Embryotoxicity Induced by Alkylating Agents.
Some Methodological Aspects of DNA Alkylation Studies in Murine Embryos Using Ethylmethanesulfonate

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Synthesis and spectroscopic analysis of some alkylated DNA purine bases are described. HPLC separation methods are developed for the determination of DNA alkylation rates in mammalian embryonic tissues. Following treatment of pregnant mice with the ethylating agent ethylmethanesulfonate (EMS), an appreciable amount of alkylation (ethylation and methylation) was found in the nuclear DNA of the embryos during organogenesis. The results are discussed in context of our thesis that a certain amount of DNA alkylation in the embryos is correlated to the teratogenic potential of alkylating agents.

Introduction

Ethylmethanesulfonate (EMS) is a directly acting monofunctional alkylating agent. In mutagenicity studies it serves as a "positive control" mutagen (reviewed by Sega [1]. It induces kidney and mammary tumors in rats at a high incidence [2, 3]. The teratogenic potential of EMS is known since the short report from Hemsworth and Jackson [4] in 1965 on embryopathic effects in rats. The teratogenic potency of EMS in mice (Han: NMRI) was investigated in our group in a large-scale dose-response study [5]. DNA alkylation studies using EMS have been performed *in vitro* [6], as well as *in vivo* [7, 8].

The genotoxic potential of alkylating agents is generally believed to be initiated by a specific DNA alkylation in the target cells at the O^6-guanine site [9]. The aim of our studies is to help answer the question whether there is a quantitative correlation between the toxic effect "teratogenicity" and the DNA alkylation rate in the embryo. With the sensitive analytical method developed in our group it has become possible to study the DNA *methylation* rates in embryos [10].

In previous DNA alkylation studies we found a good correlation between the teratogenic potency of *methylating* agents and the DNA *methylation* rate at the O^6-guanine site in the DNA of the embryos during organogenesis following administration of methylmethanesulfonate (MMS), methylnitrosourea or acetoxymethyl-methylnitrosamine to the dams [11−15]. This study intends to establish a method for the determination of DNA *ethylation* rates in mouse embryos. This should enable us to prove whether such a correlation (between teratogenicity and DNA alkylation) might also be valid for ethylating agents.

The procedure described in this paper was as follows:
1. synthesis of alkylated purine bases,
2. spectroscopic analysis,
3. HPLC separations,
4. DNA alkylation experiments with EMS (*in vitro* and *in vivo*).

Experimental Part

1. Synthesis of alkylated guanine and adenine derivatives

The synthesis of the following compounds was performed:

3-methyladenine (3-MA), 3-ethyladenine (3-EA), 7-methyladenine (7-MA), 7-ethyladenine (7-EA), 7-methylguanine (7-MG), 7-ethylguanine (7-EG), O^6-methylguanine (O^6-MG), O^6-ethylguanine (O^6-EG), O^6-*n*-propylguanine (O^6-PG), O^6-*n*-butylguanine (O^6-BG).

The synthesis of 3-MA, 3-EA, 7-MA, 7-MG, O^6-MG, O^6-EG, O^6-PG, O^6-BG were performed using methods described in the literature which were slightly modified (Table I). The method of Brookes

Table I. Synthesis of alkylated guanine and adenine bases.

Base	Abbr.	Method	Yield [%][a]
3-Methyladenine	3-MA	Denayer, 1962	25.1[c]
3-Ethyladenine	3-EA	Denayer, 1962	26.4[c]
7-Methyladenine	7-MA	Denayer, 1962	15.4[c]
7-Ethyladenine	7-EA	Platzek et al.[b]	16.3[c]
7-Methylguanine	7-MG	Jones, Robins, 1963	21.5
7-Ethylguanine	7-EG	Platzek et al.[b]	13.0
O^6-Methylguanine	O^6-MG	Balsiger, Montgomery, 1960	97.4
O^6-Ethylguanine	O^6-EG	Balsiger, Montgomery, 1960	44.8
O^6-n-Propylguanine	O^6-PG	Balsiger, Montgomery, 1960	41.8
O^6-n-Butylguanine	O^6-BG	Balsiger, Montgomery, 1960	32.2

[a] Actually obtained in this laboratory.
[b] See experimental part.
[c] Two reaction steps.

and Lawley [16] for preparing 7-EG involved large-scale chromatographic isolation. Farmer et al. [17] synthesized 7-EG in a rather troublesome way, using 2'-deoxyguanosine and diazoethane.

We modified the procedure of Jones and Robins [18] for the preparation of 7-MG and obtained 7-EG by the reaction of guanosine and diethylsulfate in either dimethylacetamide or hexamethylphosphoramide. The latter solvent was used because of its well-known polar and cation-solvating properties [19]. Synthesis of 7-EA was achieved by modification of the method of Denayer [20] for the preparation of 7-MA.

1.1.   Synthesis of 7-ethylguanine (7-EG)
1.1.1. with dimethylacetamide as solvent

1.4 g (5 mmol) of guanosine were stirred with 7.9 g (50 mmol) of diethylsulfate in 20 ml dried dimethylacetamide for 48 h at ambient temperature. After centrifugation, acetone was added to the clear solution. The precipitate was removed by filtration and the residual guanosine was precipitated with ether. The guanosine was removed by filtration and the filtrate was diluted with ether and extracted with water. The collected waterphases were washed with methylene chloride and brought to pH 1 with hydrochloric acid. After hydrolysis (3 h, 100 °C) the solution was neutralized with ammonia, the precipitate collected by centrifugation and recrystallized from water to yield 120 mg (13.4%) of 7-EG.

1.1.2. with hexamethylphosphoramide (HMPA) as solvent

1.4 g (5 mmol) of guanosine were stirred with 1.54 g (10 mmol) of diethylsulfate in 10 ml distilled HMPA for 40 h at ambient temperature. After adding 300 mg of "Kieselgel 60 Merck" the pH was adjusted to 8 with ammonia, some water was added and the HMPA was removed with methylene chloride. The precipitate that formed in the aqueous phase was collected, washed with acetone and then hydrolyzed (3 h, pH 1, 100 °C) and yielded after crystallization from water 184 mg (20.4%) of 7-EG.

1.2.   Synthesis of 7-ethyladenine (7-EA)
1.2.1. 4,6-Diamino-5-(N-ethylformyl)-
        aminopyrimidine

1 g (6.5 mmol) of 4,6-Diamino-5-formylaminopyrimidine [20] and 222 mg (7.4 mmol) of sodium hydride 80% were stirred in 40 ml dimethylacetamide (dried over molecular sieve) 15 min at 50 °C. After adding 1.15 g (7.4 mmol) of ethyl iodide to the clear solution, the mixture was kept at 90 °C for 4 h, then filtered and allowed to stand overnight at 4 °C. 750 mg of 4,6-Diamino-5-(N-ethylformyl)-aminopyrimidine were obtained, the mother-liquor yielded a further 330 mg. After crystallization from ethanol/water 450 mg (38.1%) of 4,6-Diamino-5-(N-ethylformyl)-aminopyrimidine were obtained (m.p. 244 °C, not corrected).

1.2.2. 7-Ethyladenine (7-EA)

220 mg (1.2 mmol) of 4,6-Diamino-5-(N-ethyl-formyl)-aminopyrimidine were stirred for 2 h in formamide at 200 °C. After evaporation in vacuo and sublimation of the crude product, 7-EA was purified by crystallization from isopropanol with charcoal to yield 85 mg (42.7%; m.p. 261.1 °C, not corrected).

Elementary analysis
for $C_7H_9N_5$
(calculated) : C = 51.52%; H = 5.56%; N = 42.92%.
(found)   : C = 51.50%; H = 5.54%; N = 42.58%.

2. Spectroscopy

Infrared spectra: Perkin-Elmer spectralphotometer 421.

Mass spectra: CH 7 Varian Mat (70 eV, 200 °C).
PMR spectra: WP-60 Bruker.
Carbon-13 spectra: CFT-20 Varian.

