the first experiment, was adjusted to pH 2 by addition of
dilute sulphuric acid and was stored at -20°.
Fig 13 Schematic diagram of the apparatus used to generate
and expose the rats to the atmosphere of [Methyl-14C] dichlorvos
The apparatus used for the $[^{14}\text{C}]\text{MMS}$ experiment was, with the exception of the atmosphere generator, identical to that described above (Fig. 13). The generator consisted of a single paper-wick type saturator contained in a glass tube 25.5cm x 3.0cm (Fig. 14). A solution (5.0ml) of $[\text{Me}^{14}\text{C}]\text{MMS}$ in di-(2-ethyl hexyl)-adipate (0.106mg.ml$^{-1}$) was applied to the wick. The atmosphere generated from this wick contained 0.064µg.l$^{-1}$ MMS (22º, flow rate 0.355 l.min$^{-1}$) calculated from determination of radioactivity. This atmosphere was then used to supply five individual rat holders, in replicate experiments, as above.

In vivo alkylation studies with $[\text{methyl}^{14}\text{C}]\text{methanesulphonate}$

Two in vivo intraperitoneal alkylation studies were performed. In the first study 10 male rats were injected intraperitoneally with 24µg $[^{14}\text{C}]\text{MMS}$ per kg body weight (2.5µCi per rat) in isotonic saline and in the second experiment 10 male rats were injected intraperitoneally with 2.4mg $[^{14}\text{C}]\text{MMS}$ per kg body weight (250µCi per rat). At the end of the four hour exposure period the animals were killed by decapitation and the brain, heart and lungs, liver, spleen, kidney, testes and alimentary canal, were rapidly dissected from the carcasses. The tissues were pooled according to type and were frozen in liquid nitrogen. These tissue pools were stored at -20ºC until required.

In vivo alkylation study with di$[\text{methyl}^{14}\text{C}]\text{ sulphate}$

A single study using 5 male rats was performed. Each animal received 530µg per kg body weight (125µCi per rat) by intraperitoneal injection and was killed four hours later. The soft tissues were removed and treated as described above.
Fig 14  Paper wick-type saturator used to generate the atmosphere of $[^{14}\text{C}]\text{MMS}$
Labelling of neonatal rat DNA with sodium $^{14}$Cformate

Litters of CFE rats, generally 14-15 per litter, were reduced in size to 10 in order to ensure maximum survival amongst the treated neonates. Commencing 24 hours after birth the pups were injected, subcutaneously for the first 5 days and then intraperitoneally, with 2.5μCi of sodium $^{14}$Cformate in saline (sterilised by filtration) daily for 3 weeks. The total cumulative dose of radioactivity was 105μCi per rat. The litters were killed by decapitation 1 hour after the last injection. The brains, heart and lungs, livers, spleens, kidneys and, where applicable testes were rapidly dissected from the carcasses, pooled according to type, frozen in liquid nitrogen and stored at -20°.

Labelling liver DNA following partial hepatectomy

A total of 50μCi of sodium $^{14}$Cformate or [methyl-$^{14}$C]methionine was administered to each partially hepatectomised rat by means of a series of intraperitoneal injections (each of 2.5μCi) at 4 hourly intervals commencing 16 hours after the operation. In all cases the animals were killed by decapitation 4 hours after the final injection.

Isolation of DNA

After thawing, the tissue pools were blotted to remove excess moisture and weighed. These, and (unless stated otherwise) all subsequent procedures, were carried out at 4°. Each pool was then passed through a pre-cooled stainless steel tissue-press into a pre-cooled, pre-weighed glass homogenising vessel. Homogenising medium, sodium p-aminosalicylate/butan-2-ol/tri-isopropynaphthalene sulphonate/distilled water (6:6:1:87 w/w),
was then added (5ml.gm$^{-1}$ pressed tissue weight). After filtering through a nylon mesh, the homogenate was adjusted to 10% w/v, by addition of homogenising medium.

An equal volume of phenol reagent (phenol/water/m-cresol/8-hydroxyquinoline; 500:55:70:0.5w/w) was added and the mixture stirred vigorously at room temperature for 20min using a magnetic stirrer. The resultant emulsion was broken by centrifugation at 10,000 x g for 45min at 4° in a Sorvall RC2-B refrigerated centrifuge equipped with a GS-3 rotor. The phenol phase was separated from the aqueous phase.

The aqueous phase, retained after the phenol extraction, was adjusted to 1% with respect to sodium chloride, washed with one half of its volume of phenol reagent and re-centrifuged as described above. After carefully removing the aqueous phase, it was mixed with an equal volume of 2-ethoxyethanol which resulted in the formation of a voluminous fibrous precipitate of impure DNA. This was recovered by winding onto a glass rod. After washing in 75% aqueous ethanol the impure DNA was dissolved in 2% aqueous sodium acetate containing 1.5% sodium chloride (ca 25ml).

Ribonuclease, previously heated to 80° for 10 min and slowly cooled to destroy deoxyribonuclease activity, was then added (1mg.ml$^{-1}$ DNA solution). The mixture was incubated at 37° for 30 min. Contaminating protein was removed by incubating with pronase (1mg.ml$^{-1}$ DNA solution) for 1h at 37°. The DNA was re-precipitated with 2-ethoxyethanol, washed in 75% aqueous ethanol and then redissolved in 2% aqueous sodium acetate containing 1.5% sodium chloride. Residual polysaccharide material
was removed by centrifugation at 106,000 x g for 1h at 4° in an MSE super-speed 75 refrigerated centrifuge.

The purified DNA was precipitated from solution with 2-ethoxy ethanol, washed successively with 75%, 85%, 90%, 95% and 100% ethanol, and finally with diethyl ether before drying in vacuo over phosphorus pentoxide. Samples of approximately 100μg of each DNA fraction were removed for the determination of DNA content and radioactivity.

**Estimation of DNA**

DNA was estimated using a modification of the diphenylamine procedure (Burton, 1956) described by Giles and Myers (1965). Calf thymus DNA, (high molecular weight) was used in the construction of the standard curve.

**Recovery of 7-methylguanine from the scintillation mixture**

To the pool of scintillation mixture containing the 7-methylguanine fraction derived from the first experiment, and in part the second experiment, was added 1M silver nitrate (20ml) and the mixture stored at room temperature in the dark for 2 days. At the end of this period, an equal volume of redistilled acetone was added and the mixture stored at 4° for 3 days. The resulting "off-white" precipitate was collected by centrifugation in an MSE minor centrifuge (10min; 1200rpm), washed four times with redistilled acetone and then hydrolysed in 0.1M HCl (20ml; 30min; 100°). After removal of the insoluble silver salt by filtration, the clear filtrate was taken to dryness in a stream of dry nitrogen. After redissolving in 0.1M HCl (5ml) the "recovered 7-methylguanine" was purified by
chromatography on Sephadex G-10.

**Paper chromatography**

The u.v. absorbing and the radioactive peaks were subjected to paper chromatography on Whatman No.1 or 3MM paper developed in the following solvents: methanol/hydrochloric acid/water (7:3:1, v/v); isopropanol/hydrochloric acid/water (170:41:39, v/v); t-butanol/methylethyl ketone/ammonia/water (4:3:2:1, v/v).

### 3.3 Results

**In vitro studies**

Two methods of acid hydrolysis were investigated, with a view to employing column chromatography on Sephadex G-10 or Dowex 50, to resolve the alkylated bases. Figs. 15 and 16 show the resolution of mixtures of reference bases in these systems. Samples of calf thymus DNA, alkylated *in vitro* with [methyl-\(^{14}\)C]MMS, were hydrolysed in 0.1M HCl or 12N HClO\(_4\), and the hydrolysates were chromatographed on Dowex 50 columns. The chromatographic profiles are shown in Figs. 17 and 18 respectively. In both cases the major portion of the radioactivity was associated with the marker 7-methylguanine, accounting for 66% and 70.97% respectively of the recovered radioactivity. No attempt was made in these early experiments to separate 3-methyladenine and 7-methylguanine which are not resolved by chromatography on Dowex 50 columns. Based on the amount of radioactivity recovered in the 7-methylguanine fractions, (uncorrected for 3-methyladenine) and assuming that guanine
Fig 15  Chromatography of Purine bases on Sephadex G-10
Eluted with 0.05 M ammonium formate pH 6.8
Fig 16  Chromatography of DNA bases on Dowex 50 [H+] eluted with a 1-4 M HCl gradient
Fig 17 Dowex 50\([\text{H}^+]\) chromatography of the 0.1m HCl hydrolysate of DNA alkylated \textit{in vitro} with [Methyl\(—^{14}\text{C}\)] MMS
Fig 18  Ion-exchange chromatogram of DNA alkylated *in vitro* with [Methyl-$^{14}$C] MMS hydrolysed 72% perchloric acid
contributes 10% of the weight of DNA, the yields of 7-methyl-
guanine represented 0.12% and 0.13% of the available DNA guanine respectively.

A further sample of in vitro alkylated DNA was hydrolysed
in 0.1M HCl and the hydrolysate was chromatographed on Sephadex
G-10 in order to investigate the resolution of 3-methyladenine
and 7-methylguanine. The chromatographic profile is shown in
Fig. 19. Of the radioactivity recovered, 75.69% was associated
with the 7-methylguanine marker, 9.96% with the 3-methyladenine
marker and approximately 0.4% with the $^0$-methylguanine marker.
Making the same assumptions as above, 0.157% of the available
guanine in this sample of DNA had been alkylated. Attempts to
fractionate perchloric acid hydrolysates (neutralised
with potassium hydroxide) on Sephadex G-10 failed to provide
adequate resolution. The resolving power decreased still further
when the amount of hydrolysate (both hydrochloric acid and
perchloric acid) applied to the Sephadex G-10 column was increased.
Because of the relatively large amounts of DNA to be analysed
in the in vivo experiments, the technique chosen
was Dowex 50 chromatography of 72% perchloric acid hydrolysates.

Chromatography of the perchloric acid hydrolysate of a
sample of calf thymus DNA, alkylated in vitro with [methyl-$^{14}$C]
dichlorvos gave the profile shown in Fig. 20. In marked contrast
to the elution profiles obtained with [methyl-$^{14}$C]MMS alkylated
DNA, the profile from the dichlorvos-alkylated DNA demonstrated
the presence of large quantities of radioactivity eluting with
the void volume of the column. Using paper chromatography
Fig 19 Separation of 0.1M HCl hydrolysate of DNA alkylated \textit{in vitro} with [Methyl$-^{14}$C]MMS on Sephadex G-10 eluted with 0.05M ammonium formate pH 6.8
Fig 20  Dowex 50[\( ^{+} \text{H} \)] elution profile of the acid hydrolysate of calf thymus DNA alkylated \textit{in vitro} with [Methyl\(-^{14}\text{C}\)]dichlorvos
(Hutson et al., 1971) (Whatman 3MM developed in butanol/acetic/water, 11:5:4, v/v), this radioactivity was shown to be largely associated with dimethylphosphate and desmethyl dichlorvos. Radioassay of the 7-methylguanine fraction indicated that 0.004% of the total DNA guanine had been methylated by dichlorvos.

