Table I. Hunter L Values of Control and Treated Potato Chips[a]

replicate	control	dipped in aspartic acid	dipped in glutamic acid
1	39.0[b]	47.6	44.2
2	38.3	44.6	44.7
3	38.2	45.3	43.0
4	39.0	47.0	43.0
5	37.9	46.8	43.7
6	36.7	46.6	43.9
7	39.5	47.2	43.6
mean	38.4 ± 0.57[c]	46.4 ± 1.00	43.7 ± 0.57

[a] The treatment consisted in freeze-drying potato slices and dipping them in 0.04 M aspartic or glutamic acid before frying. [b] Each L value is the average of three readings obtained by rotating the sample at 120° angles. [c] The differences between control and treated samples are significant at the 99% probability level. The difference between treated samples is not significant.

In this demonstration, the potato slices were freeze-dried in order to facilitate the absorption of the amino acid solution. In commercial practice, slight drying of the slice surfaces might be sufficient to accomplish the desired solution absorption.

Registry No. L-Aspartic acid, 56-84-8; L-glutamic acid, 56-86-0; L-lysine, 56-87-1; D-glucose, 50-99-7; D-fructose, 57-48-7.

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Methylation of Deoxyribonucleic Acid in the Rat by the Mushroom Poison Gyromitrin

Gyromitrin (acetaldehyde *N*-methyl-*N*-formylhydrazone) is the most preponderant of a group of hydrazone compounds found in the false morel mushroom *Gyromitra esculenta*. It decomposes to products considered to be metabolized to methylating agents in vivo. [*N*-methyl-^{3}H]Gyromitrin was synthesized from formylhydrazine by reaction with acetaldehyde in absolute alcohol and methylation of the resulting hydrazone with tritiated methyl iodide in tetrahydrofuran. [^{3}H]Gyromitrin was administered orally to young adult male rats. After 6 h, DNA was isolated from liver and lung. Although all DNA samples were radiolabeled, analysis by HPLC of the purines liberated from the DNA upon acid hydrolysis revealed that only liver DNA contained 7-methylguanine as a result of DNA methylation. Most radioactivity coeluted with the natural purines and was the result of biosynthetic incorporation of radiolabeled breakdown products of gyromitrin. Expressed as DNA damage per dose in the units of a Covalent Binding Index, CBI, gyromitrin exhibited an average value of 15 for liver DNA, while the respective value for lung (CBI < 5) was below our limit of detection. Upon comparison with the unavoidable intake of the methylating carcinogen dimethylnitrosamine (CBI = 6000), it is concluded that the false morel mushroom should only be consumed occasionally.

The false morel mushroom *Gyromitra esculenta* is poisonous when eaten fresh. Only after drying or thorough cooking is it considered edible and even then sometimes gives rise to intoxications (Franke et al., 1967). Typical symptoms are nausea, gastroenteritis, vomiting, and icterus (Franke et al., 1967; Guisti and Carnevale, 1974). Chemical analysis of the mushroom revealed that the principal toxic component was gyromitrin (List and Luft, 1967, 1969), subsequently found to be the most preponderant of a series of hydrazone compounds (Pyysalo and Niskanen, 1977). Since these compounds are highly volatile, it is not surprising that drying or cooking the mushrooms significantly reduces the toxicity. Gyromitrin is hydrolyzed in vivo to methylhydrazine (von Wright et al., 1978), a compound which was shown to be carcinogenic in the hamster (Toth and Shimizu, 1973) and mouse (Toth, 1972) after oral administration. Methylhydrazine methylates mouse liver DNA to a small extent (Hawks and Magee, 1974). Another line of research links the carcinogenic effect of gyromitrin to the formation of a nitrosamide (Braun et al., 1980; see Figure 1), a family of compounds known to be carcinogenic through their spontaneous conversion into alkylating agents.

The above considerations strongly suggested a possible genotoxic risk from gyromitrin via either methylhydrazine or nitrosamide formation and subsequent DNA methylation. Indeed, an increased lung tumor rate in Swiss mice was found after oral application of 100 mg of gyromitrin/kg of body weight weekly during their lifetime (Toth et al., 1981).