2.1. Mass spectroscopy

The mass spectra of alkylated adenines have been described (7-MA) by Deutsch *et al.* [21]; Rice and Dudek [22] (adenine and 6-methylaminoadenine), and Lawley *et al.* [6] (1- and 9-ethyladenine).

Fig. 1 shows the mass spectra of 3-EA and 7-EA. The principal fragmentation route of the ethylated adenines is the loss of ethylene to form the ion *m/z* 135, as evidenced by the metastable ion *m/z* 111.81, followed by the successive loss of two HCN molecules to give the ions *m/z* 108 and 81 (*cf.*
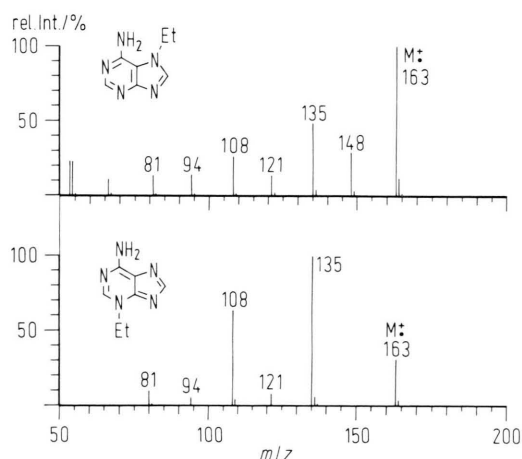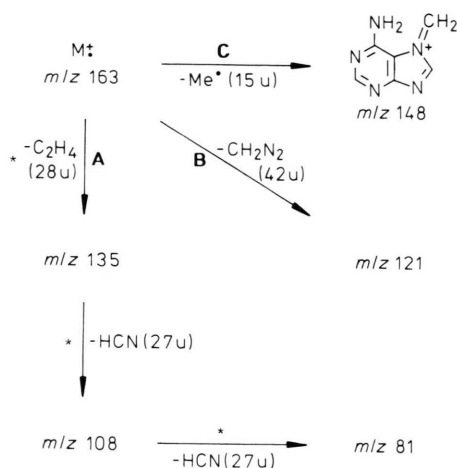
Fig. 2. Fragmentation paths of 7-EA and 3-EA.

Fig. 2A). The ion *m/z* 135 has the relative intensity of 49% and 100% for 7-EA and 3-EA respectively, and has also been reported by Lawley *et al.* [6] for 1- and 9-ethyladenine. The loss of 42 mass units from the molecular ion has been interpreted by Rice and Dudek [22] as loss of cyanamide (CH_2N_2) and we observed it in all the adenines studied (3-MA, 3-EA, 7-MA, and 7-EA; *cf.* Fig. 2B). Deutsch *et al.* [21] reported that the main fragmentation reaction of 7-MA is the loss of an H-radical, followed by the formation of an N-7-N^6-methylene-bridge. An analogous fragmentation of 7-EA has formed an ion *m/z* 148 by loss of a methyl-radical (*cf.* Fig. 2C). In the cases of 3-, 1- and 9-ethyladenine this ion has only a low intensity (about 5%). Therefore, in the case of ethyladenines an ion *m/e* 148 seems to indicate 7- (or possibly N^6-) substitution.

Rice and Dudek [22] have investigated the mass spectrometric fragmentations of 1-, 3- and 7-methylguanine in detail. Farmer *et al.* [17], Foster [23] and Lawley *et al.* [6] have described the fragmentations of the O^6- and 7-ethylated and isopropylated guanines. In addition, we investigated O^6-PG and O^6-BG, which decompose by the same mechanism. The mass spectra are shown in Fig. 3. The main fragmentation route is the loss of the alkyl substituent as an olefine (mass differences 28, 42, and 56 in the cases of O^6-EG, O^6-PG, O^6-BG, respectively), leading to the ion *m/z* 151, followed by the typical guanine fragmentation giving the ions *m/z* 135, 134, 110, 109, and 108 (Fig. 4A).

Fig. 1. Mass spectra of 7-EA and 3-EA.
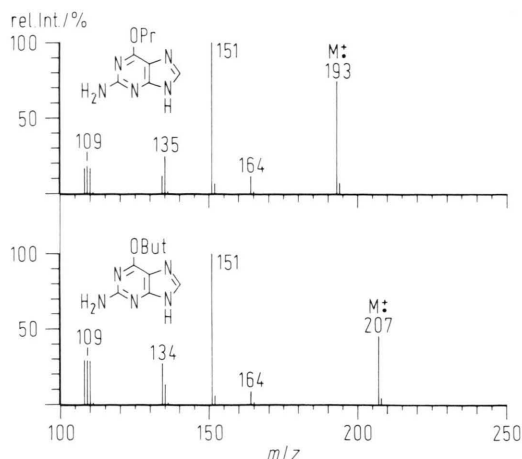
Fig. 3. Mass spectra of O⁶-PG and O⁶-BG.
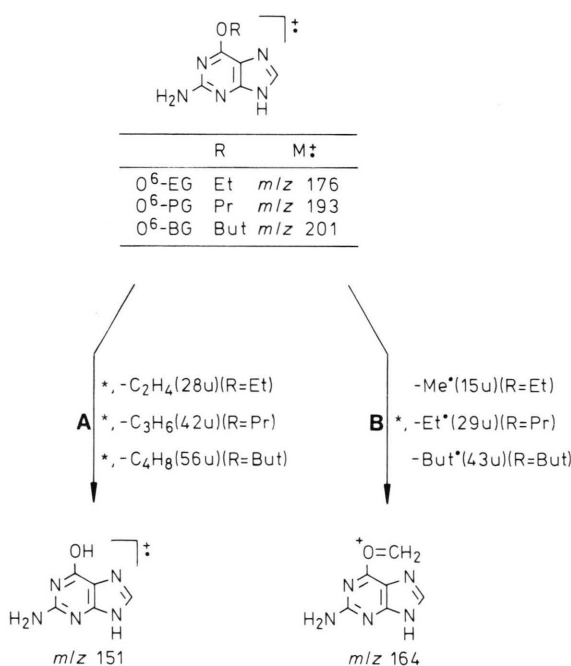
The second route is characterized by α-cleavage of an asymmetrical ether leading to the ion m/z 148 with the mass difference 15, 29, and 43 for the compounds O⁶-EG, O⁶-PG and O⁶-BG, respectively. The intensity of this ion decreases with increasing alkyl-chain length (Fig. 4B). 7-MG and 7-EG form an ion which is analogous to the ion m/z 148 in the case of adenines.

2.2. Infrared spectroscopy

The main infrared absorption bands between 3500 and 1500 cm⁻¹, which are discussed in the following, are listed in Table II.

In the region above 3100 cm⁻¹ the stretching vibrations of the amino-group give rise to two or three bands between 3500 and 3300, as well as some others in the region 3200−3100 cm⁻¹ [24]. The bands in the region 3100−2700 cm⁻¹ can be assigned to the vibrations of aromatic and aliphatic C-H bonds. The broad shoulder between 1700 (7-MG) and 1690 cm⁻¹ (3-MA, 7-EG) were interpreted by Pal and Horton [25] as uncancelled water-vapour bands. The shoulders in the spectra of 7-MG and 7-EG could be due to the six-membered lactam ring-structure, which appear in the case of guanine as resolved bands near 1700 and 1680 cm⁻¹ indicating carbonyl and N-H-group deformation vibrations [24]. The band near 1670 cm⁻¹ (1669 ± 7 in the cases of 3-MA, 3-EA, 7-MA and 7-EA) was cited by Pal and Horton [25] as evidence for the existence of an exocyclic primary amino-group in the molecule of 3-MA. The bands in the region 1640 ± 10 cm⁻¹ that we found in the spectra of the O⁶-alkylated compounds (O⁶-MG, -EG, -PG, -BG) were attributed to the C-2 amino-group by Montgomery and Holum [26] for similar compounds. Angell [24] assigned the two bands in the

Fig. 4. Fragmentation paths of O⁶-PG and O⁶-BG.