**In vivo studies**

The proposed inhalation studies with [methyl-\(^{14}\)C]dichlorvos represented a departure from the classical studies. The atmospheric concentration employed was low; equating with that of a typically high practical use concentration. A series of experiments with low doses of [methyl-\(^{14}\)C]MMS and di[methyl-\(^{14}\)C]sulphate were therefore performed as positive controls.

Table 6 shows the yields and specific radioactivities of the DNA fractions isolated from the soft-tissues of rats 4 hours after a single i.p. injection of [methyl-\(^{14}\)C]MMS (24\(\mu\)g per kg body weight). Radioactivity was associated with the DNA of all of the soft tissue pools. The chromatographic profile (Dowex 50) of the acid hydrolysate of the total soft tissue DNA is shown in Fig. 21. In addition to 7-methylguanine, radioactivity was associated with the naturally occurring bases thymine, adenine and guanine. Based on the total radioactivity associated with the 7-methylguanine peak, and assuming that all of the radioactivity resided in the methyl group and also that there was no contamination with 3-methyladenine, the overall extent of alkylation was \(387 \times 10^{-9}\) mol 7-methylguanine mol\(^{-1}\)DNA-P, \(\mu\)mol\(^{-1}\)MMS.kg\(^{-1}\) body weight.
Table 6 - Yields and specific radioactivities of DNA obtained from the soft tissues of rats exposed to 24\mu g [14C]MMS kg\(^{-1}\) body weight by intraperitoneal injection

<table>
<thead>
<tr>
<th>Organ</th>
<th>Yield DNA mg gm(^{-1}) fresh weight</th>
<th>Specific radioactivity Dpm mg(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>1.95</td>
<td>2.68</td>
</tr>
<tr>
<td>Heart and lung</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>2.14</td>
<td>1.77</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.74</td>
<td>5.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.57</td>
<td>3.92</td>
</tr>
<tr>
<td>Testis</td>
<td>1.95</td>
<td>3.24</td>
</tr>
</tbody>
</table>
Fig 21  Dowex 50 [\( ^{3}H \) ] elution profile of a DNA hydrolysate from the soft tissues of 10 rats treated with 2.5 $\mu$Ci/rat [Methyl-$^{14}$C]methanesulphonate
The yields and specific radioactivities of the DNA from the soft tissue pool DNA, from rats injected with [methyl-\(^{14}\)C] MMS (2.4 mg per kg body weight), are shown in Table 7. Chromatography of the total pooled DNA hydrolysate on Dowex 50 gave the elution profile shown in Fig. 22. Heavy labelling of the 7-methylguanine fraction was obtained, but this was accompanied by an elevated background radioactivity. Calculation of the extent of the reaction based on total radioactivity in the 7-methylguanine peak indicated an overall extent of reaction of 5.1 x 10^{-9} mol 7-methylguanine mol\(^{-1}\) DNA-P, \(\mu\)mol\(^{-1}\) MMS, kg\(^{-1}\) body weight.

During this particular experiment attempts were made to isolate a sample of DNA from the alimentary tracts of the exposed animals. These attempts were not entirely successful. Thus the addition of 2-ethoxy-ethanol to the aqueous phase of the phenol extraction produced a flocculent precipitate rather than the normal fibrous strands. Hydrolysis of the precipitate and subsequent chromatography (Dowex 50) gave the profile shown in Fig. 23. Compared with the DNA from the total soft tissue pool much higher amounts of radioactivity were associated with the naturally occurring bases of the alimentary tract DNA. This result was consistent with the rapid turnover of the cells of this latter tissue. The extent of alkylation was estimated to be 9.3 x 10^{-9} mol 7-methylguanine.mol\(^{-1}\) DNA-P, \(\mu\)mol\(^{-1}\) MMS, kg\(^{-1}\) body weight.

An analogous experiment was carried out with another alkylation agent, di-[\(^{14}\)C]methyl sulphate (530\(\mu\)g, kg\(^{-1}\), 125\(\mu\)Ci
Table 7 - Yields and specific radioactivities of DNA obtained from the soft tissues of rats exposed to 2.4 mg [14C] MMS kg⁻¹ body weight by intraperitoneal injection

<table>
<thead>
<tr>
<th>Organ</th>
<th>Yield DNA mg.g⁻¹ fresh weight</th>
<th>Specific radioactivity Dpm.mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>2.11</td>
<td>111</td>
</tr>
<tr>
<td>Heart and lung</td>
<td>4.41</td>
<td>4.44</td>
</tr>
<tr>
<td>Liver</td>
<td>2.04</td>
<td>195.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.01</td>
<td>28.4</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.92</td>
<td>268</td>
</tr>
<tr>
<td>Testis</td>
<td>2.3</td>
<td>17.37</td>
</tr>
</tbody>
</table>
Fig 22  Dowex 50 chromatography of the soft tissue DNA hydrolysate of 10 rats following i.p. injection of 250 μCi/rat [Methyl-14 C]methanesulphonate
Fig 23  Dowex 50 [H+] chromatogram of the acid hydrolysate of the "total" nucleic acid fraction derived from the alimentary canal of 10 rats each treated i.p. with 250 μCi [Methyl-14C] MMS.
per rat), the tissue distribution is shown in Table 8. Examination of the DNA hydrolysate by chromatography on Dowex 50 gave the profile shown in Fig. 24. Radioactivity was associated with the naturally occurring purines as well as the 7-methylguanine fraction. Based on the radioactivity associated with the 7-methylguanine fraction the overall extent of methylation was $356 \times 10^{-9} \text{mol } 7\text{-methylguanine } \text{mol}^{-1} \text{DNA-P, } \text{mol}^{-1} \text{DMS, kg}^{-1}$ body weight.

Having established that low levels of radiolabelled alkylating agents administered by the i.p. route lead to measurable alkylation of DNA, the inhalation experiments were performed.

The yields and specific radioactivities of the DNA fractions, isolated from the individual soft tissue pools of 10 rats exposed to an air concentration of $0.64 \mu g. l^{-1} [\text{methyl-}^{14}\text{C}] \text{MMS}$ for 12 hours are shown in Table 9. Ion exchange chromatography of the pooled DNA hydrolysates gave the elution profile shown in Fig. 25. Incorporation of considerable amounts of radioactively labelled carbon into thymine, guanine and adenine was obtained. There was also an indication of radioactivity associated with the 7-methylguanine marker peaks. The fractions comprising the 7-methylguanine peak from the Dowex column were pooled and dried in a stream of dry nitrogen. After redissolving the residue in $0.1M \text{HCl}$ it was rechromatographed on Sephadex G-10. The elution profile obtained in this experiment is shown in Fig. 26. Radioactivity was associated with the 7-methylguanine peak as well as with the adenine and guanine peaks. In addition a small amount of radioactivity was associated with the
Table 8 - Yield and specific radioactivities of DNA obtained from the soft tissues of rats exposed to 530ug [14C]DMS kg⁻¹ body weight by intraperitoneal injection

<table>
<thead>
<tr>
<th>Organ</th>
<th>Yield DNA mg.gm⁻¹ fresh weight</th>
<th>Specific radioactivity Dpm.mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>2.19</td>
<td>95</td>
</tr>
<tr>
<td>Heart and lung</td>
<td>4.57</td>
<td>3.1</td>
</tr>
<tr>
<td>Liver</td>
<td>1.97</td>
<td>172.5</td>
</tr>
<tr>
<td>Spleen</td>
<td>5.24</td>
<td>21.3</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.13</td>
<td>140</td>
</tr>
<tr>
<td>Testis</td>
<td>2.07</td>
<td>9.06</td>
</tr>
</tbody>
</table>
Fig 24  Dowex 50\[^{14}H\] elution profile of the acid hydrolysate of rat soft tissue DNA alkylated \textit{in vivo} with \[^{14}C\]dimethyl sulphate
Table 9 - Yields and specific radioactivities of DNA obtained from the soft tissues of rats exposed for 12 h to an atmosphere containing 0.064 μg [14C]MMS l⁻¹

<table>
<thead>
<tr>
<th>Organ</th>
<th>Yield DNA mg.gm⁻¹ fresh weight</th>
<th>Specific radioactivity Dpm.mg⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>2.41</td>
<td>3.1</td>
</tr>
<tr>
<td>Heart and lung</td>
<td>4.35</td>
<td>3.95</td>
</tr>
<tr>
<td>Liver</td>
<td>2.23</td>
<td>1.05</td>
</tr>
<tr>
<td>Spleen:</td>
<td>4.95</td>
<td>3.15</td>
</tr>
<tr>
<td>Kidney</td>
<td>3.26</td>
<td>3.04</td>
</tr>
<tr>
<td>Testis</td>
<td>2.24</td>
<td>0.93</td>
</tr>
</tbody>
</table>
Fig 25: DNA hydrolysate from the soft tissues of 10 rats exposed to 0.064 µg l⁻¹ [Methyl-14C] Methyl methanesulphonate by the inhalation route for 12 h, chromatographed on Dowex 50 [H].

DNA hydrolysate from the soft tissues of 10 rats exposed to 0.064 µg l⁻¹ [Methyl-14C] Methyl methanesulphonate by the inhalation route for 12 h, chromatographed on Dowex 50 [H].
Fig 26  Rechromatography on sephadex G10 of the "7-Methylguanine" peak from Dowex 50[H+] chromatography of the DNA from the soft tissue pool of 10 rats exposed to 0.064 μg l⁻¹ [¹⁴C]MMS
3-methyladenine peak. The extent of alkylation produced in this experiment was calculated to be $28.5 \times 10^{-9}$ mol 7-methylguanine. mol$^{-1}$DNA-P. umol$^{-1}$ MMS kg$^{-1}$ body weight.

The yields and specific radioactivities of the DNA fractions, isolated from the individual soft tissue pools, from both the first and second [methyl-$^{14}$C]dichlorvos experiments are shown in Table 10. These results show a good agreement, with respect to both the yields of DNA and the specific radioactivities of the DNA, between the two experiments. All of the soft tissue DNA pools were found to contain radioactivity. In both experiments the DNA from spleen had the highest specific radioactivity whilst that from liver had the lowest specific radioactivity.