The aim of our work was to determine the DNA-methylating potency of gyromitrin in vivo and to compare

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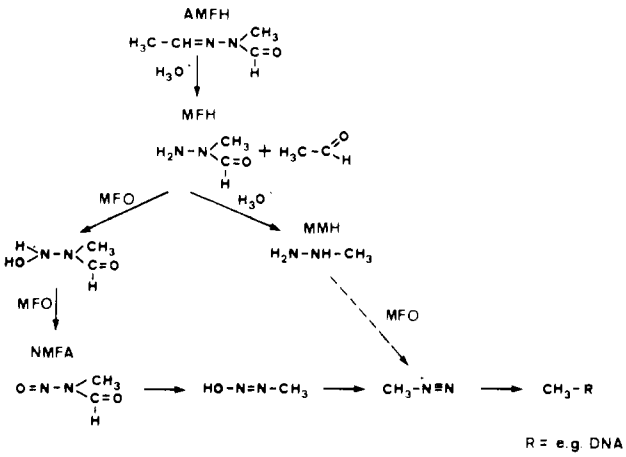

Figure 1. Possible metabolic degradation of gyromitrin to methylating agents, modified after Braun et al. (1980). AMFH, gyromitrin; MFH, methylformylhydrazine; MMH, methylhydrazine; NMFA, nitrosomethylformamide; MFO, mixed-function oxidase.

it with a known carcinogenic methylating agent occurring in food, dimethylnitrosamine (DMNA). The methylation of DNA of rat liver and lung was determined by using radiolabeled gyromitrin, synthesized with a ^3H marker in the *N*-methyl group.

EXPERIMENTAL SECTION

Apparatus and Reagents. Gas chromatographic analyses were performed on Hewlett-Packard 5840A and Carlo Erba Fractovap 4160 models equipped with a Duran capillary column, 17 m × 0.32 mm; 0.12 μm of SE-52. The stationary phase used for liquid chromatography was Merck silica gel 60, particle size 0.063–0.200 mm. GC/MS spectral analyses were carried out on Varian M.A.T. 112 and Finnigan M.A.T. 4500 models. NMR spectra were recorded on a Varian A-100 spectrograph. Radioactivity was measured on a Packard Tricarb 460 CD liquid scintillation counter in a ^3H channel, 0–12 keV. The HPLC system consisted of a Kontron pump, a rheodyne injector, and a Perkin-Elmer UV spectrophotometer set to 254 nm. [^3H]Methyl iodide was from the Radiochemical Centre, Amersham.

Synthesis of [^3H]Gyromitrin. Formylhydrazine was prepared as described by List and Luft (1968). Reaction with acetaldehyde in absolute alcohol followed by column chromatographic purification and recrystallization afforded a pure sample of acetaldehyde *N*-formylhydrazone whose physicochemical data were in accord with the structure: mp 57–62 °C (2-propanol/hexane); mass spectrum *m/e* (rel intensity) 86 (M^+, 6.7), 71 (4.5), 57 (7.5), 42 (100); NMR (100 MHz; Me_2SO-d_6; ppm from Me_4Si) 1.83 (d, 3 H), 7.33, 7.50 (2 q, 1 H), 7.87, 8.47 (two br s, 1 H), 10.94, 11.20 (two br s, 1 H); IR (KBr) 3175 (N–H), 1670 (C=O), 1635 cm^{-1} (C=N); mixture of isomers. Anal. Calcd for $C_3H_6N_2O$: C, 41.86; H, 6.98; N, 32.56;. Found: C, 42.07; H, 7.05; N, 32.71%. Our literature search revealed no recorded data for this compound.

Acetaldehyde *N*-formylhydrazone (80.12 mg, 0.932 mmol), sodium hydride (81.20 mg, 50% suspension; 1.692 mmol; 1.815 equiv), and a magnetic stirring bar were placed in dry tetrahydrofuran (THF) (1.5 mL) under N_2 in a round-bottomed flask fitted with a septum through which two needles (13 × 0.45 mm) were pierced, one equipped with a valve for escape gases and one attached to a N_2-filled balloon. The mixture was stirred for 5 min at ice-bath temperature and then between 50 and 55 °C for 1 h. Into this mixture was subsequently distilled at liquid N_2 temperature and a pressure of 6×10^{-5} torr a sample of [^3H]methyl iodide (30 μL, 0.48 mmol, 25 mCi, ca. 52 mCi/mmol) whereafter the reaction vessel was closed. Repeated sequences of warming to room temperature, stirring, and freezing were employed to ensure maximal contact between the reagents. The methylation reaction was completed with an excess of unlabeled methyl iodide. Subsequent distillation of the solvent (−50 to −60 °C, 5×10^{-4} torr) left a residue which was suspended in dry benzene and filtered through a Celite pad. The benzene solution (8 mL), containing 6.2 mCi of crude [^3H]gyromitrin (25.1% yield), was stored at −20 °C. A pure sample was isolated immediately before use by silica gel chromatography using dichloromethane/2-propanol (20/1 v/v) to elute 1.1 mCi of the required compound, exhibiting a radiochemical purity of 98.9% and a specific activity of approximately 23 mCi/mmol, as determined by colorimetric comparison with authentic gyromitrin on TLC sprayed with a solution of cinnamaldehyde and concentrated hydrochloric acid (HCl) in methanol. Two-dimensional radio-TLC with autoradiographic analysis was employed to compare the nature of the radioactivity with an authentic unlabeled sample. The solvent pairs were (A) benzene/THF/absolute ethanol, 50/50/2.5, and (B) dichloromethane/absolute alcohol, 20/1.