Table II. Principal infrared absorption bands between 3500 and 1500 cm⁻¹ of alkylated guanines and adenines.

3-Methyladenine	3325	3190	3020	1690, 1675	–	1625, 1565
3-Ethyladenine	3380	–	3010	1675, 1665	–	1615, 1560
7-Methyladenine	3300	3170	3080	1662	–	1610, 1550
7-Ethyladenine	3350, 30	3150	2990, 60	1665	–	1605, 1550
7-Methylguanine	3320	3150	2720	1770−1650	–	1615, 1560
7-Ethylguanine	3315	3150	2990, 2870, 2750−10	1690−1655	–	1612, 1560
O⁶-Methylguanine	3490, 40, 3330	3180	3025, 2965, 2775	–	1650−35	1605, 1520
O⁶-Ethylguanine	3490, 40, 3330	3200, 3120	2950	–	1645−30	1590, 20
O⁶-Propylguanine	3490, 3310	3180	2980	–	1635	1595, 15
O⁶-Butylguanine	3370−30	3190	2970	–	1635	1595, 20

regions 1615 ± 10 and 1555 ± 5 cm^{-1} to the purine nucleus, which we also identified in the spectra of 7-MG and 7-EG. An analogous pair was found around 1598 ± 8 and 1515 ± 5 in the cases of O^6-MG, -EG, -PG and -BG.

2.3. Proton nuclear magnetic resonance spectra

The proton NMR spectra were obtained in deuterated dimethyl sulphoxide, with the exception of 7-MG because of its poor solubility in this solvent. The chemical shift data are listed in Table III.

The assignment of the resonance signals of the protons located at C-6, C-2 and C-8 of simple purines was initially incorrect [27, 28]. Later, by use of deuterated analogues, the correct order could be defined: C-6, C-2, C-8 (increasing field), with the exception of adenine, where the proton at C-2 is shifted downfield by influence of the C-6 amino-group [29]. The shift data of the C-8 proton reported by various authors are in poor agreement. The tendency to form hydrogen bonds increases with increasing proton-acceptor property of the solvent used, thus shifting the resonance signal downfield. Additionally, the chemical shift depends on concentration. By forming stack complexes the anisotropic effect results in a downfield shift, especially in aqueous solutions.

The spectrum of 3-EA is representative for purines and consists of a simple feature of five distinct signal groups (Fig. 5). The quartet of the ethyl group is centred at 4.42 ppm and the triplet at 1.53 ppm ($J = 7$ Hz). The primary amino-group at C-6 gives rise to a signal at 7.94 ppm (exchangeable with deuterium oxide), while the sharp singlets of the C-2 and C-8 protons are located at 7.84 and 8.43 ppm. The PMR spectral data of 7-EA as listed in Table III are in good agreement with those reported by Hino *et al.* [30]. He had obtained 7-EA from desulfurization of a 4-aminodihydrothiazolopurine.

In connexion with the 3-substituted adenines special interest arises because of the problem of amine-imine-tautomerism. By comparing the UV spectra of 1- and 3-methyladenine, Brookes and Lawley [31] concluded that while the former exists in the imino form, the latter exists in the amino form. This conclusion was supported by the infrared spectroscopic investigations of Pal and Horton [25] who assigned the amine form to 3-methyladenine. The PMR spectra of 3-MA and 3-EA (Table III; Fig. 5) confirm their hypothesis: the protons of the amino-group at C-6 give rise to a broad singlet at 7.83 and 7.94 ppm respectively that can be exchanged with deuterium oxide.

2.4. Carbon-13 nuclear magnetic resonance

Chang and Lee [32] showed that it is possible to investigate the reaction of methylating agents such as

Table III. P.m.r. shift data of alkylated adenines and guanines (DMSO$_{d6}$) in ppm from TMS.

Compound	proton at C-8	C-2	N^6	$-CH_2-$	$-CH_3$
3-MA	8.29	7.77	7.83	–	3.91
3-EA	8.43	7.84	7.94	4.42	1.53
7-MA	8.12	8.12	6.78	–	3.99
7-EA	8.23	8.18	6.84	4.42	1.36

	N-1	C-8	N^2	$-CH_2-$	$-CH_3$
7-MG a) in NaOD	–	7.66	–	–	3.86
b) in CF$_3$COOH	–	8.72	–	–	4.26
7-EG a) in NaOD	–	7.86	–	4.32	1.45
b) in DMSO$_{d6}$	10.79	7.89	6.00	4.24	1.41

	N-9	C-8	N^2	$-CH_2-$	$-CH_2-$	$-CH_2-$	$-CH_3$
O^6-MG	12.65	7.81	6.17	–	–	–	3.96
O^6-EG	12.60	7.81	6.13	–	–	4.46	1.36
O^6-PG	12.54	7.83	6.14	–	4.37	1.67	0.98
O^6-BG	12.36	7.79	6.10	4.39	1.8	-1.2	0.94

δ/ppm
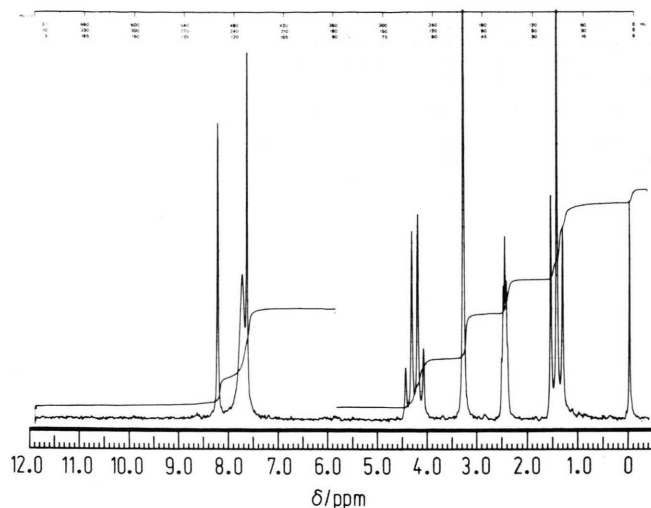
Fig. 5. PMR spectrum of 3-EA ($DMSO_{d6}$).

MMS with nucleic acids *in vitro* using carbon-13 NMR spectroscopy. To achieve a correct assignment of the resonance signals, the use of model compounds is essential. Because of the poor solubility of the purine bases, carbon-13 NMR spectra were only obtained in the cases of 7- and 9-methyladenine by Chenon *et al.* [33]. Additionally, we determined the spectrum of 7-EA using noise decoupling and off-resonance conditions. The compound was dissolved in dry spectroquality dimethylsulfoxide (DMSO). The chemical shifts were calculated relative to the internal reference (DMSO) and converted to the TMS scale: 17.34 (CH_3) − 41.30 (CH_2) − 110.67 (C-5) − 145.17 (C-8) − 151.38 (C-6) − 152.18 (C-2) − 160.06 (C-4).

3. HPLC separations

3.1. Apparatus

For all the separations described a 1010 B or 1084 B Hewlett-Packard HPLC chromatograph was used, equipped with a Waters U6 K loop or an automatic syringe injection system. Accessories: Hewlett-Packard 3380 A integrator, Infrotonics CRS-100 A integrator, H.-P. 1010 B gradient programmer, 1010 B fraction collector. Detection system: Hewlett-Packard (Schoeffel) 1030 B UV detector. The wavelength 286 nm was chosen to be best suited for the absorption maxima of the alkylated bases. Fluorimetric detection was performed with a Schoeffel FS 970 LC or Perkin Elmer 3000 fluorescence detector measuring the intrinsic fluorescence (excitation wave-length 286 nm, emission wave-length 374 nm). We used commercial stainless steel columns (250×4 mm) equipped with Swagelock fittings and exchangeable frits. They were slurry-packed with the cation-exchange resin Dionex DC-6A, a divinylbenzene-polystyrol cross-linked polymer (Pierce Inc.).