The total organ pool DNA hydrolysate from each experiment was examined by ion-exchange chromatography. The elution profiles are shown in Fig. 27 (experiment 1) and Fig. 28 (experiment 2). Both columns revealed radiolabelled material eluting with the void volume. However, in no case was there evidence of a specific association of radioactivity with any u.v.-absorbing component, e.g. 7-methylguanine. The fractions comprising the 7-methylguanine peak from the Dowex columns were pooled according to the experiment and dried in a stream of dry nitrogen. After re-dissolving in 0.1M HCl, the 7-methylguanine was rechromatographed on Sephadex G-10. The elution profiles are shown in Fig. 29 (experiment 1) and Fig. 30 (experiment 2). Recoveries of the 7-methylguanine applied to these columns were 93% and 94% respectively. As with the Dowex columns, no radioactivity
Table 10 - Yields and specific radioactivities of DNA obtained from the soft tissues of rats exposed for 12h to an atmosphere containing 0.064μg [methyl-\(^{14}\)C]dichlorvos l\(^{-1}\) (Experiments 1 and 2)

<table>
<thead>
<tr>
<th>Organ</th>
<th>Experiment 1</th>
<th></th>
<th>Experiment 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield DNA mg gm(^{-1}) fresh weight</td>
<td>Specific radioactivity Dpm mg(^{-1})</td>
<td>Yield DNA mg gm(^{-1}) fresh weight</td>
<td>Specific radioactivity Dpm mg(^{-1})</td>
</tr>
<tr>
<td>Brain</td>
<td>3.33</td>
<td>2.32</td>
<td>3.05</td>
<td>2.2</td>
</tr>
<tr>
<td>Heart and lung</td>
<td>4.99</td>
<td>1.59</td>
<td>4.5</td>
<td>1.61</td>
</tr>
<tr>
<td>Liver</td>
<td>1.99</td>
<td>0.21</td>
<td>2.2</td>
<td>0.225</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.05</td>
<td>9.88</td>
<td>3.95</td>
<td>7.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.04</td>
<td>8.84</td>
<td>1.2</td>
<td>7.8</td>
</tr>
<tr>
<td>Testis</td>
<td>2.01</td>
<td>0.83</td>
<td>2.1</td>
<td>0.79</td>
</tr>
</tbody>
</table>
Fig 27 Chromatography on Dowex 50 of the acid hydrolysate of soft-tissue DNA derived from rats exposed to [Methyl-\(^{14}\)C]dichlorvos (Experiment 1)
Fig 28 Chromatography on Dowex 50 of the acid hydrolysate of soft tissue DNA derived from rats exposed to [Methyl-^{14}C] dichlorvos (Experiment 2)
Fig 29  Rechromatography on Sephadex G–10 of the "7-Methylguanine" fraction derived from Dowex chromatography of DNA from [Methyl–$^{14}$C]dichlorvos treated rats (Experiment 1)
Fig 30  Rechromatography on Sephadex G-10 of the "7-Methylguanine" fraction derived from Dowex chromatography of DNA from [Methyl-¹⁴C] dichlorvos treated rats (Experiment 2)
was specifically associated with the 7-methylguanine fractions.

The 7-methylguanine recovered from the Sephadex columns (experiments 1 and 2; in part recovered from scintillator) was repurified by Sephadex G-10 chromatography. The overall recoveries of 7-methylguanine, expressed as a percentage of that added to the DNA fractions prior to hydrolysis, were 72.98% and 74.55% respectively, corresponding to 496mg and 650mg DNA respectively. Fractions comprising the 7-methylguanine peaks from both experiments were pooled, transferred to a scintillation vial, dried in a stream of dry nitrogen and assayed for the presence of radioactivity by repeated 7 hour counts. The blank for this procedure comprised an equivalent quantity of unlabelled 7-methylguanine from the same batch sample that was added as marker to the original DNA fractions. The results of this radio-assay are shown in Table 11. No significant difference was detected between test and blank sample. Statistical analysis of the data, using a one-sided test, indicated that a difference of 0.532 c.p.m. would have been significant at the 5% level. Based on this figure, the limits of detection of methylation of nucleic acids in the current study were estimated to be one methyl group per \( 5.7 \times 10^{11} \) nucleotide units (< \( 1.79 \times 10^{-12} \) mol 7-methylguanine, \( \text{mol}^{-1} \text{DNA-P.} \mu\text{mol}^{-1} \text{dichlorvos kg}^{-1} \text{body weight} \)).

One of the interesting facets of the above experiments was the incorporation of radioactivity into the purine ring. It seemed that such incorporation only occurred in the experiments that also yielded radiolabelled 7-methylguanine. These
Table 11 - Mean counts for the pooled 7-methylguanine fraction isolated from 1.146g DNA from soft tissues of 20 rats exposed to an atmosphere containing [methyl-14C]dichlorvos for 12h

<table>
<thead>
<tr>
<th></th>
<th>Number of 7h replicates</th>
<th>Mean count (cpm)</th>
<th>S.E.</th>
<th>Confidence limit (P&lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>20</td>
<td>21.14 (18.2-24.3)</td>
<td>0.222</td>
<td>± 0.45</td>
</tr>
<tr>
<td>Blank</td>
<td>20</td>
<td>21.23 (18.1-24.6)</td>
<td>0.222</td>
<td>± 0.45</td>
</tr>
</tbody>
</table>

Results in parentheses give the range of values obtained during repeated counting.

The mean background count of the vials prior to addition of test and control 7-methylguanine was 20.95 cpm (18.3-23.9)

The difference between the test sample and the control was 0.095 cpm with a standard error of 0.3143 on 32 degrees of freedom
findings raised certain doubts as to the natural occurrence of this base in normal DNA and, in order to probe this area, partially hepatectomised rats were treated with sodium $[^{14}\text{C}]$ formate or $[\text{methyl-}^{14}\text{C}]$methionine. Dowex 50 elution profiles of the DNA from the livers of these rats are shown in Figs. 31, 32 and 33 respectively. Treatment with both sodium $[^{14}\text{C}]$ formate and $[\text{methyl-}^{14}\text{C}]$methionine gave rise to ring labelling of thymine, guanine and adenine. With the methionine significant amounts of radioactivity were also associated with the 5-methylcytosine marker. The fractions comprising the 7-methylguanine (marker) peaks from the Dowex column were concentrated and were then rechromatographed on Sephadex G-10 (Figs. 34 and 35 respectively). No radioactivity was found to be specifically associated with the 3-methyladenine or 7-methylguanine markers. Based on the specific radioactivity of the isolated guanine ($86\text{mCi.mol}^{-1}$) from the liver DNA of rats exposed to sodium $[^{14}\text{C}]$formate, and assuming that 1 d.p.m. would have been significant, the 7-methylguanine accounted for less than 0.00024% by weight of the DNA. Based on the specific radioactivity of guanine ($5.03\text{mCi.mol}^{-1}$) from the liver DNA of rats exposed to $[\text{methyl-}^{14}\text{C}]$methionine, 7-methylguanine accounted for less than 0.0006% of the DNA by weight.

DNA, isolated from a group of rats that were treated neonatally with sodium $[^{14}\text{C}]$formate, gave the elution profile shown in Fig. 36. Radiolabelling of thymine, guanine and adenine was evident, but no radioactivity was associated with the 7-methylguanine fraction. Following concentration, rechromatography
Fig 31  Dowex 50[H+] chromatography of the liver DNA hydrolysate of an adult female rat dosed with [14C] formate following partial hepatectomy.
Fig 32: Dowex 50[H⁺] chromatography of the liver DNA hydrolysate of an adult male rat dosed with [¹⁴C] formate following partial hepatectomy.
Fig 33 DNA isolated from the soft tissues of rats labelled with [Methyl-\(^{14}\)C] methionine following partial hepatectomy.
Fig 34  Sephadex G-10 Rechromatography of the "7—Methylguanine fraction" from the Dowex 50[H+] chromatography of the soft-tissue pool derived from 10 rats treated neonatally with [14C] formate
Fig 35  Rechromatography on Sephadex G-10 of the "7-Methylguanine" fraction derived from Dowex chromatography of DNA from [Methyl-\textsuperscript{14}C] methionine—treated partially hepatectomised rats
Figure 36: Chromatography on Dowex 50[H+] of the acid hydrolysate of soft-tissue DNA derived from rats injected neonatally with sodium [14C] formate.

- Adenine
- 7-Methylguanine
- Guanine
- Thymine

Dpm ml⁻¹ vs. Fraction No.
of the 7-methylguanine containing fractions, on Sephadex G-10 gave the profile shown in Fig. 37. Again, no radioactivity was associated with either the 3-methyladenine or the 7-methylguanine fractions. The specific radioactivity of the guanine isolated from these tissues was 4.94 mCi/mol\(^{-1}\) guanine. In this study 7-methylguanine accounted for less than 0.00004% of the DNA by weight.
Fig 37  Rechromatography on Sephadex G–10 of the "7–Methylguanine" fraction derived from Dowex chromatography of DNA from sodium $^{14}$C formate treated partially hepatectomised rats
CHAPTER 4

Methylated Purines in Urine

4.1 Introduction

The detection of spontaneous \textit{in vivo} alkylation reactivity of a methylating agent necessitates the unequivocal demonstration of non-enzyme mediated transfer of an intact methyl group from the methylating agent to a tissue nucleophile. The analytical procedure must therefore discriminate between spontaneous methyl transfer reactions and natural incorporation of the partially or completely dehydrogenated electrophilic methyl groups into target nucleophiles via one carbon (1-C) pools. Such differentiation is most readily achieved by choosing targets that yield products, e.g. 7-methylguanine moieties in normal mammalian DNA, for which no natural biosynthetic pathway exists.

This definitive technique has been employed to demonstrate that those methylating agents that are established mammalian mutagens or carcinogens directly methylate a variety of nucleophilic centres in the DNA of mammalian tissues \textit{in vivo} (Swann and Magee, 1971; Craddock, 1973; Maitra and Frei, 1975; Frei and Lawley, 1975; 1976). With a view towards simplifying the analytical procedures, some studies have recently been conducted to assess the utility of monitoring urinary excretion products for the detection of \textit{in vivo} alkylation reactivity (Chu and Lawley, 1973, 1975a, 1975b). In this context, Löfroth and Wennerberg (1974) and Löfroth \textit{et al} (1974), have proposed that the excretion of radiolabelled 7-methylguanine and 3-methyladenine
constitutes evidence for the spontaneous alkylation of guanine and adenine moieties by methyl labelled dichlorvos and dimethyl-sulphate in vivo. More recently Dedek et al (1976) have drawn similar conclusions from analogous experiments with trichlorphone and butonate. For such a conclusion to be valid, significant amounts of radioactivity must not be incorporated into these methylated purines via natural metabolic pathways.

In the experiments described in this chapter the transfer of radiolabelled methyl groups from biologically active methylating agents and dichlorvos is examined. In addition the existence of a natural biosynthetic mechanism is demonstrated whereby the methyl carbon atoms of dichlorvos can become incorporated, with partial retention of hydrogen, into the heterocyclic rings and into the methyl group of urinary 7-methylguanine after entering 1-C pool in vivo. Preliminary data indicating a similar pathway for 3-methyladenine is also presented.

4.2 Methods

Animal experiments

A litter of neonates was dosed with sodium [\(^{14}\)C]formate as described previously (Section 3.2). Urine was collected for three weeks commencing immediately after weaning.

Partially hepatectomised rats were dosed with a total of 160\(\mu\)Ci of sodium [\(^{14}\)C]formate or [methyl-\(^{14}\)C]methionine equivalent to 194\(\mu\)g and 426\(\mu\)g respectively. The i.p injections, each of 10\(\mu\)Ci, were administered at 9 a.m., 12 noon, 3 p.m. and 5 p.m. on four consecutive days, commencing 16 hours after the operation.
Adult rats were dosed intraperitoneally with [methyl-\(^{14}\)C] MMS (250μCi, 1.57mCi.mmol\(^{-1}\)), [methyl-\(^{14}\)C]dichlorvos (250μCi, 3.25mCi.mmol\(^{-1}\)). [8-\(^{14}\)C]Adenine and [8-\(^{14}\)C]guanine (56mCi.mmol\(^{-1}\)) were administered to adult rats at a dose level of 50μCi per rat. S-[methyl-\(^{14}\)C]glutathione (46mCi.mmol\(^{-1}\)) was injected i.p. into adult male rats by a series of four injections (15μCi) at hourly intervals.