A preliminary attempt to methylate the hydrazone with [^3H]dimethyl sulfate in dimethylformamide (DMF) was less successful, and the resulting sample of 55 μCi (54 mCi/mmol) was used for a preliminary DNA-binding experiment on a low-dose level.

DNA-Binding Experiment. Male rats (Sprague-Dawley-derived SIV 50) weighing about 250 g were administered by oral gavage [^3H]gyromitrin dissolved in about 1 mL of water (doses are given in Table I). The animals were kept in metabolism cages during 6 h with free access to water and food. Urine was collected and counted for ^3H activity in Aquasol (New England Nuclear). The animals were bled by open heart puncture under ether anaesthesia, and liver, kidneys, and lung were excised. Blood was solubilized with Soluene-350 (Packard) and decolored with hydrogen peroxide.

DNA of liver and lung was isolated according to Markov and Ivanov (1974) with some modifications (Viviani and Lutz, 1978). In short, the tissues were homogenized and deproteinated by phenol/chloroform/isoamyl alcohol extraction. DNA was purified on hydroxylapatite columns (DNA-grade Bio-Gel HTP, Bio-Rad). After dialysis against 0.2 M NaCl, the DNA was precipitated with ethanol, dried, and dissolved in a 0.014 M phosphate buffer. The specific activity of the pure DNA was determined by counting the ^3H activity in Insta-Gel (Packard) and spectrophotometric determination of the DNA amount at 260 nm (absorbance = 20 at 260 nm for a solution of 1 mg/mL). The specific activity of DNA was expressed per unit dose as (dpm/mg of DNA)/(dpm/kg of body weight).

An aliquot of the DNA was hydrolyzed with 0.1 N HCl to liberate the purines (Lawley, 1976). 7-Methylguanine standard (Sigma) was added to the hydrolysate and the mixture was separated on a reverse-phase μBondapak C_{18} column, 300 × 7.8 mm (Waters) with 0.01 M ammonium phosphate buffer containing 1% methanol at pH 4. The flow was 3.5 mL/min. The elution was followed spectrophotometrically at 254 nm. Radioactivity of the fractions was determined in Insta-Gel. The amount of ^3H activity in the 7-methylguanine peak was expressed as a fraction of the total ^3H activity injected. The level of DNA methylation was then converted to the molar units of the Covalent Binding Index, CBI = (micromoles of chemical bound per mole of DNA nucleotide)/(millimoles of chem-

Table I. Methylation of DNA Isolated from Different Organs, 6 h after Oral Treatment of Four Rats with a Low or High Dose of [^{3}H]Gyromitrin

	dose			DNA				deg of methylation, act. in 7-methylguanine	
rat no.	mg/kg	dpm/kg	organ	total act., cpm	amount in vial, mg	sp. act.,[a] dpm/mg	sp act./dose (dpm/mg)/(dpm/kg)	%	CBI[b]
1	0.24	2.9×10^8	liver	35.7	2.98	17.8	6.2×10^{-8}	ND[d]	<19
			lung	25.8	0.91	17.4	6.1×10^{-8}	ND	<18
2	0.15	1.8×10^8	liver	32.5	2.15	19.4	1.1×10^{-7}	ND	<33
			lung	27.0	1.02	20.0	1.1×10^{-7}	ND	<34
3	10.0	5.7×10^9	liver	100.0	0.54	413.3	7.3×10^{-8}	53	12
			lung	143.4	0.79	425.7	7.5×10^{-8}	<18[c]	<4.1
4	7.8	4.5×10^9	liver	117.2	0.57	469.8	1.1×10^{-7}	57	18
			lung	127.8	0.76	386.1	8.7×10^{-8}	<18	<4.9
mean ± SD (no. of animals)			liver				$(8.9 \pm 2.5) \times 10^{-8}$ (4)		15 ± 4 (2)
			lung				$(8.3 \pm 2.1) \times 10^{-8}$ (4)		<4.5 (2)