3.2. Chemicals, buffers, separation conditions

Aqueous buffers used as mobile phases were made up in water, which has been distilled twice in an all glass still. Ammonium formate was prepared from distilled formic acid and ammonia (pro analysi "Merck"). Guanine and its 1-, 3-, 7- and 9-methyl derivatives, adenine and 1-methyladenine were obtained from Fluka (Switzerland). Ten alkylated derivatives were synthesized in our laboratory (Table I).

We routinely separate guanine, adenine and the methylated derivatives 7-MG, O^6-MG and 3-MA following DNA alkylation *in vivo* using a two-step pH gradient elution mode with 0.2 M ammonium formate buffer (Fig. 6 for details see [10, 34]).

After modification of our separation conditions we are now able to solve some other separation problems. The optimal method for the separation of the purines proved to be elution with a binary pH gradient on a Dionex DC-6A column. The gradient starts with 0.2 M ammonium formate pH 3.5 and

ends at 0.2 M ammonium formate pH 8.5. The pressure was about 30 at, flow-rate 1 ml/min and the conductivity was 17 mS. Firstly we were able to separate the seven methylated derivatives 1-, 3-, 7- and O^6-methylguanine and 1-, 3- and 7-methyladenine (Fig. 7). In addition, separation of the O^6-alkylated guanines O^6-MG, -EG, -PG and -BG was performed (Fig. 8). In order to detect these derivatives the use of a fluorimetric instead of a UV detection system has proved to be more efficient. For example, the detection limit for the measurement of O^6-MG is 25 pmol per injection. This represents a ten-fold increase in sensitivity as compared to UV detection.

In order to study ethylating agents, a separation method of guanine, adenine and the most important ethylated derivatives 3-EA, 7-EA, 7-EG and O^6-EG was elaborated (Fig. 9).

Furthermore, a separation method for methylated and ethylated purines was developed (compounds guanine, adenine, 3-MA, 3-EA, 7-EA, 7-MG, 7-EG, O^6-MG, O^6-EG; Fig. 10).

4. DNA alkylation experiments

The DNA alkylation experiments were performed using [1-^{14}C-ethyl] EMS at a specific radioactivity of 13.1 mCi/mmol (custom synthesis, Amersham Inc.).

4.1. DNA alkylation *in vivo*

For this part of the study pregnant mice were used (NMRI: Han, Zentralinstitut für Versuchstierkunde, Hannover, FRG) kept under spf-conditions and a constant day-night cycle (dark period from 8:00 p.m. to 9:00 a.m.) at 24 °C and 50% relative humidity. The animals were fed Altromin® 1324 and tap water ad libitum. The mating period was 2 h (7:00−9:00 a.m.). When vaginal plugs were detected the 24 h period following the mating period was designated day 0 of pregnancy [35].

On day 11 of pregnancy, eight dams were treated once i.p. with 210 mg/kg ^{14}C-labelled EMS in saline (10 μl/g body weight). 4 h after treatment the mother animals were sacrificed by cervical dislocation and
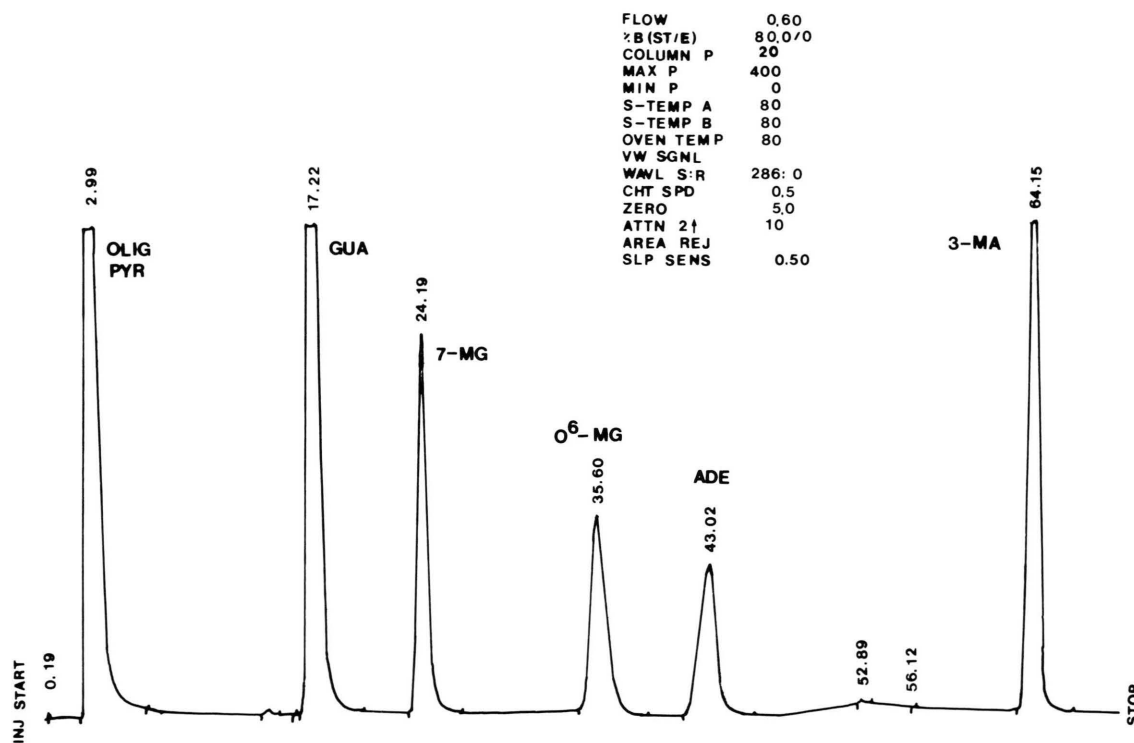

Fig. 6. HPLC separation of a DNA hydrolysate for the determination of 7-MG, O^6-MG and 3-MA.