**Collection of urine and extraction of urinary purines**

In all of the urinary purine experiments conducted with adult rats, the animals were housed singly in all-glass metabolism cages (Metabowl, Jencons Ltd.) as described by Wright et al (1965). These cages are designed for the separate collection of urine and faeces. In the experiments with animals treated for 6 weeks neonatally with sodium \(^{14}\)C-formate, the animals were weaned at three weeks of age and urine was collected during the following three weeks of the exposure period. These animals were maintained in pairs during the urine collection. In all cases the rats had free access to food and water.

Urine was collected over toluene and in the presence of 1M sulphuric acid (2ml). The collecting flasks were changed at 9 a.m. every day, and the urine samples stored at \(-20^\circ\) until required.

After thawing, urine samples from each experiment were filtered to remove any insoluble material that had precipitated during storage. Small quantities (1-10mg) of unlabelled xanthine, hypoxanthine, adenine, guanine and 7-methylguanine were added as markers to each urine pool, followed by 20ml of
1M silver nitrate solution (Craddock and Magee, 1967). These mixtures were stored for two days in the dark at room temperature, to precipitate the silver purinates and then, for two days in the dark at 4° to ensure complete precipitation. The silver purinates were harvested by centrifugation and washed (x 3) with distilled water. The purines were recovered by hydrolysis in 0.5M HCl for 20min at 100°. This hydrolysis step was repeated twice.

**Fractionation of urinary purines**

The initial chromatographic separation of the urinary purines was, unless stated otherwise, effected on a Dowex AG50 Wx8 (200 mesh, H+ form) column (15cm x 1.5cm or 35cm x 1.8cm), developed with a linear gradient of HCl (1 to 4M). Fractions of 99 drops (6.4ml) were collected using an LKB fraction collector. Aliquots (1ml) of each fraction were removed for the determination of radioactivity. The absorbance of each fraction was determined at 254nm using a Unicam SP 800 recording spectrometer.

In some experiments the remainder of each of the eluant fractions from the Dowex 50 column corresponding to the marker 7-methylguanine were pooled, dried in vacuo and redissolved in a minimum quantity of 0.1M HCl. This solution was then applied, together with marker 3-methyladenine and 1-methyladenine, to a Sephadex G-10 column (85cm x 1.5cm) and eluted with 0.05M ammonium formate, pH 6.8. Individual fractions of 99 drops (5.9ml) were collected and the absorbance of each fraction determined as described above.
Determination of radioactivity

Samples (1ml) of each fraction were blended with 10ml of scintillator solution (NE 260) and radioactivity was determined in a Packard (Model 526) liquid scintillation spectrometer. Counting efficiencies were determined by the Channels ratio method, and were in the range of 60-65%. The presence of hydrochloric acid from the Dowex column had no significant effect on the counting efficiency.

Paper chromatography


Thin-layer chromatography

Cellulose thin-layer chromatography was performed using the following systems: System I: t-butanol/methylethylketone/formic acid/water (40:30:15:15, v/v). System II: methanol/hydrochloric acid/water (7:2:1, v/v). System III: t-butanol/methylethylketone/ammonium hydroxide (sp.gr. 0.88)/water (4:3:2:1, v/v).
4.3 Results

At the dose levels employed in this experiment all of
the MMS treated rats survived, but four out of six dichlorvos
treated rats showed symptoms of acute organophosphate poisoning
and died within 10 minutes of dosing.

The Dowex 50 elution profile of the urinary purine fractions
from rats treated with [Me-$^{14}$C]MMS and [Me-$^{14}$C]dichlorvos are
shown in Figs. 38 and 39 respectively. In both cases radioactivity
was associated with marker xanthine, hypoxanthine, 7-methylguanine
and in the position of 1-methyladenine (this marker was not
added). The radioactivity recovered in the 7-methylguanine fraction
represented 0.055% of the administered radioactivity (8.8 x
$10^{-8}$ mol. methyl carbon) following treatment with [Me-$^{14}$C]MMS
and 0.025% of the administered radioactivity (3.78 x $10^{-8}$ mol.
methyl carbon) following treatment with [Me-$^{14}$C]dichlorvos.
Re-chromatography on Sephadex G-10 of the pooled "7-methylguanine
fractions" from the two surviving rats treated with [Me-$^{14}$C]
dichlorvos is shown in Fig. 40. In addition to the radioactivity
eluted with the void volume (measured using Dextran blue),
a peak of radioactivity was eluted with the marker 1-methyladenine.
A third peak of radioactivity eluted marginally ahead of marker
3-methyladenine. This material (labelled 3-methyladenine in
Fig. 40) was further examined by thin-layer chromatography
on cellulose plates. When developed with Systems I and III
(R$_f$ 0.56 and 0.61 respectively) it was indistinguishable from
the 3-methyladenine marker. However, in System II it ran ahead
of the marker (R$_f$ 0.94). In this particular fractionation, the
Fig 38 Urinary purines from a rat treated with [Methyl-\(^{14}\)C]MMS chromatographed on Dowex 50 [H\(^+\) ]
Fig 39 Urinary purine fraction from a rat treated with [Methyl-14C]Dichlorvos chromatographed on Dowex 50[H+]
Fig 40 Sephadex G–10 chromatography of the urinary purine fraction derived from two rats dosed with [Methyl$-^{14}$C] dichlorvos
resolution of radioactivity in the area of the 7-methylguanine region was poor. Thus marker 7-methylguanine eluted earlier than would have been predicted on the basis of the results of previous fractionations.

Rechromatography of the urinary "7-methylguanine" fractions (including the 1-methyladenine fraction) from rats treated with [Me-$^{14}$C]MMS gave the profile shown in Fig. 41. In this instance the resolution was slightly better than with the [Me-$^{14}$C]dichlorvos study. Radioactivity was associated with marker 1-methyladenine, 3-methyladenine and 7-methylguanine.

Further investigation of the 7-methylguanine peak, from Dowex 50 chromatography of the urinary purines from rats treated with [Me-$^{14}$C]MMS, by paper chromatography is shown in Fig. 42. In addition to 7-methylguanine, radioactivity was associated with the 3-methyladenine marker. In order to investigate the origin of the 3-methyladenine two rats, pre-labelled with $[^{14}$C]formate as previously described (Section 3.2), were maintained on a normal diet for nine months to allow the level of radioactivity not associated with DNA to decay. Paper chromatography of the urinary purine fraction from these animals, after treatment with non-radioactive MMS, by paper chromatography gave the profile shown in Fig. 43. Radioactivity was clearly associated with the 7-methylguanine marker, but little if any, was associated with the 3-methyladenine marker. The peak of radioactivity running in front of the 3-methyladenine marker ($R_f$ 0.7 in system III) remains to be identified.
Fig 41 Sephadex G-10 chromatography of the urinary purine fraction derived from a rat dosed with [Methyl-\textsuperscript{14}C]MMS.
Fig 42  Urinary purines from rat treated with [14C]MMS chromatographed on Whatman 3 MM
Solvent system III

*SShows position of reference standards
Fig 43  Urinary purines from [14C] labelled rat treated with MMS chromatographed on Whatman 3MM System III
Analysis of the DNA, from the animals used in the experiments described above, by the methods described in Section 3.2 gave the profiles shown in Fig. 44 (following [Me-\(^{14}\)C]dichlorvos; Fig. 45 (following [Me-\(^{14}\)C]MMS) and Fig. 46 (following unlabelled MMS to a pre-labelled rat). In the case of [Me-\(^{14}\)C]dichlorvos no radioactivity was associated with the 7-methylguanine or 3-methyladenine markers, and this was confirmed by paper chromatography. However, labelling of the bases thymine, guanine and adenine, each of which proved to be homogenous on paper chromatography, indicated the incorporation of the methyl carbon from dichlorvos into the 1-C pool.

Labelling of naturally occurring bases via the 1-C pool was also noted in the case of [Me-\(^{14}\)C]MMS Fig. 45. A small quantity of radioactivity was found to be associated with the 7-methylguanine peak. This was calculated to be equivalent to \(483 \times 10^{-10} \text{ mol.CH}_3\text{mol}^{-1} \text{ DNA-P.}\mu\text{mol}^{-1} \text{ MMS.kg}^{-1} \text{ body weight.}\) Assuming a biological half-life for 7-methylguanine of three days would indicate an initial extent of reaction of approx. \(230 \times 10^{-9} \text{ mol.CH}_3\text{mol}^{-1} \text{ DNA-P.}\mu\text{mol}^{-1} \text{ MMS.kg}^{-1} \text{ body weight, no 3-methyladenine could be detected. In the final profile that from the pre-labelled rat treated with un-labelled MMS (Fig. 46) radioactivity was again associated with the 7-methylguanine region but no 3-methyladenine was detected.}

In the first inhalation experiment with [Me-\(^{14}\)C]dichlorvos a urine sample, uncontaminated with faeces, was obtained. Fractionation of this sample produced the profile shown in Fig. 47. More than 98% of the radioactivity applied to the column was eluted in the first 15 fractions. No evidence was obtained
Treatmen with [Methyl-\(^{14}\)C] Dichlorvos

**Fig. 44** Dowex 50[H\(^{+}\)] elution profile of soot tissue DNA from rats 7 days after
Fig 45  Dowex 50[H] chromatography of the soft tissue DNA hydrolysate derived from a rat 7 days after dosing with [Methyl-14C]MMS
Fig 46 Dowex 50[+H] elution profile of the acid hydrolysate of the soft tissue DNA from pre-labelled rats treated with 50 mg kg⁻¹ MMS (killed after 7 days)
Fig 47 Dowex 50 chromatogram of the urinary purine fraction from rats exposed to 0.064 μg l$^{-1}$ [Methyl$^{-14}$C]dichlorvos for 12 h
for the presence of radioactivity associated specifically with 7-methylguanine. The early eluting material was identified as predominantly dimethyl phosphate, but small quantities of desmethyl dichlorvos were also present (see Section 3.3).

The results generated in the above studies indicated that ring labelling of the DNA bases occurred following the application of methylating agents. The pattern of labelling closely followed that described in Section 3.3. 7-Methylguanine is a known component of normal urine, whereas the occurrence of 3-methyladenine in urine has not been reported. Studies on the natural occurrence of these bases in urine were therefore initiated in order to gain a better understanding of the significance of their excretion in animals treated with alkylating agents.

The Dowex 50 elution profiles of the urinary purines obtained from rats dosed with [8-14C]adenine and [8-14C]guanine are shown in Figs. 48 and 49 respectively. In both cases radioactivity was associated with the xanthine, hypoxanthine, guanine and 7-methylguanine markers. In the case of [8-14C]adenine but not with [8-14C]guanine, radioactivity was associated with the urinary adenine fraction. Rechromatography on Sephadex G-10 of the radiolabelled 7-methylguanine fraction isolated by Dowex chromatography of the urinary purines from [8-14C]adenine treated rats, revealed the presence of a significant quantity of radioactivity associated with the 1-methyladenine, 3-methyladenine and 7-methylguanine markers (Fig. 50). Examination of fractions 15-45 from this elution profile by paper chromatography (system III) gave the profile shown in Fig. 51. Again radioactivity
Fig 48 Urinary purine profile of two male rats following a single i.p. injection of 50 μCi [8-14C] adenine
Fig 49 Urinary purine profile of two male rats following a single i.p. injection of 50 $\mu$Ci [8-14C] guanine
Fig 50 Rechromatography on Sephadex G-10 of the 7-Methylguanine fraction from a Dowex 50(H⁺) chromatogram of rat urine following i.p. injection of [8-¹⁴C] adenine
Fig 51 3-Methyladenine fraction from Sephadex G10 chromatography of urine from a rat injected i.p. with [8-14C]adenine chromatographed on Whatman 3MM paper

Solvent system III
was associated with 1-methyladenine, 3-methyladenine and 7-methylguanine markers. Examination of the soft tissue DNA hydrolysate from animals used in the above study (Fig. 52) failed to reveal any radioactivity specifically associated with the 7-methylguanine or 3-methyladenine fractions. However, adenine and guanine were labelled.

