

Alkylation of Deoxyribonucleic Acid *in vivo* in Various Organs of C57BL Mice by the Carcinogens *N*-Methyl-*N*-Nitrosourea, *N*-Ethyl-*N*-Nitrosourea and Ethyl Methanesulphonate in Relation to Induction of Thymic Lymphoma

SOME APPLICATIONS OF HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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1. Methods were developed for analysis of alkylpurines, *O*²-alkylcytosines, and representative phosphotriesters [alkyl derivatives of thymidylyl(3'-5')thymidine], in DNA alkylated *in vivo*, using high-pressure liquid chromatography. 2. The patterns of alkylation products in DNA *in vivo* at short times were closely similar to those found for reactions *in vitro*. Alkylation by the nitrosoureas was complete *in vivo* within 1 h, but with ethyl methanesulphonate was maximal at 2-4 h. 3. The time course of persistence of alkylation products *in vivo* was determined for several tissues. In addition to the rapid loss of 3- and 7-alkyladenines reported previously for all tissues, a relatively rapid loss of *O*⁶-alkylguanines from DNA of liver was found which was more rapid at lower doses. In brain, lung and kidney, excision of *O*⁶-alkylguanine was much less marked, but was not entirely excluded by the data. In thymus, bone marrow and small bowel, all alkylated bases were lost with half-lives of 12-24 h, at non-cytotoxic doses of alkylation. 4. No evidence for any marked excision of other minor products from alkylated DNA *in vivo* was found; thus 1-methyladenine, *O*²-ethylcytosine (found in appreciable amount only with *N*-ethyl-*N*-nitrosourea), 3-methylguanine, and dTp(Alk)dT persisted in alkylated DNA, including DNA of liver. 5. The induction of thymic lymphoma was determined over the range of single doses by intraperitoneal injection up to about 60% of the LD₅₀ values, and related to the extent of alkylation of target tissues thymus and bone marrow. With *N*-methyl-*N*-nitrosourea over 90% tumour yield was attained at 60 mg/kg, and with *N*-ethyl-*N*-nitrosourea up to 52% at 240 mg/kg, but with ethyl methanesulphonate at up to 400 mg/kg only a few per cent of tumours were obtained. 6. The carcinogenic effectiveness of the agents was positively correlated with the extents of alkylation of guanine in DNA of target tissues at the *O*-6 atom. On the basis that at doses giving equal carcinogenic response these extents of alkylation would be equal, the chemical analyses showed that the ratio of equipotent doses to that for *N*-methyl-*N*-nitrosourea would be, for *N*-ethyl-*N*-nitrosourea, 5.3, for ethyl methanesulphonate about 21, and for methyl methanesulphonate [Frei & Lawley (1976) *Chem.-Biol. Interact.* 13, 215-222] about 144. These predictions were in reasonably good agreement with the observed dose-response data for these agents.

The principal effect of directly acting alkylating carcinogens of simple chemical structure administered by single intraperitoneal injection to adult

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; dTp(Alk)dT, alkyl ester of thymidylyl-(3'-5')thymidine, where Alk is Me, methyl, or Et, ethyl.

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(8-12-week-old) mice of the CFW/D strain (Frei, 1971; Joshi & Frei, 1970) or of the C57BL strain (Frei & Lawley, 1977; see the review by Lawley, 1976a) is the induction of thymic lymphoma. This induction shows a marked dependence on the nature of the alkylating agent, particularly its chemical reactivity, as well as a dependence on dose.

A further aim of the experiments is to relate tumour yields with effective doses of these carcinogens as measured by modes and extents of alkylation of DNA. Methods were devised to achieve this by using conventional chromatographic pro-

cedures, which included paper chromatography, cation-exchange chromatography on Dowex 50 and chromatography on Sephadex G-10 (Lawley & Shah, 1972; Frei & Lawley, 1975; Lawley, 1976b). These methods were applied to the determinations of methylation products in DNA in various organs of C57BL mice after injection of the carcinogen *N*-methyl-*N*-nitrosourea at a relatively high dose of 80mg/kg (Frei & Lawley, 1975). This dose consistently induced thymic lymphomas in high yields, with a minimum latent period of about 70 days, and with an average latent period (50% of mice with tumours) of about 110 days.

The results of these studies so far (Frei & Lawley, 1977) support the hypothesis (Loveless, 1969) that tumour induction by this group of alkylating agents depends on their chemical reactivities, and particularly their abilities to react *in vivo* in the target organs, thymus and bone marrow, at the O-6 position of guanine residues in DNA.

During the present studies, a report appeared on certain products of methylation of DNA in liver and brain of mice of strains A/J and C3He/FeJ (Buecheler & Kleihues, 1977). Several other reports of analogous measurements on alkylation *in vivo* in various organs of rats and hamsters are available for comparison (Goth & Rajewsky, 1974; Kleihues & Margison, 1974, 1976; Margison *et al.*, 1976, 1977; Nicoll *et al.*, 1975; Pegg, 1977). These reports generally support the view that formation and persistence of O⁶-alkylguanine residues in DNA may be necessary, but not sufficient, conditions for induction of the neoplastic state by alkylating carcinogens.

During investigation of the quantitative relationship between tumour yields and alkylation at specific sites of DNA, it became clear that the existing methods for measurements of the latter were somewhat unsatisfactory, in that they were relatively insensitive and time-consuming. It was therefore decided to investigate the application of other methods, and we now report results of some studies using high-pressure liquid chromatography.

The silica-based cation exchanger Partisil 10-SCX was found to be useful in these studies; this type of column was also found useful for isolation of phosphotriesters from alkylated DNA (Swenson & Lawley, 1978). The more conventional type of support such as the reversed-phase column, μ Bondapak C₁₈, has so far proved more useful for re-chromatography of DNA-alkylation products after preliminary separation on the cation exchanger.

These high-pressure liquid-chromatographic methods are developments from previous procedures (Lawley & Shah, 1972; see the review by Lawley, 1976b), particularly the use of cation-exchange chromatography. Results of analyses of alkylated

DNA *in vivo* obtained by these methods are included for comparative and confirmatory purposes.

Another aim of the present work was to extend measurements of alkylation of DNA *in vivo* to a lower range of administered dose than was feasible previously. As noted (Frei & Lawley, 1975), it was expected that lower doses of the potent carcinogen *N*-methyl-*N*-nitrosourea would not cause marked necrosis of target organs (Frei, 1970; Joshi & Frei, 1970), and that persistence of various alkylation products might therefore differ from those observed at the high, toxic and carcinogenic dose of 80mg/kg body wt. The persistence of base-alkylation products after the rapid methylation of DNA *in vivo* was therefore investigated. Since analytical chemical methods had been devised for their determination, the persistence of phosphotriesters in DNA was also studied and compared with the estimates from physicochemical measurements (Shooter & Merrifield, 1976; Shooter & Slade, 1977).

Experimental

Radioactive carcinogens

N-[¹⁴C]Methyl-*N*-nitrosourea (11 mCi/mmol) and [¹⁴C]ethyl methanesulphonate (8.7 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.; *N*-[³H]ethyl-*N*-nitrosourea (25.6 mCi/mmol) and *N*-[¹⁴C]ethyl-*N*-nitrosourea (4.6 mCi/mmol) were obtained from [³H]- or [¹⁴C]-ethylamine hydrochloride as described previously (Swenson & Lawley, 1978).

Marker alkylated bases

Methylpurines (Lawley & Shah, 1972) and ethylpurines (Lawley *et al.*, 1975) were prepared as previously described. O²-Methylcytosine was a gift from Dr. D. J. Brown, Australian National University, Canberra, Australia (Brown & Lyall, 1962).