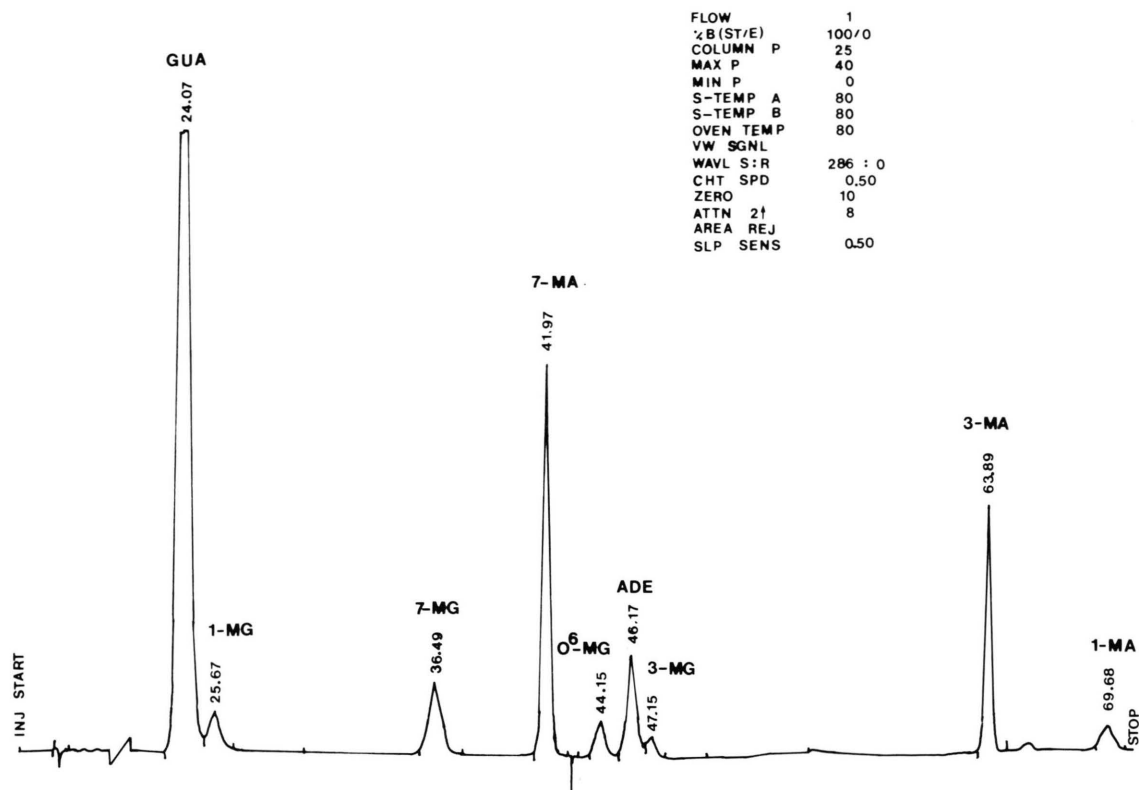Fig. 7. HPLC separation of guanine and adenine and seven methylated derivatives.
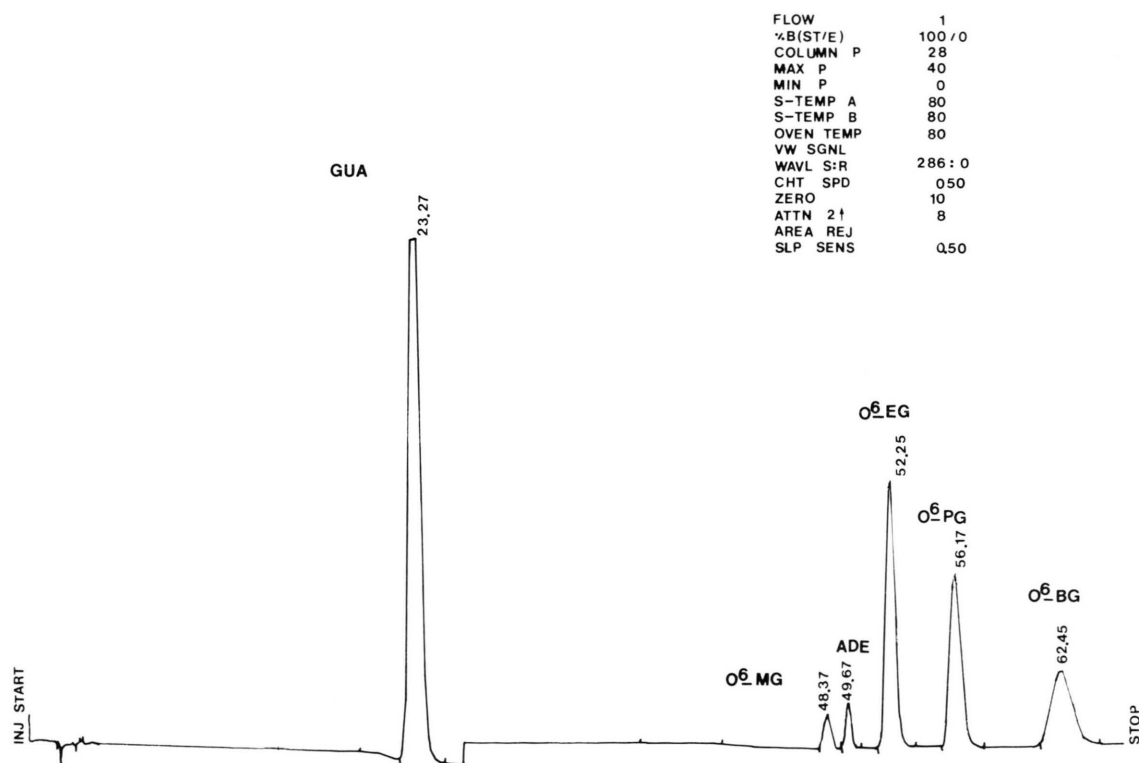
Fig. 8. Separation of O⁶-alkylated guanines.
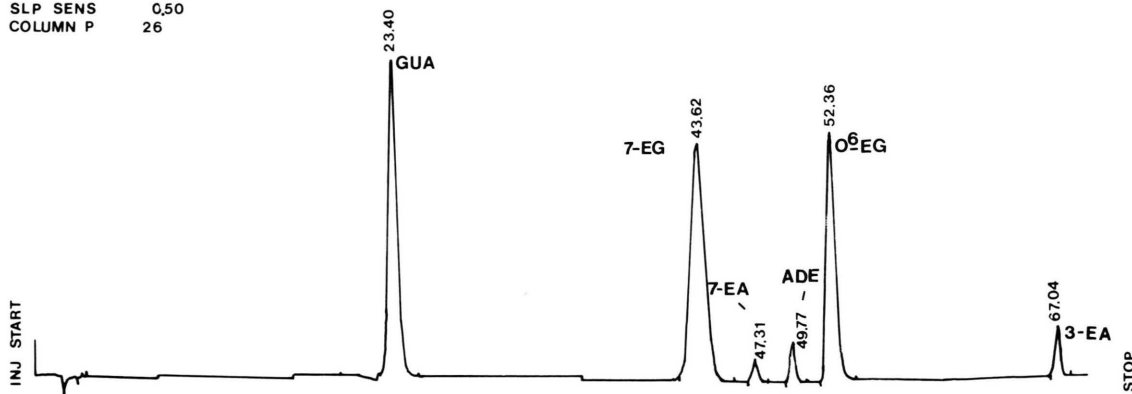
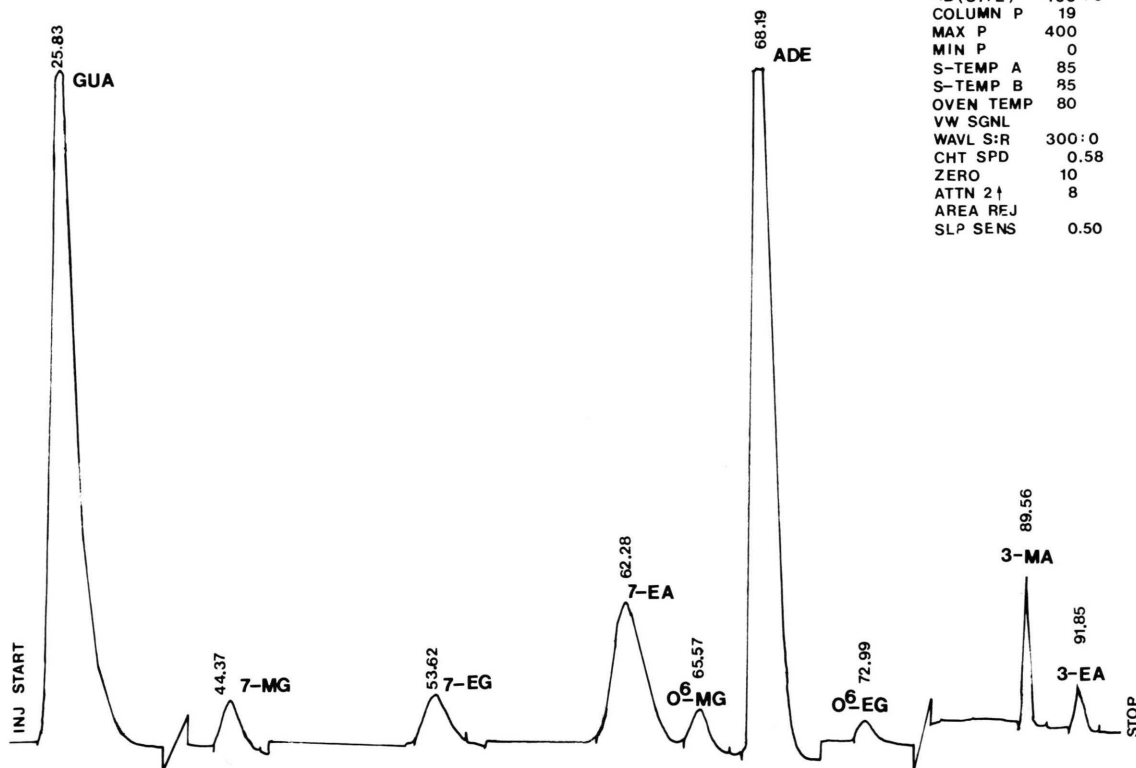Fig. 9. HPLC separation of guanine, adenine and the ethylated purines 3-EA, 7-EA, 7-EG and O^6-EG.