Thus it seemed that both 3-methyladenine and 7-methylguanine could occur naturally in rodent urine. If this were the case, then they must have been synthesised by methylation of the pre-formed purine. A series of experiments with formate and methionine (natural contributors to the 1-C pool) were therefore performed. Two model systems, designed to examine the urinary purine level during rapid cell division, were used. In the first, urinary purines from neonatal rats injected with sodium $[^{14}\text{C}]$formate (as described in Section 3.2) was examined by ion-exchange chromatography on Dowex 50. This profile is shown in Fig. 53. Radioactivity was associated with the 7-methylguanine and guanine, and, in addition, radioactivity eluted in the area of 1-methyladenine. Rechromatography of the pool of fractions 145-180 on Sephadex G-10 reaffirmed the distribution of radioactivity in these bases (Fig. 54). In the second system, partially hepatectomised rats, treated with either [methyl-$^{14}\text{C}$]methionine or sodium $[^{14}\text{C}]$formate gave rise to radiolabelled 7-methylguanine in their urine (Figs. 55 and 56 respectively). The presence of 3-methyladenine was confirmed by paper chromatography.

Radioactivity from the 1-C pool can therefore be incorporated into the urinary 7-methylguanine fraction and possibly 3-methyladenine fraction. Organophosphorus esters and alkylating agents both give rise to S-methylglutathione during their metabolic
Fig 52  Dowex 50[+H] chromatography of the acid hydrolysate of DNA isolated from soft tissues of rats dosed i.p. with [8—14C]adenine
Fig 53 Dowex 50 [\textsuperscript{1}H] chromatography of the urinary purine fraction from 10 rats treated neonatally with [\textsuperscript{14}C]formate (\textasciitilde 3 week urine sample)
Fig 54  Sephadex G10 chromatogram of the 7-Methylguanine fraction from the Dowex 50[H+] separation of urinary purine fraction from rats treated neonatally with [14C]formate
Fig 55  Dowex 50 $[^{14}\text{H}]$ chromatography of the purine fraction derived from the urine of 1 female and 1 male rat treated with [Methyl-$^{14}\text{C}$] methionine following partial hepatectomy.
Fig 56. Dowex 50[H+] chromatogram of the urinary purine fraction derived from 1 male and 1 female rat dosed with [14C]formate following partial hepatectomy.
degradation. The methyl group of material when injected into rats is largely excreted as CO₂ (Hollingworth, 1970). Intraperitoneal injection of S-methylglutathione (60μCi) into male rats gave rise to the excretion of 65% of the dose as ¹⁴CO₂ within the first three days. Analysis of the urinary purine fraction gave the elution profile shown in Fig. 57. Radioactivity was associated with the 7-methylguanine fraction which when examined by paper chromatography, were also found to contain radioactivity running with the 1-methyladenine marker, but not with the 3-methyladenine marker. Examination of the soft tissue DNA from this experiment (Fig. 58) revealed labeling of thymine, guanine and adenine moieties via the 1-C pool.

The "3-methyladenine" produced in the above studies were heavily diluted with marker 3-methyladenine and were therefore not suitable for mass spectrometry, and the time available did not permit the collection of sufficient material to obtain a positive identification. However, the mass spectral correlations shown in Fig. 59 were obtained with a range of methylated derivatives of adenine. The spectra of these reference standards showed sufficient individual differences to allow their positive mass spectrometric identification.
Fig. 57. Dowex 50 (H+) elution profile of the urinary purine fraction from 4 male rats treated i.p. with \( S^-[\text{Methyl-}^{14}\text{C}] \) glutathione (7 day urine sample).
Fig 58 Dowex 50 chromatogram of the hydrolysate from soft-tissue DNA of 4 male rats treated i.p. with $\beta$-[Methyl-14C]glutathione.
Fig 59 Mass spectra of adenine and methylated adenines
(8 most intense peaks are indicated)
5.1 Introduction

Damage to DNA, produced by chemical or physical agents, is repairable in most types of cell. During the excision of the damaged region a "patch" of up to 100 bases is removed (Roberts et al, 1971; Cleaver, 1975), and replaced using the undamaged strand as the template. Single-strand breaks in the DNA will have a transient existence during the excision repair process. The presence of these breaks may be detected by using alkaline sucrose gradients (McGrath and Williams, 1966) to determine the molecular weight of single-strand DNA. Lett et al (1967) employed this technique to monitor X-ray damage to DNA in mammalian cells in culture. More recently the technique has been applied to measure DNA strand damage in vivo (Cox et al, 1973; Damjarov et al, 1973).

In this Chapter the results of a series of experiments are described in which this technique has been applied to measure the comparative reactivity of an organophosphorus triester and MMS. The triester chosen for these studies was dimethyl-naphthylphosphate which like dichlorvos has proven alkylating reactivity in 4-(4-nitrobenzyl)pyridine test system (Bedford and Robinson, 1972). However, unlike dichlorvos, this model compound does not have the added complication of the possible reactivity of a vinyl grouping.
5.2 Methods

Labelling of liver DNA

Liver DNA was labelled by injecting [methyl-$^3$H]thymidine during the period of maximum DNA synthesis following partial hepatectomy. A total of 500μCi of radiolabelled thymidine was administered to each rat in a series of four hourly i.p. injections (each of 50μCi), commencing 16 to 17 hours after the operation.

These rats were utilised for strand damage experiments after a minimum recovery period of 14 days, by which time the liver had returned to its quiescent state (Bucher, 1963). At this time the levels of acid soluble radioactivity in the liver had fallen to insignificant levels i.e. less than 0.1% of total recoverable radioactivity (Wooder and Holmes, 1975; unpublished observations).

Labelling of liver protein and RNA

Liver proteins were labelled with [$^{14}$C]leucine two weeks after partial hepatectomy. The [$^{14}$C]leucine was administered by i.p. injection at a dose level of 100μCi per rat every hour for four hours. Animals were killed by decapitation one hour after the last injection.

RNA was labelled with [$^{14}$C]orotic acid administered by i.p. injection commencing 19 hours after partial hepatectomy. Each rat received 100μCi of [$^{14}$C]orotic acid every hour for four hours. The animals were killed by decapitation 30min after the last injection.
Treatment of animals

MMS was diluted with 0.9% NaCl solution (1ml) and administered immediately by i.p. injection at 30mg.kg\(^{-1}\); 60mg.kg\(^{-1}\); 120mg.kg\(^{-1}\) and 200mg.kg\(^{-1}\) body weight. The animals were killed by decapitation 1, 4 or 16 hours after administration of the MMS. The vehicle control for these experiments was 0.09% NaCl (1ml). In addition, the relevance of the methyl group in this reaction was investigated using methane sulphonic acid administered as a saline solution (1ml) by the i.p. route at 500 and 1000mg.kg\(^{-1}\) body weight.

Rats were injected with a solution of dimethylnaphthylphosphate in corn oil (0.3ml) at dose levels of 100mg per kg, 500mg per kg and 1000mg per kg body weight. The rats in the 100mg per kg and 500mg per kg groups were killed by decapitation at 1, 4 and 16 hours, and those in the 1000mg per kg group at 1 and 4 hours after administration of the compound. The control experiments for these studies were performed using 0.3-1.0ml of corn oil.

Preparation of liver cell suspension

After decapitation, the liver was rapidly removed from the carcass. A 2g sample of the liver was placed into a small beaker with 2ml of ice-cold homogenising medium (0.024M Na\(_2\)EDTA; 0.075M NaCl (pH 7.5). The tissue was gently squashed with the blunt-end of a spatula and the resulting suspension centrifuged, for 1min at 200 rev/min in a MSE minor bench centrifuge, to sediment the connective tissue. Dilution of the supernatant 1:10 with ice-cold homogenising medium yielded
the cell suspension for gradient analysis (equivalent to 2.0-10μg DNA per 1.0ml).

**Sedimentation analysis**

Linear alkaline sucrose gradients (5ml; 5-20%; containing 0.9M NaCl and 0.3N NaOH) were prepared over a 1ml shelf of 2.3M sucrose in 16ml polypropylene centrifuge tubes (MSE). The sucrose solutions used to prepare the gradient were treated with diethylpyrocarbonate to destroy nuclease activity. Gradients were prepared using a modified Technicon Autoanalyser pump (Fig. 60). The shape of this gradient, as measured in terms of refractive index is shown in Fig. 61.

Onto each gradient was carefully layered 0.3ml of lysing solution (0.3M NaOH, 0.03M EDTA, 0.1M Tris-HCl buffer and 0.5% sodium dodecyl sulphate, pH 10). To this was added 0.1ml of cell suspension (equivalent to 0.2-1.0μg DNA) and, to ensure complete lysis, a further 0.1ml of lysing solution was added (Fig. 62) (Cox et al, 1973a). After filling the remainder of the tube with liquid paraffin, the tubes were centrifuged at 110,000 x g for 45min at 20° in an MSE SS75 refrigerated centrifuge equipped with a 6 x 15ml aluminium swing-out rotor. In all cases the centrifuge was decelerated to a stop without the use of the brake.

Each gradient was fractionated into 48 fractions each of 10 drops by upward displacement with 2.5M sucrose. The fractionation apparatus consisted of a MSE displacement stand assembly in conjunction with an LKB 1200 Vario-Perpex peristaltic pump (Fig. 63). The fractions were collected directly into scintillation vials.
Fig 60  Gradient forming apparatus
Fig 61 The shape of the alkaline sucrose gradient as determined by refractive index measurement
Fig 62 Centrifuge tube and contents before centrifugation
Fig 63  Gradient fractionating apparatus
Determination of radioactivity

Each fraction was blended with 10ml of scintillator mixture (NE 260). The quantity of radioactivity present in each fraction was then determined by liquid scintillation counting in a Packard Tricarb 2450 spectrometer. Such samples regularly counted with an efficiency for $[^3]$H of 38 to 40% as determined by the Channels ratio method.

Investigation of the rapidly sedimenting material

In an experiment using rat liver cells labelled with $[^3]$H thymidine, the rapidly sedimenting material isolated from 20 alkaline sucrose gradients was pooled and dialysed for 16 hours against 2% aqueous sodium acetate containing 1.5% sodium chloride. The total volume was then reduced by forced dialysis against polyethylene glycol (>220,000 m.wt). After adjusting the total volume, to a half of the original volume of cell suspension applied to the gradient, with 0.01M phosphate buffer pH 7.5, the material was divided into four equal fractions. After the addition of an equal volume of buffer, fraction one was incubated at $37^\circ$ for 1 hour. To fractions two, three and four was added an equal volume of either pronase (1mg.ml$^{-1}$, previously autodigested Section 3.2) ribonuclease (1mg.ml$^{-1}$, previously heated to destroy deoxyribo nuclease, Section 3.2) or deoxyribonuclease (1mg.ml$^{-1}$ in buffer containing 0.015M MgCl$_2$) and the mixtures incubated at $37^\circ$ for 1 hour. At the end of the incubation period, samples of the four fractions were examined, in duplicate, by alkaline gradient centrifugation. The remainder of each fraction was
then examined for retention on millipore cellulose acetate filters (i.e. high molecular weight DNA) eluted with 0.05M phosphate buffer pH 7.6 (Kohn et al, 1976).