O²-Ethylcytosine was prepared by the action of diazoethane on cytosine as suggested in the report by Singer (1976). Diazoethane was prepared in ether by the general procedure of Arndt (1943), dried over KOH and stored at 0°C in the dark. Before use, the concentration of diazoethane was determined by titration with benzoic acid (Fieser & Fieser, 1967). To a rapidly stirred suspension of cytosine (3.0 mmol) in 100 ml of methanol maintained at 0°C was added diazoethane (60 mmol) in ether (120 ml) over a period of about 30 min. When the orange colour from the diazoethane was discharged (about 2 h) the solution was adjusted with conc. acetic acid so that a 1:10 (v/v) dilution with water gave a pH of about 6. The neutralized solution was evaporated *in vacuo* (35°C) and chromatographed on 0.5 mm-thick layers of silica gel in methanol/methylene chloride

(3:7, v/v); *O*²-ethylcytosine (R_F 0.62; 1.66mmol) was eluted with methanol.

The product was further purified by high-pressure liquid chromatography on the ODS- μ Bondapak column, which was eluted with aq. 10% (v/v) methanol for 2min at 2ml/min, followed by a linear gradient that brought the methanol concentration to 100% (v/v) in 15min (retention time, *O*²-ethylcytosine, 10.8min).

The u.v. absorption of this compound in water, pH7.0, showed maxima at 270nm ($\epsilon = 6780$) and 225nm ($\epsilon = 7250$) and a minimum at 244nm ($\epsilon = 2040$). In 0.1M-HCl the maxima were at 260nm ($\epsilon = 8410$) and 299nm ($\epsilon = 8050$) and the minimum was at 244nm ($\epsilon = 6560$). These values are in agreement with those found for this compound by Singer (1976) and closely similar to those found for the methyl analogue, *O*²-methylcytosine, by Brown & Lyall (1962). The identity of our product was confirmed by its mass spectrum, which showed a molecular ion at m/e 139, with prominent ion fragments at m/e 111 ($M - C_2H_4$)⁺, 95 ($M - C_2H_4O$)⁺ and 95 ($M - C_2H_5O$)⁺.

Treatment of animals and isolation of DNA

Treatment of mice was as described previously (Frei & Lawley, 1975). The mice were injected by the intraperitoneal route with single doses of the carcinogens in 0.9% (w/v) NaCl (0.02ml/g body wt.). At various times, from 0.5h to 122h, the animals were killed by cervical dislocation and the organs were removed and frozen immediately in liquid N₂.

The isolation of DNA from the tissues followed Kirby's (1957) procedure with modifications as described previously (Lawley & Thatcher, 1970; Frei & Lawley, 1975). In some cases, glycogen was removed from the DNA, after the treatment with ribonuclease (Sigma Chemical Co., Poole, Dorset, U.K.) in 0.25M-sodium acetate, by addition of 4M-NaCl (0.1 vol.) followed by centrifugation for 30min at 4°C and 82000 g (r_{av} , 5.9cm).

Hydrolysis and chromatography of DNA

DNA was heated at pH6.5, 100°C for 30min to liberate 3- and 7-alkylpurines (Frei & Lawley, 1975) and, for ethylated DNA, *O*²-ethylcytosine (Singer, 1976). The residual polynucleotide was precipitated at 0°C by addition of 1M-HCl (0.1 vol.), washed with 0.1M-HCl, and then hydrolysed in 0.1M-HCl at 70°C for 20min to liberate purines as described previously (Frei & Lawley, 1975).

To simplify presentation of data, previous methods for chromatography are denoted in the Tables as: D, Dowex 50 (NH₄⁺ form); S, Sephadex G-10 (described by Frei & Lawley, 1975).

In addition to these systems, h.p.l.c. (denoted in the Tables as H) was used for determination of alkylation products. Before solutions were injected

on to high-pressure liquid-chromatographic systems, they were filtered through sintered glass. Enzymic digests (Swenson & Lawley, 1978) were adjusted to about pH4 by addition of formic acid (0.04ml/ml digests), whereas the neutral or acid hydrolysates, in 0.1M-hydrochloric acid, were chromatographed directly.

DNA hydrolysates containing up to 10 μ mol of DNA P (by u.v. absorption with $\epsilon_p = 8900$; Frei & Lawley, 1975) were injected together with marker bases in total volumes less than 1.8ml on to a Waters ALC/GPC 204 liquid chromatograph equipped with a model U6K injector and a model 440 u.v. detector (Waters Associates, Northwich, Cheshire, U.K.). The instrument was fitted with a column (9.20mm \times 250mm) of Partisil 10-SCX cation exchanger with a pre-column (4.6mm \times 100mm) of the same material (Whatman Lab Sales, Bromley, Kent, U.K.).

To separate purine bases from methylated DNA, the column was eluted at 4ml/min with 0.02M-ammonium formate, pH4.0, containing 6% (v/v) methanol for 2min, then continued with a gradient (25min, programme curve 9) to 0.2M-ammonium formate, pH4.0, in 8% (v/v) methanol. The retention times (R_T) of the purines were as follows (after initial elution at 5–6min of the pyrimidine nucleotide-containing material): guanine, 7–8min; 7-methylguanine, 12–14min; adenine, 16–18min; 3-methylguanine, 17–19min; *O*⁶-methylguanine, 20–22min; 7-methyladenine, 23–25min; 1-methyladenine, 27–29min; 3-methyladenine, 31–34min. A marker of *O*²-methylcytosine (R_T 30–31min) was eluted immediately before 3-methyladenine, but in no case with hydrolysates of [¹⁴C]methylated DNA was significant radioactivity associated with it. This system was most useful for purine separations if the 3- and 7-methylpurines (from the hydrolysis at pH6.5 and 100°C) were chromatographed separately from the acid hydrolysate (0.1M-HCl, 70°C) of the residual polynucleotide.

In experiments with ethylated DNA, procedures were analogous, but the ethylpurines were eluted at 4ml/min for 2min with 0.016M-ammonium formate pH4.0, in 12% (v/v) methanol, followed by a gradient (35min, programme curve 8) which brought the buffer to a final concentration of 0.18M-ammonium formate, pH4.0, in 12% (v/v) methanol. The purines from ethylated DNA chromatographed with the following R_T values: guanine, 8–10min; 7-ethylguanine, 13–15min; adenine, 14–16min; 3-ethylguanine, 18–20min; *O*⁶-ethylguanine, 21–24min; 7-ethyladenine, 25–28min; 1-ethyladenine, 28–30min; 3-ethyladenine, 34–38min; *O*²-ethylcytosine chromatographed at 31–34min.

For determination of representative alkyl phosphotriesters, of thymidyl(3'-5')thymidine, dTp-(Alk)dT, the residual polynucleotide from the

neutral hydrolysis (pH 6.5, 100°C) of DNA was redissolved in 0.1M-sodium borate, pH 8.5, and digested with deoxyribonuclease followed by two additions of venom phosphodiesterase and alkaline phosphatase. The dTp(Alk)dT was isolated from the digests by chromatography on the Partisil 10-SCX column and the ODS- μ Bondapak column as previously described (Swenson & Lawley, 1978).

Induction of thymic lymphoma

Female C57BL mice at 8–10 weeks of age (generally 40 or more animals per group) were injected by the intraperitoneal route with *N*-ethyl- or *N*-methyl-nitrosourea or with ethyl methanesulphonate in 0.9% NaCl, as described previously (Frei & Lawley, 1976). The range of doses was up to 60mg/kg body wt. for *N*-methyl-*N*-nitrosourea, up to 240mg/kg for *N*-ethyl-*N*-nitrosourea, and up to 400mg/kg for ethyl methanesulphonate. At the maximal doses fewer than 10% of mice died within 3 weeks of injection.