Fig. 10. HPLC separation of methylated and ethylated purines (guanine, adenine, 7-MG, 7-EG, 7-EA, O^6-MG, O^6-EG, 3-MA and 3-EA).

the livers and the uteri were taken. The livers were immediately homogenated, the embryos of each 4 dams were pooled. DNA isolation and purification, as well as DNA hydrolysis and LSC counting, was performed as described earlier [10]. The separation by HPLC was performed using the method shown in Fig. 9. Because of the small amount of radio-labelled bases, the alkylated bases were added as markers prior to separation. In addition to the eluate fractions associated with the ethylated bases, further radioactive fractions were found in both maternal liver and embryonic DNA. By using the separation method as shown in Fig. 10, these fractions could be identified as the methylated derivatives 7-MG, O^6-MG and 3-MA. The DNA alkylation rates are compiled in Table IV.

4.2. DNA alkylation experiments *in vitro*

Alkylation of calf thymus DNA

5 mg calf thymus DNA (Serva Inc.) were dissolved in 5 ml buffer (sodium acetate 0.08 M, EDTA-Na 0.03 M, pH 7.1). ^{14}C-labelled EMS was added (0.2 mCi, 3 mM) and allowed to react 1, 2, 4, and 6 h, respectively, at 37 °C. The reaction was termi-

nated by precipitation of the DNA with icecold ethanol. After washing, the DNA was redissolved and precipitated two times before hydrolysis was performed (0.1 N HCl, 37 °C, 16 h). The separation of the methylated and ethylated purine bases was performed as described in Fig. 10.

In a second series, calf thymus DNA was alkylated by [^{14}C]EMS and mouse liver homogenate/NADPH was added. 4 mg c.t. DNA in buffer (sodium phosphate 150 mM, EDTA-Na 150 mM, pH 7.4), addition of EMS (165 µCi, 2 mM), 6 mg NADPH and mouse liver homogenate (30 mg protein), total volume 6 ml, incubation 1 h at 37 °C. The DNA was isolated and purified using a modified phenol extraction. Hydrolysis and separation were performed as described above. The results are compiled in Table V.

DNA alkylation of liver slices

Adult male NMRI mice were sacrificed by cervical dislocation. The livers were removed. They were dissected into 1 mm slices using two scalpels. Incubation was performed using the method of Nau *et al.* [36]. Sealed bottles were rotated in a roller device in HAMF medium (HAM's F 12, 5% FCS, preincubated 10 min/56 °C, P/S, glut., bma aa, bicarbonate).

Table IV. DNA alkylation by EMS *in vivo*, 210 mg/kg, i.p., day 11 of pregnancy, 4 h.

Site	Liver Ethylation[a]	Methylation[a]	Embryo Ethylation[a]	Methylation[a]
N-7 GUA	477.2	48.0	357.2	32.4
O-6 GUA	15.0	1.9	14.3	1.5
N-3 ADE	13.5	1.4	9.9	1.0
N-7 ADE	1.6	n.d.[b]	0.6	n.d.[b]

Ratios		Liver	Embryo
ethylated bases	O^6-EG/7-EG	0.03	0.04
	3-EA/7-EG	0.03	0.03
	7-EA/7-EG	0.004	0.002
methylated/	7-MG/7-EG	0.11	0.09
ethylated bases	O^6-MG/O^6-EG	0.13	0.10
	3-MA/3-EA	0.10	0.10
methylated	O^6-MG/7-MG	0.04	0.05
bases	3-MA/7-MG	0.03	0.03
ratios MMS[c]			
methylated	O^6-MG/7-MG	0.007	0.008
bases	3-MA/7-MG	0.03	0.04

[a] The alkylation rates were calculated as pmol alkylated base per µmol guanine.
[b] n.d. = not determined.
[c] Bochert *et al.*, 1978 [12].

[^{14}C]EMS was added (0.15 mCi, 2 mM) and incubated 2 h at 37 °C. DNA isolation, as well as HPLC, was described above. The data are shown in Table V.

5. Gas chromatography

5.1. Apparatus and separation conditions

2300 Carlo-Erba fitted with a FID detector, column: 2 m × 2 mm, OV 17 (50% coated, Appl. Sci.), 120 °C isotherm, carrier gas: 40 ml N_2/min, injection temperature: 220 °C, detection temperature: 300 °C.

5.2. Isolation and determination of EMS and MMS from standards

To about 0.2 ml mouse serum of adult non-pregnant female NMRI mice 1 µmol EMS and MMS were added. Protein was precipitated by addition of 0.4 ml acetonitrile. After centrifugation (550 × g, 2 min) 0.4 ml of the supernatant were mixed with 0.4 ml ethyl acetate and 0.4 g dried sodium sulfate. The upper layer was dried with sodium acetate, evaporated and redissolved in hexane. The recovery rate was 71 ± 14% (EMS) and 61 ± 9% (MMS).

5.3. Determination of EMS in serum

Adult mice were treated once i.p. with 300 mg/kg EMS. 2 or 4 h following treatment the animals were sacrificed and the serum concentration of the individuals was determined to be 2.6 mM and 2.8 mM (2 h) and 2.5 mM and 2.2 mM (4 h). No MMS was detected.

In a second experiment sera of 9 animals were pooled 1 h following a single i.p. dosage of 300 mg/

kg EMS. The EMS concentration was determined (2.4 mM). No MMS was found. Using this method a concentration of 15 µm MMS in the serum would have been detected representing less than 1% of the EMS concentration.

Discussion

Before starting DNA alkylation studies it is necessary to have the main alkylated bases available. Because the amount of radio-labelled alkylated bases obtained following DNA alkylation *in vivo* is small, it is necessary to add the non-labelled alkylated bases as markers prior to HPLC separation. In previous communications dealing with the *ethylation* of DNA the following four bases were reported to be the principal reaction products: 7-ethylguanine (7-EG); O^6-ethylguanine (O^6-EG); 3-ethyladenine (3-EA) and 7-ethyladenine (7-EA) [6, 7, 37]. By revising the literature, the synthetic methods, as well as the analytical data reported were not convincing in our opinion. This holds true especially for 7-EA and 7-EG. We achieved the synthesis of 7-EG from the reaction of diethylsulfate and guanosine by a simple procedure using either dimethylacetamide or hexamethylphosphoramide as solvent. Furthermore, synthesis of 7-EA was performed by modifying the method of Denayer [20] for the preparation of 7-MA. In Table I the alkylated guanine and adenine bases are compiled which were synthesized in our group.

The spectroscopic data have been discussed in the experimental part of this paper in detail. The identity of the compounds was confirmed by infrared, mass and NMR spectroscopy. The principal IR absorption

Table V. DNA alkylation data obtained by alkylation with [^{14}C]EMS *in vitro*.

| | | pmol alkylated base per µmol guanine | | | | Alkylation rates per 7-ethylguanine | | |
		7-EG	O^6-EG	3-EA	7-EA	O^6/N-7	N-3/N-7	7-EA
calf thymus								
DNA *in vitro*	1 h	106 ± 3	5.0 ± 0.4	7.1 ± 0.7	1.5 ± 0.4	0.05	0.07	0.01
2 mM EMS	2 h	220 ± 9	9.0 ± 1	13.3 ± 0.6	2.4 ± 0.3	0.04	0.06	0.01
	4 h	369 ± 11	13.7 ± 1	23.6 ± 1	4.9 ± 0.6	0.04	0.07	0.01
	6 h	550 ± 13	19.7 ± 2	33.3 ± 2	6.8 ± 0.3	0.04	0.07	0.01
c.t. DNA, homogenate, NADPH								
2 mM EMS	1 h	67	3.5	4.2	< 1	0.05	0.06	–
liver slices, 3 mM EMS	1 h	79	5	3	< 1	0.06	0.04	–

bands are compiled in Table II. Fig. 1−4 show the mass spectra and fragmentation pathways of 3-EA and 7-EA, as well as of O^6-PG and O^6-BG. Table III contains the PMR shift data.