**Repair of single-strand damage**

To follow the time course of the in vivo removal of single-strand damage (repair) a group of eight rats were injected intraperitoneally with 60mg MMS per kg body weight. This dose was chosen because, in addition to producing a measurable degree of damage, it was consistent with the survival of the animals. Pairs of animals were examined for liver DNA damage at 4, 16, 25 and 48 hours after dosing.

5.3 Results

Alkaline sucrose gradient profiles of lysed liver cells derived from rats pre-labelled with [methyl-\(^3\)H]thymidine indicated that the bulk of the radioactivity associated with the DNA was concentrated in a compact peak at the interface of the 5-20\% gradient and the 2.3M shelf (Fig. 64). Elution profiles of liver cell lysates from rats treated with \(^{14}\)C orotic acid or \(^{14}\)Cleucine are shown in Figs. 65 and 66 respectively. The radioactivity associated with both the RNA and the protein was located in the upper regions of the gradient and did not overlap with the control DNA peak. Therefore neither protein nor RNA should significantly affect the quantitation of the DNA peak. Treatment of the rapidly sedimenting material with pronase and ribonuclease (both pretreated as in Section 3.2) caused no significant change in sedimentation characteristics.
Fig 64: Alkaline sucrose gradient profile of liver DNA from an untreated male rat.
Fig 65  Alkaline sucrose gradient profile of rat liver cells labelled with $[^{14}\text{C}]$-Orotate
Fig 66. Alkaline sucrose gradient profile of rat liver cells labelled with $[^{14}\text{C}]$—Leucine
In contrast deoxyribonuclease caused a shift to the top of the gradient (Table 12).

Treatment of rats with MMS produced a broadening of the DNA peak accompanied by a change in its position in the gradient, indicative of the formation of a heterogenous population of polynucleotide material. This effect was dose related in the range 30-200mg MMS per kg body weight (Fig. 67). At the lower dose the effect was manifested in a biphasic distribution of radioactivity, part of which sedimented with the characteristics of control DNA. At the higher dose, 200mg MMS per kg body weight a complete reduction in molecular weight was seen. This degree of damage proved to be the maximum that could be detected with these gradients. Thus increases in dose above 200mg MMS per kg body weight whilst acutely toxic to the rats, failed to produce any further shift in the position of the radioactive peak. Methanesulphonic acid, the demethylation product of MMS, failed to produce measurable strand damage at dose levels up to 1000mg MMS per kg body weight and for exposure periods of up to 16 hours. The full range of results are given in Table 13.

The reversibility of this damage, to liver cell DNA, induced by MMS, was shown using groups of rats exposed to 60mg MMS per kg body weight. At four hours after dosing the presence of damage was clearly visible, but this disappeared with time until at 48h no difference between treated and control animals was discernable. Figs. 68-71 show points in the time course of this "repair process". As judged by examination of similarly
Table 12 - Nature of the Rapidly Sedimenting Material

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Deviation from control on alkaline gradient</th>
<th>Retained on Millipore filter</th>
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<tr>
<td>Pronase</td>
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<td>Positive</td>
</tr>
<tr>
<td>RNAse</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>DNAse</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Fig 67 ALKALINE SUCROSE GRADIENT PROFILE OF LIVER CELL DNA FROM RATS TREATED WITH MMS
Fraction of radioactivity recovered

(iii) 60 mg kg\(^{-1}\) MMS

(iv) 120 mg kg\(^{-1}\) MMS

(v) 200 mg kg\(^{-1}\) MMS

Fraction No.

Fig 67 cont.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>Route</th>
<th>Exposure time</th>
<th>Effect</th>
</tr>
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<td>i.p.</td>
<td>4 h</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>1 ml kg(^{-1})</td>
<td>Oral</td>
<td>4 h</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>1 ml kg(^{-1})</td>
<td>i.p.</td>
<td>16 h</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>1 ml kg(^{-1})</td>
<td>Oral</td>
<td>16 h</td>
<td>Negative</td>
</tr>
<tr>
<td>MMS</td>
<td>30 mg kg(^{-1})</td>
<td>i.p. (saline)</td>
<td>4 h</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>60 mg kg(^{-1})</td>
<td>i.p. (saline)</td>
<td>4 h</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>120 mg kg(^{-1})</td>
<td>i.p. (saline)</td>
<td>4 h</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>200 mg kg(^{-1})</td>
<td>i.p. (saline)</td>
<td>4 h</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>120 mg kg(^{-1})</td>
<td>Oral</td>
<td>4 h</td>
<td>Positive</td>
</tr>
<tr>
<td>Methanesulphonic acid</td>
<td>500 mg kg(^{-1})</td>
<td>i.p. (saline)</td>
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</tr>
<tr>
<td></td>
<td>1000 mg kg(^{-1})</td>
<td>i.p. (saline)</td>
<td>1, 4, 16 h</td>
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</tr>
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<td>Dimethyl-</td>
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<tr>
<td>Corn oil</td>
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Fig 68  Alkaline sucrose gradient profile of liver cell DNA from rats treated i.p. with 60 mg kg$^{-1}$ MMS – 4 h after dosing
Fig 69  Alkaline sucrose gradient profile of liver cell DNA from rats treated with 60 mg kg$^{-1}$ MMS – 16 h after dosing
Fig 70  Alkaline sucrose gradient profile of liver cell DNA from rats treated with 60 mg kg⁻¹ MMS — 25 h after dosing
Fig 71 Alkaline sucrose gradient profile of liver cell DNA from rats treated with 60 mg kg⁻¹ MMS - 48 h after dosing.
treated animals the repair was stable for at least three weeks (Wooder, 1976, unpublished observations).

Dimethylnaphthylphosphate at dose levels of 100 and 500mg per kg body weight caused no measurable DNA damage at 1, 4 or 16 hours. At a dose level of 1000mg per kg body weight, which caused death within 12 hours of dosing, no damage was detectable at 1 or 4 hours. Figs. 72 and 73 show typical alkaline gradient profiles of liver cell DNA following administration of dimethylnaphthylphosphate. The full range of the results obtained are shown in Table 13. No attempt was made in any of the above studies to determine the molecular weight of the DNA strands.
Fig 73  Alkaline sucrose gradient profile of liver DNA from a male rat
dosed with 500 mg kg\(^{-1}\) dimethylnaphthylphosphate
(Killed after 4 h)
CHAPTER 6

Discussion

6.1 Direct interactions with DNA.

Assuming that all of the radioactivity in the 7-methyl-guanine fraction was associated with the methyl group, the extents of methylation at N-7 of DNA guanine moieties in groups of rats exposed intraperitoneally to 24μg and 2.4mg \(^{14}\text{C}\)MMS per kg body weight were \(387 \times 10^{-9}\) and \(511 \times 10^{-9}\) mol methyl.mol\(^{-1}\).DNA-P.\(\mu\)mol\(^{-1}\)MMS.kg\(^{-1}\) body weight, respectively. These results, obtained with pooled tissue DNA, were not dissimilar to those reported for the DNA of individual tissues. For example, Kleihues and Magee (1973) reported an overall extent of reaction of \(312 \times 10^{-9}\) mol methyl.mol\(^{-1}\).DNA-P.\(\mu\)mol\(^{-1}\) MMS.kg\(^{-1}\) body weight in the DNA from rat brain following a single intravenous injection of 100 mg MMS per kg body weight. In an analogous experiment to those described above for \(^{14}\text{C}\)MMS, \(^{14}\text{C}\)dimethylsulphate gave an overall extent of reaction of \(356 \times 10^{-9}\) mol methyl.mol\(^{-1}\).DNA-P.\(\mu\)mol\(^{-1}\)DMS.kg\(^{-1}\) body weight following a single intraperitoneal injection of 530μg per kg body weight. As was predictable significant amounts of radioactivity were present in the DNA bases as a consequence of natural incorporation. The proportional contribution of this incorporation increased with increasing dose levels. Thus, using incorporation into the guanine molecule as an example, a specific radioactivity 11.04\(\mu\)Ci.mol\(^{-1}\).DNA.guanine
was obtained with the low doses of $^{14}$C]MMS while the corresponding result obtained at the high exposure was 76.7μCi per mol guanine. Whilst this incorporation did not mask the detection of alkylation it was at least partly responsible for the different extents of reaction measured in the two MMS experiments.

Following a 12h inhalation exposure to an atmosphere containing 0.064μg l$^{-1}$ [methyl-$^{14}$C]methanesulphonate, the degree of alkylation of soft tissue DNA was $28.5 \times 10^{-9}$ mol. methyl/mol$^{-1}$DNA-P.$\mu$mol$^{-1}$MMS.$kg^{-1}$ body weight. The maximum cumulative inhaled dose, assuming a minute volume ($V_m$) of 0.13 l.min$^{-1}$ (Rodericks, H. personal communication) and quantitative abstraction of MMS from the lung would have amounted to 6μg per rat (i.e. 24μg MMS per kg body weight). A comparison of the total extent of reaction at DNA in the inhalation study with those obtained by intraperitoneal injection at the same dose level, shows a significantly greater reaction in the latter case. Even allowing for that proportion of the 7-methylguanine depurinated during the exposure period, using the following equation (Löfroth, G. personal communication).

$$\frac{K \times t}{1-e^{-Kt}}$$

where $cf =$ correction factor

$K = 0.231$ d$^{-1}$ (based on $t_\frac{1}{2}$ 7-methylguanine of 3 days Margison et al., 1973).
The extent of reaction only amounts to $30.2 \times 10^{-9}$ mol methyl.

$\text{mol}^{-1}\text{DNA-P.}\mu\text{mol}^{-1}\text{MMS.kg}^{-1}$ body weight, i.e. 7.8% of that found following intraperitoneal exposure (9.7% of representative
literative values).

In calculating the total inhaled dose, it was assumed that all of the compound entering the respiratory tract would have been absorbed. By analogy with the results obtained with sarin where approximately 90% of the dose was retained in rabbit, monkey and man (Ainsworth and Shephard, 1961), it seems unlikely that poor absorption was the major factor accounting for the disparate extents of alkylation obtained. It seems more likely that administration of MMS by the intraperitoneal route allowed facile penetration, and hence alkylation, of the major organs in the peritoneal cavity. Certainly, at least during the initial phase of exposure, the MMS reaching the non-hepatic tissues in the peritoneal cavity would not have passed through the liver which is, quantitatively the most important site of MMS metabolism (Pillinger et al, 1968). In contrast, exposure via the inhalation route might be expected to yield a quantitative disposition of MMS (in tissues other than lung) similar to that resulting from continuous intravenous infusion of the compound. However, it is possible that some detoxification of MMS occurs in lung cells (Grover, 1974; Hook and Bend, 1976). The distribution of MMS in the blood stream would provide adequate opportunity for the generation of S-methylglutathione during its transportation to other organs. This concept may gain some support from the fact that the extent of ring labelling following the inhalation exposure was
greater than that after the same total dose applied as a single intraperitoneal injection. Thus specific radioactivities of DNA guanine of 11.04 and 20.8μCi.mol⁻¹ were obtained following intraperitoneal injection and inhalation exposure respectively.