[a] Background value for DNA isolated from control animals that had not been administered radiolabels: 21.0 ± 0.5 cpm. Counting efficiency: no. 1 and 2, 28%; no. 3 and 4, 36%. [b] Covalent Binding Index (CBI) = (μmol of 7-methylguanine/mol of DNA nucleotides)/(mmol/kg). [c] Background HPLC fractions of base analysis: 18 ± 1.3 cpm. Counting efficiency: 25%. [d] ND = no data.

ical administered per kilogram of body weight) by using the specific activity of DNA divided by the dose and an average molecular weight of a DNA nucleotide of 309 (Lutz, 1979). CBI = (dpm/mg of DNA)/(dpm/kg of body weight)(309)(10^6).

RESULTS AND DISCUSSION

Six hours after the oral administration of [^{3}H]gyromitrin to rats, ^{3}H radioactivity was distributed as follows: 16–18% was excreted in the urine, 5.1–5.4% was measured in the whole blood, 2–3% in the liver, 0.3–0.5% in the kidney, and 0.1–0.3% in the lung. DNAs isolated from liver and lung were all radiolabeled (Table I). The specific activity of the DNA isolated from the two rats which received the low dose of gyromitrin (no. 1 and 2) was about 20 dpm/mg. This was too low to allow a degradation of the DNA to its constituents in order to determine whether the radioactivity was due to methylations or to biosynthetic incorporation of radiolabeled precursors of DNA synthesis. The higher dose administered to rats no. 3 and 4 resulted in specific activities of the DNA of about 400 dpm/mg. Here, purine base analysis of acid-hydrolyzed DNA of liver and lung allowed a differentiation between methylation of DNA and biosynthetic incorporation of ^{3}H activity from precursors into natural bases (Figure 2). As is illustrated in the second last column of Table I, 7-methylguanine was detectable only in the liver DNA but not in the lung where the lower amount of DNA available for base analysis resulted in nonsignificant radioactivities in the 7-methylguanine fraction.

For a comparison of the values obtained from the single animals with each other, a normalization to the dose administered was performed and the third last column of Table I shows that the radiolabeling of whole DNA was proportional to the dose administered. This is an indication that the processes involved followed first-order kinetics and that no saturation of any type was reached with the high dose.

For the comparison of the methylating potency of gyromitrin with other methylating agents, an expression of the above data in the units of the "Covalent Binding Index" (Lutz, 1979) seemed appropriate. For such, only those DNA samples were taken where 7-methylguanine had been detected (liver DNA of rats no. 3 and 4). Based on the percentage of the radioactivity collected in the 7-methylguanine peak as a fraction of the injected, a mean Covalent Binding Index, CBI = 15, could be calculated for DNA of the liver (Table I). This CBI of 15 places gyromitrin among the weakly genotoxic agents (Lutz, 1979), in

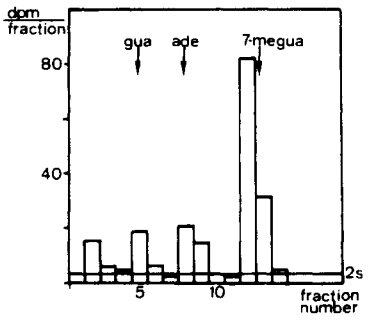

Figure 2. HPLC–radiochromatogram of purine bases released from acid-hydrolyzed liver DNA (sp act. 413 dpm/mg). gua, guanine; ade, adenine; 7megua, 7-methylguanine; 2s, two standard deviations of the mean of the total counts of background fractions 16–20.

agreement with the low carcinogenic potency (Toth et al., 1981). High dose levels were needed for tumor induction, viz., approximately 100 mg/kg of body weight once weekly was administered in their study.

Methylation of lung DNA was below our limit of detection, although methylhydrazine, *N*-methyl-*N*-formylhydrazine (MFH), and gyromitrin have been shown to increase the lung tumor rate in mice (Toth, 1972; Toth and Nagel, 1978; Toth et al., 1981). It has been shown recently that lung tissue is comprised of a large number of different cell types, only some of which contain appreciable amounts of cytochrome P-450 (Boyd, 1981) thought to be required for the activation of gyromitrin or its degradation products to a methylating agent. DNA methylations in these cells could therefore be diluted below limit of detection by the DNA contributed by all the other cells.