Over the last years, NMR spectroscopic analysis of intact biomolecules has gained much interest. Using ^{13}C-enriched MMS, DNA *methylation* experiments had been performed and the DNA was analyzed by ^{13}C NMR spectroscopy [32]. In preparation for similar studies on DNA *ethylation* we reported the spectroscopic data of 7-EA (*cf.* Experimental part).

Hitherto, the influence of alkylating agents on embryonic development has been mainly investigated with morphological methods, and in only a few cases with biochemical methods. This is probably due to the small amount of material available from embryonic tissue. Cation-exchange HPLC is the most efficient method for separating natural and alkylated purine bases. Shaikh *et al.* [38] reported a fast HPLC separation on a Partisil 10-SCX column with ammonium phosphate buffer. With the sensitive analytical method developed in our group, it has become possible to study the alkylation of nuclear DNA in fetal and embryonic tissues. The routinely used methods are: alkylation *in vivo* with a ^{14}C-labelled alkylating agent, DNA isolation, hydrolysis of DNA to purine bases, chromatographic separation and determination of the radioactivity of the frac-

tions containing the alkylated purine bases. The methods and the results obtained are described in detail in previous papers. A typical separation run of a DNA hydrolysate is shown in Fig. 6 [10−12, 34].

In the beginning, the chromatographic separation had been achieved by cation-exchange chromatography on a modified amino acid analyser. Use of the advanced HPLC technique enabled us to solve even more sophisticated separation problems.

First, we excluded a possible contamination with "minor bases" of the three methylated bases which we measure routinely. As shown in Fig. 7, a separation of seven methylated purines was performed: 1-, 3-, and 7-methyladenine, 1-, 3-, 7- and O^6-methylguanine. Following a DNA alkylation experiment *in vivo* (10 mg/kg i.p. methylnitrosourea), the DNA of 11-day-old mouse embryos was analyzed. By measuring the radioactivity of the eluate in 1 ml fractions, we could confirm that the fractions which were routinely determined are not contaminated with "minor bases" (Fig. 11).

Furthermore, a separation method for the ethylated bases (3-EA, 7-EA, 7-EG, O^6-EG) was elaborated (Fig. 9).

We then applied this method to a DNA alkylation experiment *in vivo*. Pregnant mice were treated i.p. once with 210 mg/kg ^{14}C-EMS on day 11 of pregnancy. 4 h later maternal livers and the embryos were
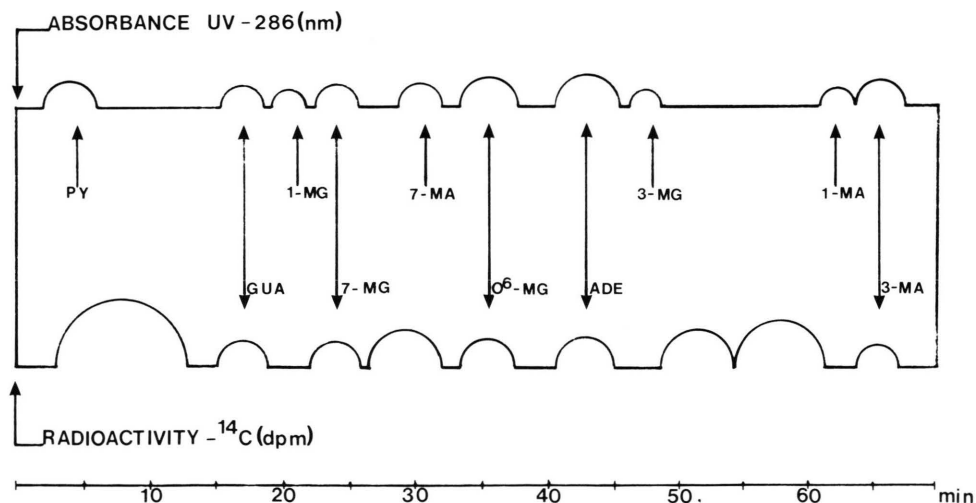

Fig. 11. Exclusion of contamination with minor bases. DNA of 11-day-old embryos was analyzed 1 h following i.p. administration of 10 mg/kg MNU to the dams. Following hydrolysis to the purine bases (37 °C, 16 h, 0.1 N HCl) the methylated derivatives were added as markers. 1 ml fractions were collected and the radioactivity was measured by LSC. The diameters of the semicircles indicate the period of UV absorption (above) and radioactivity (below) during the separation run.

taken. The DNA was isolated and hydrolyzed. First, separation of the ethylated purines was performed. They were determined by LSC of the corresponding chromatographic fractions. The data are shown in Table IV.

Krüger [39] reported that following alkylation of rat liver DNA with propyl- and butylnitrosamines he could identify 7-MG in addition to the expected propylated and butylated bases. Therefore, we established a method for separation of the most important methylated and ethylated purines in a single chromatographic run (Fig. 10).

To our surprise an appreciable amount of the methylated homologues was found in both maternal and embryonic DNA. For all analyzed alkylation sites, the amount of *methylation* was about 10% (9–13%) of the respective *ethylation* rate. This holds true for the maternal liver DNA, as well as the embryonic DNA. Therefore, the relative alkylation rates (ratios: O^6-MG/7-MG and 3-MA/7-MG) were similar to the respective ratios of the ethylated bases (O^6-EG/7-EG and 3-EA/7-EA). For comparison, the ratios calculated from DNA alkylation studies with MMS are included in Table IV. Here, the ratio O^6-MG/7-MG is five times lower.

To confirm our own findings we performed some DNA alkylation experiments *in vitro*. These should exclude possible contamination of the radioactive EMS with methylating agents and, furthermore, separation artefacts. First, calf thymus DNA was reacted with EMS *in vitro* for 1, 2, 4 and 6 h using the same commercial batch. The data are shown in Table IV, no methylated derivatives could be detected. The same negative result was obtained following addition of homogenate/NADPH to the reaction mixture, as well as the analysis of DNA of liver slices after reaction with EMS *in vitro* (Table IV). So, either the amount of formation *in vitro* is too low or the complete *in vivo* metabolising system is necessary for the formation of methylated purines.

The metabolic fate of EMS in the rat was investigated by Roberts and Warwick [40]. The metabolites found were S-ethylcysteine and carbondioxide. They postulated the latter to be derived from ethanol as an intermediate. The findings do not indicate, but also do not exclude a methylating metabolic intermediate.

Following this idea we tried to detect MMS in the serum of mice treated with EMS. Non-pregnant mice were treated once i.p. with 300 mg/kg non-labelled EMS. The serum concentration of EMS was found to be 2–3 mM using a GC-method. But no MMS was found. This result is in good agreement to the relative *methylation* rates induced by MMS. Therefore, an experimentally supported idea for the formation of the methylated derivatives cannot be proposed.

Krüger [39] and Krüger and Bertram [41] have proposed that *e.g.* dipropylnitrosamine is biotransformed analogously to the β-oxidation of fatty acids and hypothesized the formation of methyl-propyl-nitrosamine which he assumed to be the methylating species responsible for the methylation of guanine in RNA and DNA *in vivo*. They confirmed their thesis by additional investigations with oxidated metabolites. Further confirmation was given by work of Leung *et al.* [42]. They found O^6-MG and 7-MG in the liver DNA of rats following treatment with N-nitroso-2-oxopropyl-propylnitrosamine. Other authors proposed an ω-oxidation and, in addition, a direct alkyl-chain cleaving oxidation including hydroxylation, oxidation and decarboxylation [43, 44]. In contrast, for ethyl nitrosamines no indication exists for a similar pathway.