The thesis that the quantitative disposition of a reactive compound administered by the inhalation route would differ from that obtained when the same dose (total inhaled dose) is administered by a single intravenous or intraperitoneal injection is also supported by the following evidence. The extent of methylation of \( N\text{-}7 \) of guanine moieties in the testicular DNA of mice exposed to a high intraperitoneal dose level of \([^{14}\text{C}]\text{MMS} \) (140mg.kg⁻¹ body weight) was 137 x 10⁻⁹ mol 7-methylguanine.mol⁻¹DNA-P.μmol⁻¹ MMS.kg⁻¹ body weight. At low intraperitoneal doses of MMS (12-80μg.MMS.kg⁻¹ body weight) the extent of methylation ranged from 62 x 10⁻⁹ to 84 x 10⁻⁹ mol 7-methylguanine.mol⁻¹DNA-P.μmol⁻¹ MMS.kg⁻¹ body weight (van Sittert et al, 1977).

However, the extent of methylation of DNA in the inhalation experiment with MMS (total theoretical inhaled dose 24μg MMS kg⁻¹ body weight) was 28.5 x 10⁻⁹ mol 7-methylguanine.mol⁻¹ DNA-P.μmol⁻¹ MMS.kg⁻¹ body weight. The results of these experiments using analogous techniques for the assessment of methylation of DNA albeit in two different species and in different organs, demonstrate that protracted exposure of a low concentration of MMS by the inhalation route results in a markedly lower extent of methylation of DNA than when the total theoretical inhaled
dose is administered as a single intraperitoneal injection. This reduction was at least 75%. It seems clear that the lower extent of reaction observed in the inhalation experiment was a direct consequence of a smaller total dose of MMS arriving at the genetic material.

The absence of radiolabelled 7-methylguanine moieties in the DNA of the internal organs of rats exposed to an atmosphere containing 0.064μg [methyl-14C]dichlorvos per litre for 12 hours indicate that this phosphoric acid triester does not methylate the DNA of mammalian tissues when it is inhaled continuously at practical use concentration. This conclusion is based on two precepts. Firstly, although a variety of nucleophiles in the constituent bases of DNA can react with electrophilic methyl carbon, reaction at the N-7 atom of guanine moieties is always predominant irrespective of whether the mechanism of the nucleophile substitution is SN\textsubscript{1} or SN\textsubscript{2}. Secondly, the exposure period employed in this study constituted a significant fraction of the in vivo half-life of 7-methylguanine moieties in DNA (3 days) (Margison et al, 1973).

The limits of detection of methylation of nucleic acids in the current study were one methyl group per 5.7 x 10^{11} nucleotide units in DNA. The proportion of the administered dose that would have been consumed in such hypothetical reaction with DNA would be 0.000001%. The results of experiments in mice, although lacking the very high sensitivity of the current work, indicate that much higher doses of dichlorvos also fail to give significant methylation of nucleic acids in vivo.
Thus, no radiolabelled 7-methylguanine moieties were detected in the liver or lungs of mice exposed for 4.5 hours to an atmosphere containing \([\text{methyl-}^{14}\text{C}]\text{dichlorvos} \ (22.7\text{mCi.mmol}^{-1})\) at a concentration exceeding domestic use concentrations by at least a factor of 100. In addition no radioactivity was reported to be present in the purine fractions isolated from the lung and liver DNA of mice after i.p. injection of \([\text{methyl-}^{14}\text{C}]\text{dichlorvos} \ (3.7\text{mCi.mmol}^{-1}; >10\text{mg.kg}^{-1} \text{body weight (Wennerberg, 1973)}\) 

The apparent lack of reactivity towards mammalian DNA demonstrated in the various \textit{in vivo} studies with dichlorvos was also supported by the results of the current strand damage experiments conducted with dimethylnaphthylphosphate. In these experiments MMS was shown to produce dose related changes in the rate of sedimentation of DNA in linear alkaline sucrose gradients. No such changes were observed with dimethylnaphthylphosphate even at very high doses. The observed breaks may not actually obtain \textit{in vivo} and may be a consequence of the action of the alkaline gradient on the initial lesions generated by MMS. The fact that very high doses of methanesulphonic acid, the demethylation product of MMS, gave negative results \textit{in vivo} indicated that the methylation of sites, presumably in DNA, was in fact a prerequisite for the induction of strand damage. The damage induced in rat liver DNA by MMS does not appear to constitute a permanent effect. In the current studies the sedimentation rate of MMS-damaged DNA had returned to the control level within 48 hours of the initial damage. However, although the damage may be repaired, the fidelity of this process cannot
be assessed in this type of experimentation. In respect of the rapid repair the current data correlates with other reports that repair of carcinogen-induced damage in the DNA of target organs is less rapidly repaired than damage produced in non-target organs (Sarma et al, 1975). The liver appears not to be a target for the carcinogenic action of MMS (Magee, 1969; Clapp et al, 1968).

Similar strand damage studies conducted in E. coli and Chinese hamster cells have shown that dichlorvos is capable of inducing DNA strand damage (Bridges et al, 1973; Green et al, 1974). In the E. coli system, the greatest damage was produced in strains possessing a functional post-replication repair system (error prone), a correlation which also holds for mutagenicity in E. coli. These findings indicate that neither the initial methylation nor the ensuing strand break are ultimately responsible for the mutagenic effect. Dose related damage could not be detected in E. coli WP2. At very high concentrations, however, damage was manifested as an all or none phenomenon. Similar in characteristics to that observed with iodoacetamide a compound possessing little reactivity towards DNA. This type of response is consistent with the hypothesis that the toxic action of the compound on the cell caused release of nucleases. In the case of dichlorvos dimethylphosphorylation, probably of protein may have been responsible for the mediation of the cytotoxicity.

The results obtained in Chinese hamster cells show dichlorvos to be at least 10 times less efficient as MMS at mediating this
type of response. In this particular cell line the mutagenicity of dichlorvos could not be demonstrated even at near cytotoxic doses (Green et al., 1974).

The failure of these organophosphate esters to methylate and damage DNA of mammalian tissues is a direct consequence of the chemistry of these compounds. For example, as a mixed triester of phosphoric acid, dichlorvos possesses two methyl groups and an electron-withdrawing dichlorovinyl group. The electron withdrawing capacity of the attached oxygen atoms and of the dichlorovinyl group result in a marked residual positive charge at phosphorus. The electrophilic phosphorus atom is susceptible to nucleophilic attack, particularly by those nucleophiles classified as hard (Pearson and Songstad, 1967) (e.g. hydroxy groups, oxyanions). In addition to activating the phosphoryl centre towards nucleophilic attack, the dichlorovinyl group is a better leaving group than the methyl group. Consequently, such attack leads to scission of the \( P-O(\text{dichlorovinyl}) \) bond and to the demethylphosphorylation of the attacking nucleophile (Equation III, Section 1.3).

The reaction mechanism depicted in Equation III is fundamental to both the insecticidal action of dichlorvos and the detoxification of dichlorvos by mammals. Dichlorvos exerts its insecticidal action by attack at phosphorus by the serine hydroxyl or the corresponding oxyanion, located at the active centre of the acetylcholine esterase, leading to the dimethylphosphorylation of this group and the loss of enzyme activity. In the current context it is extremely important to recognise that this mechanism
also applies when the attacking nucleophile is water and that, in mammalian tissues and blood, such reaction is greatly accelerated by the catalytic action of esterases (Hodgson and Casida, 1962; Hutson and Hoadley, 1972a; Hutson and Hoadley, 1972b; Blair et al., 1975). As a consequence the dimethylphosphorylation of water (hydrolysis of dichlorvos) is extremely efficient and constitutes the predominant detoxification reaction undergone by this compound in vivo (Fig. 74).

The methyl carbon atom(s) constitute a second electrophilic centre in the dichlorvos molecule. However, compared with the electron-deficient phosphorus atom, these carbon atoms possess a relatively high electron density. In practice, the intrinsic structural differences between these two centres leads to marked differences in reactivity towards different nucleophiles. Whereas the phosphorus atom is particularly susceptible to attack by "hard" nucleophiles, the electrophilic carbon atom reacts preferentially with "soft" nucleophiles, i.e. groups combining high polarisability with a rather low basicity. A typical example is the sulphur atom of mercaptans. Thus, under suitable conditions, dichlorvos is able to transfer one of its methyl groups to such a reaction partner. In mammalian liver, reaction with the sulphhydryl group of glutathione is greatly accelerated by the catalytic action of a soluble S-alkyl transferase (Hollingworth, 1969; Hutson et al., 1971; Dicowsky and Morello, 1971).
Pathways of dichlorvos metabolism

Fig. 74
Dichlorvos can also transfer one of its methyl groups to the nitrogen atoms of heterocyclic ring systems, e.g. N-7 atom of guanine in vitro. In the current context this reaction was 5% of that produced by [methyl-\textsuperscript{14}C]MMS under identical conditions. But in contrast to the enzyme-catalysed reaction with glutathione, mammals do not possess enzymes capable of accelerating the reaction of an electrophilic methyl carbon atom of dichlorvos with N-nucleophiles.

Dichlorvos undergoes very rapid metabolic degradation in a wide variety of tissues including skin, lung, intestine, blood plasma, erythrocytes, liver, kidney, heart, skeletal muscle and brain (Zaika, 1972). The ubiquity and efficiency of the dichlorvos degrading enzymes are such that the parent compound cannot be detected in the tissues or blood of animals inhaling dichlorvos at up to 12 times practical use concentrations (Page et al, 1972; Blair et al, 1975).

Of the two major routes, esterase-catalysed hydrolysis is quantitatively the more important and occurs in a wide range of tissues (Hodgson and Casida, 1962). The tissue distribution of enzymes catalysing the methyl transfer from dichlorvos to glutathione is less well-defined. However, such S-alkyl transferases are known to be active in liver and kidney (Clifton et al, 1975; Kaplowitz et al, 1975; Habig et al, 1974).