The animals were observed for development of thymic lymphomas over a period of 250 days after injection. Few primary tumours of this type are induced after 250 days, and no tumours have yet been found in 138 control animals over this period (J. V. Frei & P. D. Lawley, unpublished work).

The thymoma increases in size rapidly for a few days before the death of the animal affected, filling up the chest and causing respiratory failure. Mice were therefore killed and autopsied when they became severely short of breath. The induced thymomas were examined histologically as described previously (Joshi & Frei, 1970), and designated as lymphocytic lymphomas. Previous findings (Joshi & Frei, 1970) were confirmed, that these tumours may be confined to the thymus, or may metastasize to other organs, notably lymph nodes or spleen, and may have a terminal leukaemic phase. Other lymphomas were observed to occur spontaneously in this strain of mice, well after the period of observation used in these experiments, at ages of mice greater than 400 days, but were not primary in the thymus and could generally be distinguished histologically from the induced tumours.

Results and Discussion

Assessment of the use of h.p.l.c.

The use of h.p.l.c. for analysis of alkylated DNA *in vivo* is illustrated in Figs. 1 and 2. Full analysis of 3- and 7-alkylpurines was best achieved (as found previously by Frei & Lawley, 1975) by hydrolysis of alkylated DNA in neutral solution at 100°C. This procedure was also expected to liberate *O*²-alkylcytosines, since, while the present work was in progress, Singer (1976) reported that the O-2 atoms

of pyrimidines in DNA could be alkylated, most extensively by *N*-ethyl-*N*-nitrosourea, and that *O*²-alkylcytosines were removed from DNA under mild conditions of acid hydrolysis. Our results confirmed this expectation for *O*²-ethylcytosine. *O*²-Methylcytosine was not detected in DNA methylated by *N*-methyl-*N*-nitrosourea, and *O*²-ethylcytosine was not detected in DNA ethylated by ethyl methanesulphonate, but the possible occurrence in trace amounts cannot be ruled out.

The residual DNA after neutral hydrolysis contains, as principal alkylation products, *O*⁶-alkylguanine and 1-alkyladenines (Lawley & Shah, 1973), but, *in vivo*, some isotopic label also enters the normal purines, guanine and adenine (cf. Frei & Lawley, 1975). This did not interfere with the determination of the alkylated bases by the methods used. For some tissues, particularly active in DNA synthesis, such as small bowel, bone marrow and thymus, at longer times after injection, the amounts of label in the normal purines exceeded those in alkylated bases, as previously found (Frei & Lawley, 1975). This did not prevent adequate determination of *O*⁶-alkylguanines by the h.p.l.c. method. A further advantage was that, whereas it had been found that determination of *O*⁶-alkylguanines by using Sephadex G⁻10 (Frei & Lawley, 1975) was satisfactory, adequate measurement of 1-alkyladenines required the h.p.l.c. method.

Some phosphotriesters can be determined individually by h.p.l.c. (Swenson & Lawley, 1978), notably the methyl or ethyl esters of thymidyl(3'-5')-thymidine. The residual polynucleotide, after neutral hydrolysis of alkylated DNA, can be degraded enzymically to give these products in quantitative yield. The chromatographic procedure necessitates isolation of the triesters on the cation-exchanger Partisil 10-SCX, followed by their rechromatography on the ODS- μ Bondapak reversed-phase column. The methods, devised previously (Swenson & Lawley, 1978), proved satisfactory for DNA alkylated *in vivo*.

The advantages of the newer h.p.l.c. methods over those used previously (Frei & Lawley, 1975) in addition to their ability to permit measurement of certain products not formerly feasible, such as 1-alkyladenines, *O*²-alkylcytosines and representative triesters, are that the products are obtained in considerably smaller volumes. In the present work this advantage was not exploited fully, since in order to validate the methods, more fractions were taken than were strictly necessary to obtain detailed chromatographic profiles. The sensitivity of the method, taking a limit of detection of products of about 10 c.p.m., would be 0.1 μ mol/mol of DNA P, when about 10 μ mol of DNA P was applied to the columns, and the specific radioactivity of the alkyl

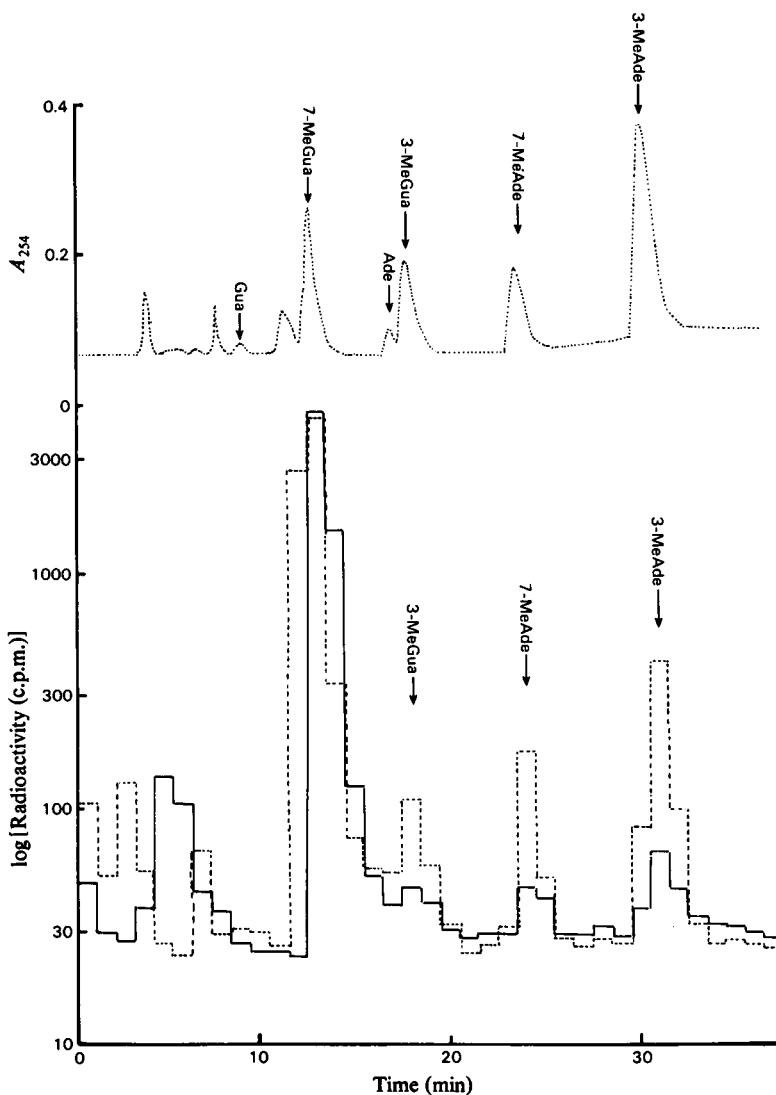


Fig. 1. H.p.l.c. of methylpurines liberated by hydrolysis from methylated DNA at neutral pH
 DNA was isolated from liver of C57BL female mice injected intraperitoneally with *N*-[¹⁴C]methyl-*N*-nitrosourea (25mg, 0.243mmol, 2.67mCi/kg). Samples of DNA (about 4mg) were heated at pH6.5, 100°C, and the liberated purines were chromatographed on Partisil 10-SCX cation-exchanger as described in detail in the text. Upper curve: *A*₂₅₄ of eluted marker bases, together with traces of guanine and adenine; lower curves (c.p.m. shown on logarithmic scale): ----, DNA isolated 0.5h after injection; —, DNA isolated 48h after injection. For chromatography of purines from residual polynucleotide, see Fig. 2.

groups was of the order 10 μCi/μmol for ¹⁴C; for ³H-labelled carcinogen, a specific radioactivity of about 30 μCi/μmol would be required to give this limit.