Special interest arises to the DNA alkylation rate of the embryo at the O^6-guanine site. In previous studies with the methylating agents methylnitrosourea, acetoxymethyl-methylnitrosamine and MMS we were able to demonstrate that a good correlation exists between the DNA methylation rate at the O^6-guanine site in mouse embryos and the teratogenic potency of the substances. The latter was determined by evaluating skeletal abnormalities. Before starting the DNA alkylation experiments with EMS, we performed large-scale dose-response studies in order to define precisely the teratogenic dose range [5]. The dose of 210 mg/kg represents approximately the 80% effect level of teratogenicity.

In the DNA alkylation experiment *in vivo* reported above following administration of the same dose of EMS the amount of O^6-EG in the DNA of the embryos was found to be 14 pmol per μmol guanine. This is about the same degree of O^6-alkylation compared to those which we obtained by administration of equitoxic doses (80% effect in teratogenicity) of the methylating agents. We interpret this result as an indication that an *ethylation* at the O^6-guanine site may be of similar toxicological significance as the *methylation* is generally believed to be. This is in good agreement with analogous find-

ings in carcinogenicity studies. We are now performing extensive dose-alkylation studies with EMS in order to obtain precise dose-alkylation relationships.

In conclusion, we have developed a method for the analysis of ethylated purine bases in the DNA of mammalian embryos. Preliminary results using EMS as ethylating agent indicate that similarly to methylating agents, the embryotoxic effect of ethylating agents is due to certain amounts of alkylation at the O^6-guanine site.

[1] G. A. Sega, Mutat. Res. **134**, 113−142 (1984).
[2] P. F. Swann and P. N. Magee, Nature **223**, 947−948 (1969).
[3] H. Ueo, R. Takaki, H. Yamagami, S. Nakano, T. Okeda, and K. Sakakibara, Cancer Lett. **79**, 79−84 (1979).
[4] B. N. Hemsworth and H. Jackson, in: A Symposium on Embryopathic Activity of Drugs (J. M. Robson, F. M. Sullivan, and R. L. Smith, eds.), pp. 116−137, Churchill Ltd., London 1965.
[5] T. Platzek, G. Bochert, W. Schneider, and D. Neubert, Arch. Toxicol. **51**, 1−25 (1982).
[6] P. D. Lawley, D. J. Orr, and M. Jarman, Biochem. J. **145**, 73−84 (1975).
[7] J. V. Frei, D. H. Swenson, W. Warren, and P. D. Lawley, Biochem. J. **174**, 1031−1044 (1978).
[8] M. S. S. Murthy, C. J. Calleman, S. Osterman-Golkar, D. Segerbäck, and K. Svensson, Mutat. Res. **127**, 1−8 (1984).
[9] H. Bartsch, B. Terracini, C. Malaveille, L. Tomatis, J. Wahrendorf, G. Brun, and B. Dodet, Mutat. Res. **110**, 181−219 (1983).
[10] G. Bochert and J. Webb, in: Methods in Prenatal Toxicology (D. Neubert, H.-J. Merker, and T. E. Kwasigroch, eds.), pp. 456−464, Georg Thieme Publ., Stuttgart 1977.
[11] G. Bochert and B. Schnieders, in: Embryotoxikologische Probleme in der Arzneimittelforschung, AMI-Berichte (B. Schnieders, G. Stille, and P. Grosdanoff, eds.), pp. 148−152, Dietrich Reimer Verlag 1978.
[12] G. Bochert, U. Rahm, and B. Schnieders, in: Role of Pharmacokinetics in Prenatal and Perinatal Toxicology (D. Neubert, H.-J. Merker, H. Nau, and J. Langman, eds.), pp. 235−252, Georg Thieme Publ., Stuttgart 1978.
[13] G. Bochert, T. Platzek, and M. Wiessler, in: Culture Techniques (D. Neubert and H.-J. Merker, eds.), pp. 223−235, Walter de Gruyter, Berlin 1981.
[14] T. Platzek, G. Bochert, and U. Rahm, Arch. Toxicol. **52**, 45−69 (1983).
[15] G. Bochert, T. Platzek, G. Blankenburg, M. Wiessler, and D. Neubert, Arch. Toxicol. **56**, 139−150 (1985).
[16] P. Brookes and P. D. Lawley, J. Chem. Soc. **1961**, 3923−3928.
[17] P. B. Farmer, A. B. Foster, M. Jarman, and M. J. Tisdale, Biochem. J. **135**, 203−213 (1973).
[18] J. W. Jones and R. K. Robins, J. Am. Chem. Soc. **85**, 193−201 (1963).
[19] J. E. Shaw, D. C. Kunerth, and S. B. Swanson, J. Org. Chem. **41**, 732−733 (1976).
[20] R. Denayer, Bull. Chim. Biol. **1962**, 1358−1364.

[21] J. Deutsch, Z. Neiman, and F. Bergman, Org. Mass Spectrom. **3**, 1219−1221 (1970).
[22] J. M. Rice and G. O. Dudek, J. Am. Chem. Soc. **89**, 2719−2725 (1967).
[23] A. B. Foster, Lab. Pract. **18**, 743−748 (1969).
[24] C. L. Angell, J. Chem. Soc. **1961**, 504−515.
[25] B. C. Pal and C. A. Horton, J. Chem. Soc. **1964**, 400−405.
[26] J. A. Montgomery and Holum, J. Am. Chem. Soc. **79**, 2185 (1957).
[27] C. D. Jardetzky and O. Jardetzky, J. Am. Chem. Soc. **82**, 222−229 (1960).
[28] G. S. Reddy, L. Mandell, and J. H. Goldstein, J. Chem. Soc. **1963**, 1414−1421.
[29] W. C. Coburn, Jr., M. C. Thorpe, J. A. Montgomery, and K. Hewson, J. Org. Chem. **30**, 1110−1113 (1965).
[30] K. Hino, A. Irie, and H. Uno, Chem. Pharm. Bull. **23**, 1696−1701 (1975).
[31] P. Brookes and P. D. Lawley, J. Chem. Soc. **1960**, 539−545.
[32] C. Chang and C. Lee, Cancer Res. **38**, 3734−3736 (1978).
[33] M.-T. Chenon, R. J. Pugmire, D. M. Grant, R. P. Panzica, and L. B. Townsend, J. Am. Chem. Soc. **97**, 4627−4635 (1975).
[34] G. Bochert, T. Platzek, U. Rahm, and J. Webb, in: Role of Pharmacokinetics in Prenatal and Perinatal Toxicology (D. Neubert, H.-J. Merker, H. Nau, and J. Langman, eds.), pp. 253−262, Georg Thieme Publ., Stuttgart 1978.
[35] I. Chahoud and T. E. Kwasigroch, in: Methods in Prenatal Toxicology (D. Neubert, H.-J. Merker, and T. E. Kwasigroch, eds.), pp. 78−91, Georg Thieme Publ., Stuttgart 1977.
[36] H. Nau, C. Liddiard, H.-J. Merker, and K. Brendel, Life Sci. **23**, 2361−2372 (1978).
[37] L. Sun and B. Singer, Biochemistry **14**, 1795−1802 (1975).
[38] B. Shaikh, S.-K. S. Huang, N. J. Pontzer, and W. L. Zielinski, Jr., J. Liq. Chromat. **1**, 75−88 (1978).
[39] F. W. Krüger, Z. Krebsforsch. **76**, 145−154 (1971).
[40] J. J. Roberts and G. P. Warwick, Biochem. Pharmacol. **1**, 60−75 (1958).
[41] F. W. Krüger and B. Bertram, Z. Krebsforsch. **80**, 189−196 (1973).
[42] K.-H. Leung, K. K. Park, and M. C. Archer, Toxicol. Appl. Pharmacol. **53**, 29−34 (1980).
[43] L. Blattmann and R. Preussmann, Z. Krebsforsch. **88**, 311−314 (1977).
[44] L. Blattmann, Z. Krebsforsch. **88**, 315−322 (1977).
[45] R. W. Balsiger and J. A. Montgomery, J. Org. Chem. **25**, 1573−1575 (1960).