Both of these major metabolic pathways are operative in rats, pigs, mice, hamsters and man (Hutson and Hoadley, 1972a,b; Hutson et al, 1971; Page et al, 1972) and there is evidence (Hutson and Hoadley, 1972a,b; Hutson et al, 1971)
that at adequate dose levels, these pathways are followed regardless of the route of administration. However, an atypically low $K_m$ value ($3.2 \times 10^{-6}$M) has been reported for the esterase in human blood plasma (Blair et al, 1975) and it is very probable that the emphasis on the hydrolytic route or scission of the $P-O$ (vinyl) bond would become even more pronounced at low doses of dichlorvos. Thus, on the basis of their findings, that dimethyl phosphate was the major degradation product and that desmethyl dichlorvos was absent from the tissues of pigs exposed to atmospheric concentrations of dichlorvos ranging from $0.05-0.5 \mu g.1^{-1}$, Page et al (1972) suggested that cleavage of the $P-O$ (vinyl) bond was the only significant pathway operative under such conditions. Furthermore, the contribution of hydrolysis to the metabolism of organophosphates relative to $O$-dealkylation increases as the dose is lowered (Plapp and Casida, 1958) and Hollingworth (1969) has reported that the proportions of radioactive carbon dioxide and desmethyl-paraoxon decreased markedly and the urinary excretion of dimethylphosphate increased as the dose of the related compound, methyl paraoxon, was decreased. Relatively high exposures to methyl-labelled dichlorvos result in the generation of carbon dioxide (Hutson and Hoadley, 1972a) and incorporation of the label into natural components such as adenine, guanine and urinary 7-methylguanine (Wennerberg, 1974; Wennerberg and Lofroth, 1974) The generation of carbon dioxide and natural incorporation of the label into these compounds is almost certainly dependent on the initial methyl transfer to glutathione (Wooder et al, 1977).
In the current study administration of S-methylglutathione to rats by the intraperitoneal route resulted in the generation of large amounts of $^{14}$CO$_2$ (approx. 70%) within the first 24-48 hours after dosing. This result was in good agreement with the results reported by Hollingworth (1970) and indicates that oxidative demethylation of S-methylcysteine is a major metabolic pathway in this species (Kuchinskas, 1965). Examination of the soft tissue DNA fraction from rats treated with S-methylglutathione revealed the incorporation of the label into adenine, guanine and thymine but as was the case in the studies with formate and methionine no 7-methylguanine could be detected in the DNA.

It was not possible to measure the output of CO$_2$ in the current dichlorvos inhalation studies. However, the failure to detect labelled adenine and guanine in the nucleic acids, or labelled 7-methyguanine in the urine of the rats employed in these experiments points to a limited production of S-methylglutathione, and thus provides additional evidence for the preponderance of reaction at the phosphoryl centre and the very weak in vivo methylating reactivity of dichlorvos, even in the case of enzyme-accelerated reactions with the favoured nucleophile, glutathione.

Thus, the alkylation data on both dichlorvos and MMS correlates well with the toxic properties of these two compounds. MMS which is known to alkylate DNA in bacteria (Lawley et al., 1974); mammalian cells in culture (Lawley et al., 1974) and the intact animal (O'Connor et al., 1973; Frei and Lawley, 1976), and is mutagenic in all three systems
(Dean, 1972; Roberts et al, 1971; Dean and Blair, 1976) and is also carcinogenic in the mouse (Clapp et al, 1968) and in the rat (Magee, 1969). Dichlorvos on the other hand alkylates DNA in, and is mutagenic to bacteria, but, although it methylates the DNA mammalian cells in culture (Lawley et al, 1974), mutagenicity tests in these systems have been consistently negative (Dean, 1972; Green et al, 1974). This compound has also given negative results in all of the mammalian mutagenicity tests conducted to date (Epstein et al, 1972; Voogd, 1972; Dean and Thorpe, 1972; Dean and Blair, 1976). Studies conducted in mammals on both the teratogenicity (Thorpe et al, 1971) and the carcinogenicity of dichlorvos (Witherup et al, 1971; Collins et al, 1971; Blair et al, 1976) have also proved to be negative.

6.2 Origins of urinary methylated purines

Radio labelled 7-methylguanine has been detected in the urine of rats and mice after high intraperitoneal doses of DMN and dichlorvos (Wennerberg, 1973; Wennerberg and Löfroth, 1974). The results of studies into the methylation of DNA by dichlorvos indicate that, at least in the case of this compound, the urinary 7-methylguanine does not originate in the DNA.

In the current studies, radioactivity was associated with both the 7-methylguanine and 3-methyladenine fractions from the urine or rats after intraperitoneal injection of high doses of [methyl-\(^{14}\)C]MMS and [methyl-\(^{14}\)C]dichlorvos. In the case of MMS the origin of the urinary 3-methyladenine was investigated using a rat with \(^{14}\)C]formate labelled DNA. Thus, while exposure to un-labelled MMS caused the excretion of radio labelled 7-methylguanine no radio labelled 3-methyladenine was detected indicating, albeit in a limited way, that the latter compound did not originate
in DNA. This hypothesis is supported by the current work and by the work of Wennerberg (1973), who failed to detect the presence of 7-methylguanine or 3-methyladenine moieties in DNA, but identified both of these compounds in the urine of rats and mice exposed to methyl-labelled dichlorvos.

The natural occurrence of methylated purines in mammalian urine was first demonstrated by Kruger and Saloman (1898a,b) who described the presence of 7-methylguanine in normal human urine. This finding has since been confirmed (Weissman et al., 1957a,b) and a daily excretion of some 65μg has been reported in the rat (Craddock and Magee, 1967). Whilst it is possible that some part of this urinary 7-methylguanine may be of dietary origin it is now clear that at least a major portion is generated by the action of methyltransferases. From a quantitative standpoint, the most important route is likely to involve the enzymatic methylation of guanine moieties in certain species of RNA and the subsequent release of 7-methylguanine during the catabolism of RNA (Scheme 1). Thus, 7-methylguanine is a natural constituent of both t-RNA (Borek and Srinivasan, 1966) and m-RNA (Rottman et al., 1974; Croner and Hurwitz, 1975; Furuich and Muira, 1975), the methyl groups being inserted at specific guanine moieties, during the post synthetic modification of these nucleic acids, by the action of specific methyltransferases. S-Adenosyl methionine is utilised as the methyl donor in these reactions (Borek and Srinivasan, 1966). Both of these RNA species, but particularly t-RNA, are turned over rapidly, and because there is no salvage pathway for 7-methylguanine (Craddock et al., 1968),
Scheme 1: Natural pathways leading, via \( S^-\text{Methylglutathione} \), to the incorporation of the methyl carbons of dichlorvos into the ring and methyl carbon atoms of 7-Methylguanine and 3-Methyladenine with partial retention of the methyl hydrogen atoms. (Emphasis is placed on the biosynthesis and degradation of ribosyl derivatives of purines. Similar pathways exist for the generation of ring-labelled purines in DNA).
the methylated purine would be expected to be excreted unchanged in the urine. However, while 7-methylguanine is a natural excretory product, 3-methyladenine has never been reported to occur naturally in mammalian tissues or urine.

The current studies have shown that the administration of both radiolabelled purines and compounds that can contribute to the 1-C pools lead to the production of radioactive urinary methylated purines. Administration of adenine or guanine to a, otherwise normal, rat gave rise to urinary 7-methylguanine and in the former case to "3-methyladenine". The incorporation of exogenously formed purines into DNA necessitates their conversion into the corresponding nucleoside triphosphate and it follows that the production of 7-methylguanine, by the mechanism outlined above, requires the conversion of both guanine and adenine into guanosine triphosphate (GTP). The metabolic reactions leading to the synthesis of GTP from these purines are well established and are illustrated in Scheme 1. Similar results to those obtained with adenine were found when sodium formate was injected into both partially hepatectomised adult rats, and neonatal rats. The implication of such findings is that any radioactively labelled material, irrespective of its alkylating properties, that is capable of donating carbon fragments to the 1-C pools will cause labelling of urinary purines. Administration of methionine, the precursor of the proposed methyl donor, to partially hepatectomised rats also results in the labelling of 7-methylguanine and "3-methyladenine".
Examination of the soft tissues or liver DNA fractions from the rats used in these experiments revealed, in every case, radioactivity associated with adenine and guanine. In addition, DNA from the methionine treated rat showed the presence of radioactivity associated with the 5-methylcytosine marker, indicating quite clearly that under these conditions DNA methyltransferase was operative. In no case was detectable radioactivity associated with the 7-methylguanine or 3-methyladenine fractions. Based on this data it was calculated that 7-methylguanine accounts for less than 0.00004% of the guanine moieties in normal mammalian DNA.

Although a proportion of the urinary methylated purines present after exposure to proven alkylating agents may well be derived from the alkylation of DNA and RNA the existence of natural pathways to 7-methylguanine and 3-methyladenine casts serious doubts as to the validity of utilising such measurements in the assessment of the \textit{in vivo} alkylating reactivity of such compounds. In this context, it has been proposed, on the basis of detection of methylated purines in the urine or rats and mice exposed to methyl-labelled dichlorvos, butonate and trichlorphone, that these organophosphorus esters directly methylate guanine and in some instances adenine moieties \textit{in vivo} (Wennerberg and Lüfroth, 1974; Lüfroth \textit{et al}, 1974; Dedek \textit{et al}, 1976). These authors have further suggested that such reaction occurs at the level of nucleic acids.

Like dichlorvos the metabolism of MMS also gives rise to CO$_2$ as an excretion product (Pillinger \textit{et al}, 1968; Cummings and Walton, 1970). Metabolism of MMS is complex, presumably as
a consequence of its high reactivity towards tissue nucleophiles but appears to involve reaction with glutathione, as judged by the excretion of S-methylcysteine and related compounds in the urine. Although it is probable that at least part is formed by the oxidative demethylation of S-methylcysteine (Horner and Kuchinokas, 1959) it has been suggested that most of the expired CO$_2$ is derived from the hydrolytic cleavage of the parent compound (Pillinger et al., 1970: Cummings and Walton, 1968). However, irrespective of the mechanism the net result would be labelling of the pool of 1-C fragments (formaldehyde). Thus in the case of both MMS and dichlorvos, the carbon atom of the 0-methyl group can therefore be donated to the 1-C pool, part of which appears as CO$_2$. On purely theoretical grounds both compounds would be expected to give rise to labelled urinary purines (Scheme 1). This theoretical expectation is borne out by the experimental findings. The data presented by Wennerberg and Löfroth (1974) shows significant amounts of radioactivity associated with urinary xanthine, hypoxanthine, guanine and, possibly adenine. The radioactivity associated with this latter group of compounds could only have been introduced during the de novo synthesis of these purine moieties. It is therefore not surprising that significant amounts of radioactivity were associated with the adenine and guanine moieties of both DNA and RNA of mouse tissues after exposure to [methyl-$^{14}$C]dichlorvos (Wennerberg, 1973). Indeed in the current urinary purine studies, examination of the soft-tissue DNA following exposure
to both MMS and dichlorvos revealed extensive labelling of adenine and guanine moieties. The probable involvement of S-methylglutathione in this process was demonstrated following the intraperitoneal injection of rats with S-[methyl-¹⁴C]glutathione. Similar patterns of labelling to those observed following formate and methionine were seen in both urine and soft tissue DNA. Re-examination of the urinary 7-methylguanine fraction, whilst not at this stage exhaustive, failed to provide evidence for the presence of "3-methyladenine". In this particular experiment the labelling of the purine ring systems was lower than observed with formate or methionine. Thus the failure to demonstrate the presence of "3-methyladenine" may be a consequence of the low extent of labelling. Clearly it is important to conduct additional studies to establish whether 3-methyladenine is a natural excretion product.

The current data demonstrates that the detection of radiolabelled 7-methylguanine, per se, in the urine of rodents exposed to methyl labelled methylating agents does not constitute evidence for the spontaneous methylation of guanine moieties at any level of organisation e.g. at the nucleotide or nucleic acid levels. It would be premature to draw a similar conclusion with respect to the detection of urinary 3-methyladenine until unequivocal evidence has been obtained for the natural occurrence of this base in mammalian urine. However, the current evidence strongly indicates a need for caution in the interpretation of such data, and it is already clear that the excretion of 3-methyladenine cannot be employed as evidence for the methylation of the adenine moieties of DNA.
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