Patterns of initial alkylation products in DNA in vivo

Extents of alkylation of DNA *in vivo*, in various

organs of C57BL mice, at various times after injection of the isotopically labelled carcinogens, are presented in Tables 1–4.

The results are conveniently discussed under two headings. First, the initial extents of reaction at short times (around 0.5–1h after injection) will be discussed in relation to the known reactivities of

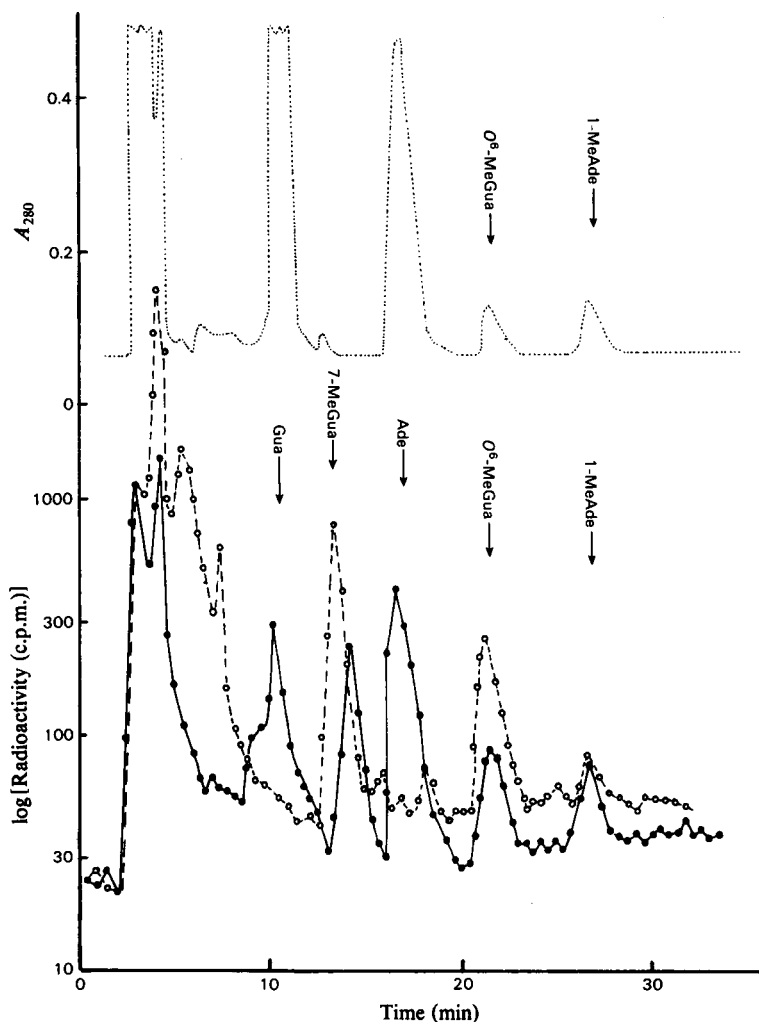


Fig. 2. H.p.l.c. of purines liberated by hydrolysis from the residual polynucleotide of Fig. 1 in 0.1 M-HCl at 70°C, after removal of methylpurines from methylated DNA by hydrolysis at neutral pH
 ····, A_{280} of liberated purines and marker bases; ----, DNA isolated from liver of mice 0.5h after injection of N -[¹⁴C]methyl- N -nitrosourea; —, DNA isolated 48h after injection. For corresponding chromatogram of other methylpurines see Fig. 1.

the alkylating agents; then the persistence of the various products in DNA for times up to about 5 days will be considered.

The extents of reaction at short times show that for the alkyl nitrosoureas, reaction of DNA is essentially complete within about 0.5h after injection (Tables 1, 2 and 4). The time course of ethylation by ethyl methanesulphonate (Table 3) is less clear. Because of the low reactivity of this agent, it was necessary to inject high doses, and the extents of alkylation in relation to dose were the least con-

sistent of the three carcinogens studied. A possible explanation is that the rate of reaction of ethyl methanesulphonate is lower *in vivo* than for the alkyl nitrosoureas, as it is *in vitro*, and that removal of ethyl groups from the alkanesulphonate by enzymic detoxification, e.g. by glutathione transferase enzymes (Boylard & Chasseaud, 1969), plays a more critical role in determining the amount of the alkylating agent *in vivo*. The greater variation between the various groups of mice injected with respect to ethylation by ethyl methanesulphonate

Table 1. Methylation of DNA of C57BL mouse brain or liver at various times after single intraperitoneal injection of N-[14C]methyl-N-nitrosourea (11 mCi/mmol) DNA was isolated at the time stated after injection and analysed by chromatography of acid hydrolysates on Sephadex G-10 (denoted S) (as described in detail by Frei & Lawley, 1975) or by high-pressure liquid chromatography (denoted H) (see Figs. 1 and 2 and the text for details); dTp(Me)dT denotes the methyl ester of thymidyl(3'-5')thymidine; — denotes below the limits of detection by this method; n.d., not determined.

Tissue ...	Brain						Liver															
	20	25	25	25	25	25	20	20	20	20	25	25	25	25	25	25	25	25	25	25		
Dose (mg/kg)																						
Method	S	H	H	H	H	H	S	S	S	S	H	H	H	H	H	H	H	H	H	H	H	
Time (t) (h)	44	3	24	48	72	96	63	25	25	122	0.5	3	24	48	72	40	29	25	25	25	25	
Extent of methylation of DNA at N-7 of guanine ($\mu\text{mol/mol}$ of DNA P)	0.106	0.004	0.120	0.122	0.207	0.222	0.055	0.032	0.028	0.024	0.069	0.016	0.014	0.024	0.014	0.018	0.043	0.027	0.043	0.027	0.044	0.31
Molar ratios of products relative to 7-methylguanine = 1.00	n.d.	0.063	0.007	0.003	0.009	0.011	n.d.	0.020	—	n.d.	0.010	0.011	0.011	0.017	0.012	0.007	0.007	0.007	0.007	0.007	0.008	0.008
1-Methyladenine	0.106	0.004	0.120	0.122	0.207	0.222	0.055	0.032	0.028	0.024	0.069	0.016	0.014	0.024	0.014	0.018	0.043	0.027	0.043	0.027	0.044	0.31
3-Methyladenine	n.d.	0.004	0.004	0.009	0.011	0.009	n.d.	0.020	—	n.d.	0.010	0.011	0.011	0.017	0.012	0.007	0.007	0.007	0.007	0.007	0.008	0.008
7-Methyladenine	n.d.	0.004	0.004	0.009	0.011	0.009	n.d.	0.020	—	n.d.	0.010	0.011	0.011	0.017	0.012	0.007	0.007	0.007	0.007	0.007	0.008	0.008
3-Methylguanine	n.d.	0.004	0.004	0.009	0.011	0.009	n.d.	0.020	—	n.d.	0.010	0.011	0.011	0.017	0.012	0.007	0.007	0.007	0.007	0.007	0.008	0.008
O ⁶ -Methylguanine	n.d.	0.004	0.004	0.009	0.011	0.009	n.d.	0.020	—	n.d.	0.010	0.011	0.011	0.017	0.012	0.007	0.007	0.007	0.007	0.007	0.008	0.008
dTp(Me)dT	n.d.	0.004	0.004	0.009	0.011	0.009	n.d.	0.020	—	n.d.	0.010	0.011	0.011	0.017	0.012	0.007	0.007	0.007	0.007	0.007	0.008	0.008
Ratio (7-methylguanine at time t)/(7-methylguanine at 0.5 or 1 h)	1.00	1.00	0.59	0.50	0.50	0.41	1.00	0.73	0.29	1.00	0.96	0.69	0.51	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44

Table 2. Methylation of DNA in various organs of C57BL mice at various times after single intraperitoneal injections of N -[^{14}C]methyl- N -nitrosourea (11 mCi/mmol)
The methods were as for Table 1.