7-Methylguanine is the most abundant methylated base formed upon reaction of DNA with methylating carcinogens, representing about 80% of all DNA methylations (Lawley, 1976), and was the only methylation product detectable in the present assay. The level of DNA methylation in the 7-position of guanine is not correlated with mutagenic or carcinogenic effects. Other methylation products, such as O^6-methylguanine, seem to be more important. The relative abundance of other methylated bases is dependent upon the reactivity of the ultimate methylating agent. The ratio of O^6- to 7-methylation of guanine has been shown to be the same for 1,2-dimethylhydrazine, *N*-methyl-*N*-nitrosourea and dimethylnitrosamine (DMNA) (Lawley, 1976; O'Connor, 1981). A direct comparison of genotoxic potencies of gyromitrin and DMNA on the basis of the level of 7-methylguanine formation

should therefore be possible.

DMNA exhibits a covalent binding index for rat liver DNA of 6000 (Meier-Bratschi et al.., 1983); i.e., it is by a factor of 400 more potent than gyromitrin with respect to liver DNA methylation. The DMNA content in the average human diet amounts to approximately 1 μg/day (Spiegelhalder et al., 1980). Thus, a daily intake of 400 μg of gyromitrin would produce the same degree of methylation of liver DNA as an intake of 1 μg of DMNA. Hereby, it must be assumed that the dose-binding relationship is linear down to environmental doses of methylating agent. Good data are available to show that it is indeed the case for the generation of 7-methylguanine from doses of DMNA as little as 10 μg/kg (Diaz Gomez et al., 1977). The relatively low methylating potency of gyromitrin does not allow the detection of DNA methylations at such low dose levels. In a preliminary experiment with the first small batch of [^{3}H]gyromitrin, two rats were treated with 0.15 and 0.24 mg/kg. We could not detect radiolabel in the 7-methylguanine fraction of any DNA sample. The corresponding limit of detection, CBI < 33 and CBI < 19, respectively, for liver DNA was near the true value measured in the main experiment using a 50-fold higher dose.

In the dose range studied, there is therefore no indication for a higher methylating potency at lower doses, so that the liver DNA damage produced by DMNA and gyromitrin can tentatively be compared. Since the use of false morels can be easily avoided in contrast to the intake of DMNA in food, a much lower risk seems to be acceptable for gyromitrin. Although no rules exist for defining such risks, we believe that the acceptable DNA damage from gyromitrin should be at least 100 times lower than that from the ubiquitous and therefore unavoidable DMNA. The acceptable daily gyromitrin intake would then maximally amount to 400/100 = 4 μg. Pyysalo and Niskanen (1977) calculated a permitted intake of 35 μg of gyromitrin man^{-1} day^{-1} derived from the no-effect level of gyromitrin in chicken (0.05 mg kg^{-1} day^{-1}) divided by a security factor of 100. It is reasonable that our approach, based on potentially irreversible effects of gyromitrin, produces a lower limit for an acceptable daily intake.

The above risk estimate has to be based upon the assumption that our data in the rat can be extrapolated to humans. An additional uncertainty has to be faced when the acceptable levels of gyromitrin intake are to be expressed in terms of the whole mushroom. Reported contents range between 10 and 3000 mg/kg of dried mushroom (Pyysalo and Niskanen, 1977; Schmidlin-Mészaros, 1974; Stijve, 1978) so that 4 μg of gyromitrin can be present in as little as 0.4 g of mushroom or less. On the other hand, the chemical instability of gyromitrin during cooking must be taken into account. A conclusive risk estimate therefore will require an analysis of the amount of gyromitrin and other methylating agents on the fork.

ACKNOWLEDGMENT

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Registry No. [*N*-methyl-^{3}H]Gyromitrin, 85650-34-6; formylhydrazine, 624-84-0; gyromitrin, 16568-02-8; 7-methylguanine, 578-76-7; acetaldehyde, 75-07-0; acetaldehyde *N*-formylhydrazone, 85650-35-7; [^{3}H]methyl iodide, 72165-55-0.

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Effect of Bioregulators on the Accumulation of Rubber in Guayule

Previous work on the use of bioregulators to increase the yield of rubber from guayule was expanded to include additional bioregulators, concentrations as low as 125 ppm, and longer treatment periods. A ^{13}C NMR analysis showed substantial increases in rubber as compared to that for the control. A 91% increase in total rubber content was obtained with 2-(3,4-dimethylphenoxy)triethylamine. No statistically significant differences in the percent rubber values were obtained.

Research interest in guayule (*Parthenium argentatum* A. Gray) continues to grow as the worldwide demand for natural rubber increases (D'Ianni et al., 1978). Previously we reported on the increase in rubber content of guayule