Tissue . . .	Bone marrow												Kidney						Lung					
	20	20	20	25	25	50	50	50	50	50	50	50	20	20	20	20	20	20	20	20	20	20	20	20
Dose (mg/kg)	S	S	S	H	H	H	H	H	H	H	H	S	S	S	S	S	S	S	S	S	S	S	S	
Method	1	25	122	0.5	3	1	24	48	72	72	72	1	25	122	1	25	122	1	25	122	1	25	122	
Time (t) (h)	30	20	2	37	37	88	22	9	3	3	3	49	34	12	43	29	12	43	29	12	43	29	12	
Extent of methylation of DNA at N-7 of guanine (μ mol/mol of DNA P)	0.073	0.023	—	0.121	0.088	0.089	0.04	—	—	—	—	0.074	0.014	0.008	0.065	0.016	0.004	0.065	0.016	0.004	0.065	0.016	0.004	
Molar ratios of products relate to 7-methylguanine =1.00	0.111	0.116	—	0.114	0.114	0.117	0.105	0.133	—	—	—	0.105	0.125	0.228	0.107	0.144	0.259	0.107	0.144	0.259	0.107	0.144	0.259	
Ratio (7-methylguanine at time t)/(7-methylguanine at 0.5 or 1h)	1.00	0.67	0.07	1.00	1.00	1.00	0.25	0.10	0.03	0.03	0.03	1.00	0.77	0.25	1.00	0.67	0.28	1.00	0.67	0.28	1.00	0.67	0.28	
Tissue . . .	Small bowel												Spleen						Thymus					
Dose (mg/kg)	20	20	20	25	25	25	25	25	25	25	25	20	20	20	20	20	20	20	20	20	20	20	20	
Method	S	S	S	H	H	H	H	H	H	H	H	S	S	S	S	S	S	S	S	S	S	S	S	
Time (t) (h)	1	25	122	0.5	3	1	24	48	72	72	72	1	25	122	1	25	122	1	25	122	1	25	122	
Extent of methylation of DNA at N-7 of guanine (μ mol/mol of DNA P)	59	29	3	69	69	69	69	69	69	69	69	47	47	47	47	47	47	33	22	2	33	22	2	
Molar ratios of products relate to 7-methylguanine =1.00	0.070	0.018	0.008	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.087	0.018	0.006	0.087	0.018	0.006	0.069	0.018	—	0.069	0.018	—	
Ratio (7-methylguanine at time t)/(7-methylguanine at 0.5 or 1h)	1.00	0.142	0.218	0.108	0.108	0.108	0.108	0.108	0.108	0.108	0.108	1.00	0.096	0.144	0.267	0.144	0.267	1.00	0.131	0.167	1.00	0.131	0.167	

Table 3. Ethylation of DNA in various organs of C57BL mice at various times after single intraperitoneal injection of ¹⁴C ethyl methanesulphonate (8.7 mCi/mmol). Methods and abbreviations were as for Table 1. 'Pooled' tissue denotes kidney, lung and spleen.

Tissue ...	Bone marrow		Brain		Liver		Lung		'Pooled'		Small bowel		Thymus	
	191	286	191	196	350	350	191	196	350	350	191	196	350	315
Dose (mg/kg)	S	S	S	H	S	S	S	H	S	S	S	H	S	S
Method	2	4	2	4	1	24	2	4	1	24	2	4	1	24
Time (h)	25	58	28	36	34	81	37	38	37	81	44	67	157	157
Extent of ethylation of DNA at N-7 of guanine (μmol/mol of DNA P)	0.093	n.d.	0.054	0.046	0.050	0.055	0.005	0.055	0.024	0.048	0.047	0.017	0.030	0.045
Molar ratios of products relative to 7-ethylguanine = 1.00	n.d.	n.d.	n.d.	n.d.	0.021	n.d.	n.d.	n.d.	n.d.	n.d.	0.021	n.d.	n.d.	n.d.
3-Ethyladenine	0.040	0.034	0.033	0.022	0.011	0.020	0.010	0.035	0.040	0.029	0.032	0.043	0.020	0.026
7-Ethyladenine	n.d.	n.d.	n.d.	n.d.	0.013	n.d.	n.d.	n.d.	n.d.	0.012	n.d.	n.d.	n.d.	n.d.
O ⁶ -Ethylguanine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
dTp(E)tT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

might thus reflect the variation in their abilities to detoxify the compound.

Overall, the patterns of reaction products in DNA *in vivo* from the carcinogens at short times were similar to those found from reactions *in vitro* (cf. Lawley *et al.*, 1975; Lawley, 1976b). Thus the ratio O⁶-alkylguanine/7-alkylguanine for alkylation of DNA by *N*-methyl-*N*-nitrosourea was 0.105 *in vitro* and the values found for methylation *in vivo* at short times were clearly the same. For ethyl methanesulphonate, this ratio was much lower (0.03) both *in vitro* (Lawley *et al.*, 1975) and *in vivo*. The highest ratio (0.68) was observed *in vitro* with *N*-ethyl-*N*-nitrosourea (Lawley & Warren, 1975) and values close to this were found for bone marrow, thymus and brain *in vivo*.

The other principal minor product, 3-alkyladenine, was known to be among the bases most rapidly removed from alkylated DNA *in vivo* (Frei & Lawley, 1975), so that values of the ratio 3-alkyladenine/7-alkylguanine observed *in vivo* even at short times were expected to be somewhat lower than those found *in vitro*. This expectation was confirmed in that values for this ratio *in vivo* at short times approached the values *in vitro* [0.12 for *N*-methyl-*N*-nitrosourea (Lawley *et al.*, 1975); 0.15 for ethyl methanesulphonate (Lawley & Martin, 1975)]. It should be noted that a previous value of 0.66 quoted for the ratio 3-ethyladenine/7-ethylguanine for *N*-ethyl-*N*-nitrosourea (Lawley & Warren, 1975) is almost certainly too high, since the chromatographic system then used did not separate 3-ethyladenine from the then unrecognized product O²-ethylcytosine. With the chromatographic systems now available, this separation was achieved, and the ratio 3-ethyladenine/7-ethylguanine for DNA ethylated by *N*-ethyl-*N*-nitrosourea *in vitro* was 0.50.

The relative amounts of other minor products *in vivo* at short times were also closely similar to those *in vitro*. For 1-alkyladenines, appreciable amounts were found only with *N*-methyl-*N*-nitrosourea with a ratio of 1-methyladenine/7-methylguanine of 0.01. The ratio 7-alkyladenine/7-alkylguanine was 0.02 for *N*-methyl-*N*-nitrosourea and ethyl methanesulphonate and 0.03 for *N*-ethyl-*N*-nitrosourea. The least relatively abundant alkylguanines were 3-alkylguanines. The ratio 3-methylguanine/7-methylguanine for *N*-methyl-*N*-nitrosourea was 0.014 at short times *in vivo*, compared with 0.015 *in vitro* (Lawley & Shah, 1973). With ethyl methanesulphonate, the amounts of 3-ethylguanine obtained were close to the limits of detection, and with *N*-ethyl-*N*-nitrosourea the ratio 3-ethylguanine/7-ethylguanine was 0.08.

O²-Alkylcytosine, an alkylation product in DNA reported by Singer (1976), was detected only with *N*-ethyl-*N*-nitrosourea (Table 4) at an initial ratio

Table 4. Ethylation of DNA in various organs of C57BL mice at various times after single intraperitoneal injection of N -[^{14}C]ethyl- N -nitrosourea (4.6 mCi/mmol; 60 mg/kg) or N -[^3H]ethyl- N -nitrosourea (26 mCi/mmol; 240 mg/kg) Methods were as for Table 1; D denotes chromatography of hydrolysates of DNA on Dowex 50 (NH_4^+ form) (Frei & Lawley, 1975).

Tissue . . .	Bone marrow		Brain		Liver			Lung	Small bowel	Thymus	
Dose (mg/kg)	60	240	240	240	60	240	240	60	60	240	240
Method	D	H	H	H	D	H	H	D	D	H	H
Time (h)	1	1	1	96	1	1	96	1	1	1	96
Extent of ethylation of DNA of N-7 of guanine ($\mu\text{mol/mol}$ of DNA P)	2.6	11.7	11.7	6.8	5.3	27.5	12.1	3.8	4.0	12.2	5.7
Molar ratios of products relative to 7-ethylguanine = 1.00											
3-Ethyladenine	0.35	0.28	0.29	0.01	0.45	0.27	0.02	0.26	0.45	0.27	—
7-Ethyladenine	n.d.	0.03	0.04	0.03	n.d.	0.04	—	n.d.	n.d.	0.04	—
O^2 -Ethylcytosine	n.d.	0.15	0.13	0.19	n.d.	0.16	0.21	n.d.	n.d.	0.13	0.18
3-Ethylguanine	n.d.	0.08	0.08	0.09	n.d.	0.10	0.12	n.d.	n.d.	0.08	0.07
O^6 -Ethylguanine	0.61	0.68	0.57	0.99	0.43	0.48	0.16	0.56	0.50	0.60	1.3
dTp(Et)dT	n.d.	n.d.	0.28	0.50	n.d.	0.25	0.74	n.d.	n.d.	0.22	n.d.

O^2 -ethylcytosine/7-ethylguanine of 0.14. This value is somewhat less than that found by us for ethylation of DNA *in vitro* of 0.20, and somewhat more than that which can be deduced from the report by Singer (1976) of 0.05. [This value was derived from the relative yield of 0.5% for O^2 -ethylcytosine (Singer, 1976), relative to 11% for 7-ethylguanine, stated for the products of ethylation of salmon sperm DNA (Sun & Singer, 1975).]

With regard to phosphotriesters, the relative proportions of the representative products from alkylated thymidyl(3'-5')thymidine sequences, dTp(Alk)dT, that we determined, were closely similar to those found *in vitro* (Swenson & Lawley, 1978). Thus the ratio dTp(Me)dT/7-methylguanine for N -methyl- N -nitrosourea *in vivo* was 0.016 in liver DNA and 0.011 in brain DNA, compared with the value 0.015 *in vitro* (Swenson & Lawley, 1978). The ratio dTp(Et)dT/7-ethylguanine was 0.013 for ethyl methanesulphonate (0.016 *in vitro*) and 0.27 for N -ethyl- N -nitrosourea (0.27 *in vitro*).

Other products that have been reported for DNA alkylated *in vitro* include 3-alkylthymidines and O -alkylthymidines (Lawley *et al.*, 1973; Singer, 1976). In view of the limited stability of these products, it was expected that milder conditions of degradation of DNA might have to be used in order to measure them accurately, and this problem has not been investigated in the present work.

With regard to the investigation of the dose-response relationships for tumour induction, it will be important to determine the relationships between extent of alkylation of target DNA and dose of carcinogen. Relevant results so far are presented in Fig. 3, for alkylation of guanine in DNA of thymus and bone marrow, at short times after injection of the

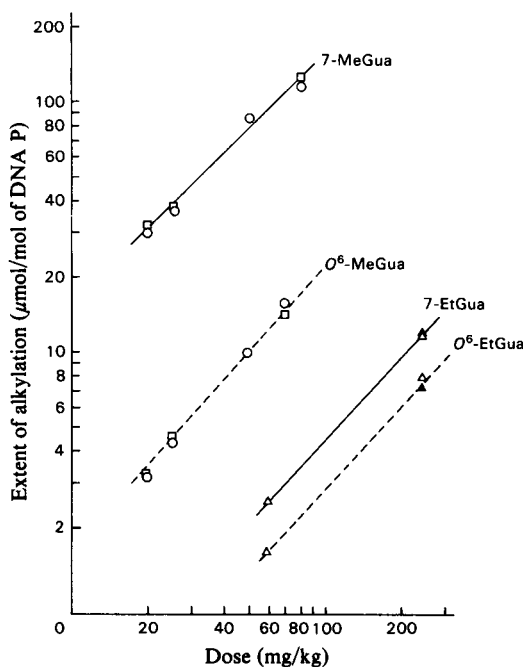


Fig. 3. Extent of alkylation of guanine in DNA of target tissues bone marrow and thymus of C57BL mice 1 h after single intraperitoneal injection of alkyl-labelled N -ethyl- or N -methyl- N -nitrosourea
 ○—○, 7-Methylguanine, bone marrow; □—□, 7-methylguanine, thymus; ○---○, O^6 -methylguanine, bone marrow; □---□, O^6 -methylguanine, thymus; ▲—▲, 7-ethylguanine, bone marrow; ▲, 7-ethylguanine, thymus; △---△, O^6 -ethylguanine, bone marrow; ▲, O^6 -ethylguanine, thymus.

carcinogens *N*-ethyl- and *N*-methyl-*N*-nitrosourea. Alkylation was proportional to dose over the carcinogenic range of doses, and was not significantly different for thymus and bone marrow.

The respective proportionalities were, expressed as means \pm s.d. of the ratios of extent of reaction ($\mu\text{mol/mol}$ of DNA P) divided by dose (mmol/kg): 7-ethylguanine, 5.6 ± 0.5 ; 7-methylguanine, 170 ± 17 ; *O*⁶-ethylguanine, 3.5 ± 0.4 ; *O*⁶-methylguanine, 18.7 ± 1.8 (with this product the results suggested that the ratio tended to somewhat lower values as the dose decreased, but this trend was not very significant).

Persistence of alkylation products in DNA *in vivo*

It was previously found that 3- and 7-methyladenines were lost from DNA methylated *in vivo* by *N*-methyl-*N*-nitrosourea (Frei & Lawley, 1975), whereas, at the relatively high dose used (80mg/kg), removal of *O*⁶-methylguanine did not occur to a significant extent within a period of 18h, although some removal from DNA of liver could be detected after 48h (Lawley, 1976b), the ratio *O*⁶-methylguanine/7-methylguanine having decreased from 0.11 to 0.05 during this period in this tissue.

With *N*-methyl-*N*-nitrosourea at lower dosages, as used here (20 and 25 mg/kg), relatively rapid removal of *O*⁶-methylguanine was found from DNA methylated in liver, in addition to the rapid removals of 3- and 7-methyladenines previously reported (Frei & Lawley, 1975).

The relatively rapid removal of *O*⁶-methylguanine from DNA of liver at lower doses of methylating carcinogens has also been reported for the rat (e.g. O'Connor *et al.*, 1973; Kleihues & Margison, 1974; Kleihues & Cooper, 1976; Pegg & Nicoll, 1976) and mouse (Buecheler & Kleihues, 1977). [It should be noted, however, that the initial ratio of *O*⁶- to 7-methylguanine reported by Buecheler & Kleihues (1977) (0.2) is much higher than the values that we have found consistently, which are the same as for methylation of DNA *in vitro* (0.11).]

The loss of 7-methylguanine from DNA of liver (Fig. 4) approximated to a first-order process of half-life about 60h. This is somewhat more rapid than the loss reported for hydrolysis *in vitro*, for which values of 79h for the half-life at pH5 and 144h at pH7 have been reported, compared with a value of 72h for liver of rats (Margison & O'Connor, 1973).

The loss of *O*⁶-methylguanine was not a first-order process, but bi- or multi-phasic (Fig. 4), as found previously for 3-methyladenine (Frei & Lawley, 1975), with a rapid initial phase. These results suggest that enzymically mediated removals of methylated bases are characterized by non-first-order kinetics, and that the loss of 7-methylguanine may be mainly by spontaneous chemical hydrolysis. The enzymic removals may thus be proceeding at

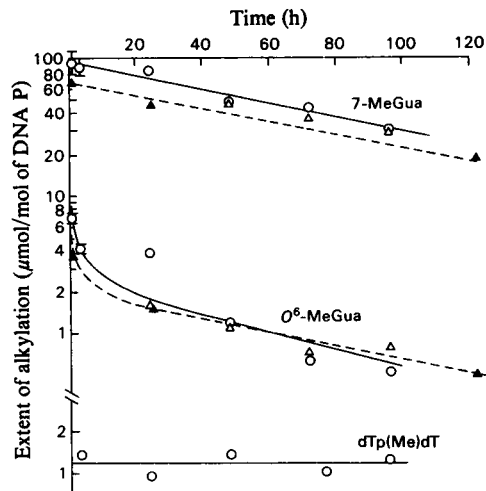


Fig. 4. Extent of alkylation of guanine and of thymidyl-(3'-5')thymidine in DNA of liver of C57BL mice at various times after single intraperitoneal injection of *N*-[¹⁴C]-methyl-*N*-nitrosourea (20 or 25 mg/kg)

Upper curves: \blacktriangle , 7-methylguanine, after dose of *N*-methyl-*N*-nitrosourea of 20mg/kg; \triangle , 7-methylguanine, 25mg/kg (by Sephadex G-10 chromatography); \circ , 7-methylguanine (by h.p.l.c.). Middle curves: \blacktriangle , *O*⁶-methylguanine, 20mg/kg; \triangle , \circ , *O*⁶-methylguanine, 26mg/kg. Lower curve (separate ordinate scale): dTp(Me)dT, 25mg/kg.

different rates in different cells within the tissues, or at different rates in different parts of cellular DNA.

The persistence of methylated bases other than 3- and 7-methyladenines in other organs of the mouse appears to depend mainly on the rate of metabolic turnover of DNA.

Thus, in bone marrow, thymus and small bowel, all methylated bases are lost with half-lives of 12–24h, which is consistent with the known rapid turnover of cells in these tissues. It should be noted that the lower doses of *N*-methyl-*N*-nitrosourea were not toxic to thymus and bone marrow, in the sense that DNA synthesis did not appear to be inhibited, whereas marked necrosis was induced at the higher dose of 80mg/kg (Frei, 1970).

In other organs, brain, lung and kidney, where metabolic turnover of DNA is expected to be small, little evidence for excision of *O*⁶-methylguanine was obtained, and the ratio *O*⁶- to 7-methylguanine increased with time. However, in no case did this ratio increase as fast as would be predicted for complete retention of *O*⁶-methylguanine, while 7-methylguanine was being lost with its observed half-life of about 60h. Therefore the possibility of some enzymic excision of *O*⁶-methylguanine cannot be ruled out in these tissues, although it is clearly not as prominent as in liver.

With regard to other minor alkylation products, the general conclusion to emerge was that 1-methyladenine was persistent in DNA even in liver and its ratio to 7-methylguanine increased with time. 3-Alkylguanines were lost at about the same rate as 7-alkylguanines.

Phosphotriesters did not appear to be subject to any marked enzymic removal. This conclusion for phosphotriesters has also emerged from the work of Shooter & Slade (1977) and Shooter & Merrifield (1976) in parallel studies using a physicochemical determination of phosphotriesters in DNA.

The apparent lack of excision of 1-methyladenine is remarkable, since alkylation to give this product causes a block to Watson-Crick hydrogen-bonding in the double helix of DNA, and therefore would be expected to distort the macromolecular structure.

*O*²-Ethylcytosine, which was detected in appreciable amounts only with *N*-ethyl-*N*-nitrosourea, also persisted in ethylated DNA, both in the liver (supposedly the most active tissue for excision) and in brain (supposedly inactive in this respect).

Tumour induction

The ultimate objective of this work is to correlate extents of alkylation of DNA in target organs with yields of tumours, and to this end the carcinogens presently under investigation were tested for their ability to induce tumours (thymomas) over appropriate ranges of dose.

The limitations of the dose range for tumorigenesis induced by single intraperitoneal injections emerged during the course of the work. For *N*-methyl-*N*-nitrosourea, tumorigenesis, as measured by proportion of animals with induced thymoma up to 250 days after injection, became appreciable at doses above 20 mg/kg (3% yield, not significant at $P < 0.05$ for a group of 50 mice; Mainland & Murray, 1952). As the dose was increased, the tumour yield increased to over 90% at 60 mg/kg (Fig. 5). This dose was well below the toxic value, being about 60% of the LD₅₀ value (Frei, 1971).

For the ethylating carcinogens, it became clear that the maximal yields of tumours would be limited by toxicity of the carcinogens. This was particularly so for ethyl methanesulphonate. Doses of 200–400 mg/kg were given, and the total yield of tumours so far is 5 from 133 mice, which is near the level of significance ($P = 0.06$). Clearly more experiments will be required to determine the tumorigenicity of this compound quantitatively.

N-Ethyl-*N*-nitrosourea is intermediate between ethyl methanesulphonate and *N*-methyl-*N*-nitrosourea. Appreciable tumorigenesis was detected at a dose of 40 mg/kg (4% yield; not significant, $P > 0.05$). Higher doses gave significant yields (Fig. 5), and at the highest dose, giving no early deaths from

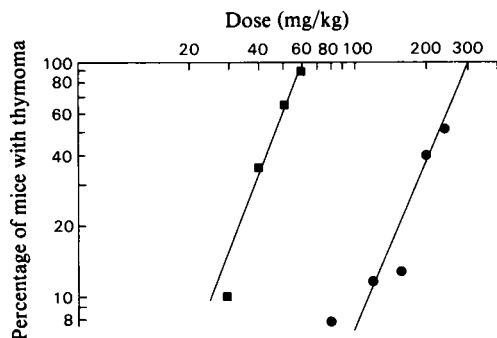


Fig. 5. Yield of thymic lymphoma in C57BL mice up to 250 days after intraperitoneal injection of (●) *N*-ethyl-*N*-nitrosourea (■) *N*-methyl-*N*-nitrosourea

The compounds were given as single intraperitoneal injections in 0.9% NaCl, and tumour yields were determined as described in the text.

toxic action of the carcinogen (240 mg/kg), the tumour yield was 52%.

In 138 control mice, and in 98 mice so far injected with methyl methanesulphonate (80–160 mg/kg), no thymomas developed in the period of 250 days after injection.

In all groups the minimal latent period for development of a tumour was 65–70 days, and the average latent periods were around 100–140 days. Lifetime studies of control and carcinogen-treated mice (J. V. Frei & P. D. Lawley, unpublished work) have so far shown that, although spontaneous lymphomas can develop in old animals (more than 400 days old), they are histologically distinguishable from the induced tumours.

The dose-response relationships for induction of thymomas by single injections of *N*-methyl- and *N*-ethyl-*N*-nitrosoureas can be discussed in provisional fashion. It is clear, despite the limitations of the data, that yield of tumours is not a linear function of dose (Fig. 5), since the slopes of the log-log plots are significantly greater than unity, over the range of tumour yields from about 10% to near 100%, and give a relationship $\text{yield} \approx (k \cdot \text{dose})^n$, where n is about 2.4 for both carcinogens, and k is a constant specific to the carcinogen used. If dose is expressed as mmol/kg body wt. the ratio $k_{\text{methyl}}/k_{\text{ethyl}}$ is about 4.5, and this reflects the relative carcinogenic effectiveness of the compounds for equimolar doses. A reasonable assumption is that the factor ($k \cdot \text{dose}$), which evidently determines the yield of tumours, is dependent on the chemical reactivity of the carcinogen, in the sense that it represents that fraction of the applied dose that reacts with a critical cellular receptor. There is now abundant evidence that DNA is an important target of chemical carcinogens, although the available evidence (reviewed for the

alkylation carcinogens by Lawley, 1976c) is not adequate to specify which specific mutations are essential for initiation of tumours.

It therefore follows that any critical reaction must occur, at a given dose, about 4.5 times as frequently for the methylating carcinogen as for the ethylating carcinogen. It is clear from Fig. 3 that the alkylation of guanine in DNA of the target organs bone marrow or thymus at the N-7 atom does not fit this requirement, since at a given dose the ratio of methylation to ethylation at this atom is about 30. However, the alkylation of O-6 of guanine fits the requirement quite well, since at a given dose the ratio of methylation to ethylation at this atom is about 5.3.

These findings also support the hypothesis of Loveless (1969) that *O*⁶-alkylation of guanine is an important cause of miscoding in DNA and hence of mutations and tumour initiation.

The possibility that other reactions contribute to tumour initiation cannot be ruled out, although it is clear that any such reaction, if predominant, should occur at a relative extent closely proportional to that of alkylation of O-6 of guanine residues, and no such coincidence has yet been reported. The discrepancy between the ratio of carcinogenic effectiveness (4.5) for methylation and ethylation by the alkylnitrosoureas and the relative alkylation at O-6 of guanine in DNA (5.3) may, however, reflect a contribution to the totality of tumour-initiating reactions by minor alkylation products, which are more prominent for ethylation than for methylation, and these may include the alternative proposed miscoding bases *O*⁴-alkylthymines (Lawley *et al.*, 1973), and presumably *O*²-alkylthymines (Kusmierek & Singer, 1976; Singer, 1976).

The observed relationship that yield of tumours is proportional to a power of dose greater than unity suggests that more than one mutagenic event is required for tumour initiation, but more data for lower dosages will be required before a comprehensive dose-response relationship adequate for any theoretical analysis can be obtained. The increase in carcinogenic effectiveness per unit dose of the carcinogens at high doses may, for example, reflect the decreased survival of target cells because of cytotoxic action of the carcinogens at these dosages. This factor may act in a manner analogous to tumour promotion, by stimulating tissue-reparative cell division of alkylated cells (Frei, 1970).

The concepts outlined here can readily account in a quantitative fashion for the lack of carcinogenic effectiveness of methyl methanesulphonate in this system. The value of *k*, in the sense defined here, for this alkylating agent is very low (so far undetectably small), and the ratio of alkylation (μmol of *O*⁶-methylguanine/mol of DNA P)/dose injected (mmol/kg) was 0.13 ± 0.08 for pooled organs (including thymus and bone marrow) of the mouse (Frei &

Lawley, 1976), compared with the value 18.7 ± 1.8 found here for *N*-methyl-*N*-nitrosourea. Thus, on the basis that at equicarcinogenic doses the extents of alkylation at O-6 of guanine in DNA are equal, equicarcinogenic doses of methyl methanesulphonate and *N*-methyl-*N*-nitrosourea would be in the ratio 18.7:0.13 or 144:1. In other words, an approximately maximal non-toxic dose of methyl methanesulphonate (150mg/kg) would be equivalent to a dose of *N*-methyl-*N*-nitrosourea of about 1mg/kg, which clearly would give an insignificantly small yield of tumours, since only at 20mg/kg did tumorigenesis reach a detectable value.

These concepts also account for the relatively small yield of tumours from ethylation by ethyl methanesulphonate. The ratio (μmol of *O*⁶-ethylguanine/mol of DNA P)/dose (mmol/kg), as determined for DNA of thymus or bone marrow, 2-4 h after injection, was 0.9 ± 0.5 , and therefore, on the basis proposed, the expected equicarcinogenic doses of ethyl methanesulphonate and *N*-methyl-*N*-nitrosourea would be, somewhat approximately, in the ratio of 18.7:0.9 or 21:1. Since the minimal dose of *N*-methyl-*N*-nitrosourea at which a detectable yield of tumours (3%) has been detected is around 20mg/kg, the corresponding expected minimal tumorigenic single dose of ethyl methanesulphonate would be of the order of 500mg/kg, which is somewhat greater than the LD₅₀ dose by single injection (430mg/kg; Frei, 1971). The results obtained so far for this carcinogen are thus in good agreement with this prediction, since a single dose of 300mg/kg gave 1% tumour yield (1 out of 89 mice), and the highest yield has so far been obtained with four weekly doses of 300mg/kg (11%, 2 out of 19 mice).

Evidently, in order to extend the quantitative correlations to the weaker carcinogens, either larger groups of animals will be required, or a more sensitive test system for carcinogenesis must be found.

In considering alkylations at sites other than O-6 of guanine in DNA as being possibly of importance with regard to carcinogenesis, it should be noted that the three positively carcinogenic agents gave detectable amounts of a typical phosphotriester derivative dTp(Alk)dT *in vivo* as *in vitro*, but there was no obvious quantitative correlation between these alkylations and induction of tumours. Thus the relatively weak carcinogen ethyl methanesulphonate gave more of this product than did the very strong carcinogen *N*-methyl-*N*-nitrosourea.

Furthermore, the triester products persisted *in vivo* in the DNA of liver, which also contra-indicates their involvement in carcinogenesis, according to arguments that excision of the supposed miscoding bases, *O*⁶-alkylguanines, from DNA of liver is associated with lack of tumour induction in this tissue (see reviews by Kleihues & Cooper, 1976;

Lawley, 1976b; Pegg & Nicoll, 1976; Rajewsky & Goth, 1976).

Nevertheless the formation of phosphotriesters as chemically relatively stable (Lawley, 1973; Shooter, 1976; Swenson *et al.*, 1976) and biochemically persistent (Shooter & Slade, 1977) detectable groups in DNA *in vivo* appears to be a useful index of action of carcinogens, even if this reaction is not itself a critical promutagenic event. In fact the lack of enzymic removal may reflect the relatively biologically inert nature of this type of chemical modification of DNA *in vivo*.

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