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Effects of Selenium on 1, 2-Dimethylhydrazine (BMH) Metabolism and DNA Alkylation

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EFFECTS OF SELENIUM ON 1,2-DIMETHYLHYDRAZINE (DMH) METABOLISM AND DNA ALKYLATION

by

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INTRODUCTION

Selenium is an essential trace element in animal and human nutrition (64, 65, 67, 69). It is found organically bound in nearly all foods including grain, meat, eggs, milk, fruits, vegetables, and seafood. Other biological properties of selenium include toxicity (53, 67, 79), mutagenicity (38, 49, 52), carcinogenicity (30, 50, 80), and anti-carcinogenicity (26, 27, 31, 32, 45, 66, 68). The latter property has led to epidemiological studies which suggest an inverse relationship between human cancer mortality and dietary intake of selenium (34, 64, 69). Therefore, investigations of the anti-carcinogenic properties of selenium in animals are necessary to understand this relationship.

Selenium has recently been shown to inhibit colon carcinogenesis induced by the chemical carcinogen 1,2-dimethylhydrazine (DMH[1]) (31, 32), which specifically induces colon tumors in rats and mice (6, 11, 13, 46, 78). Investigations of the colon-specific mechanisms of action of DMH and/or selenium may shed light on the prevalence of human colon cancer, which shows one of the highest incidences of all neoplastic diseases in the United States (85). DMH has been shown to methylate the DNA of

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[1]The abbreviations used are: DMH, 1,2-dimethylhydrazine; AM, azomethane; AOM, azoxymethane; MAM, methylazoxymethanol; 7-MeG, 7-methylguanine; O^6-MeG, O^6-methylguanine; ENU, *N*-ethyl-*N*-nitrosourea; MNU, *N*-methyl-*N*-nitrosourea; ppm, parts per million; Se, selenium as sodium selenite.

various animal tissues (28, 29, 37, 62, 77). It is believed that alkylation of particular sites in the DNA represents promutagenic events which may lead to tumor initiation. The purpose of the present work is to ascertain whether selenium affects the metabolism of DMH, and/or the alkylation of DNA by DMH, and how these effects are related to the carcinogenic activity of DMH.

LITERATURE REVIEW

Colon Carcinogenesis and 1,2-Dimethylhydrazine

The study of DMH has been of particular interest because of its high specificity for inducing colon cancer in laboratory animals. Druckrey *et al.* (13) reported that weekly s.c. doses of 7 and 21 mg DMH/kg induced intestinal adenocarcinomas in all treated rats. Weekly 20 mg/kg s.c. injections of DMH in mice induced colonic carcinomas in more than 90% of the animals after 186 days (78). In another rat study (46), weekly 20 mg/kg s.c. injections of DMH predominantly induced adenocarcinomas of the colon in 100% of the animals after 24 weeks. Induction of tumors by DMH also depends on genetic susceptibility (2, 11, 14), age, and sex (48).

Metabolic Activation of DMH

The mechanism of tumor induction by DMH has been postulated to involve metabolism to an active alkylating agent (12, 59). Alteration of DNA by alkylation is believed to be a major step in chemical carcinogenesis. In the postulated metabolic pathway (Chart 1), DMH is oxidized, probably nonenzymatically, to azomethane (AM), which may proceed to two alternate pathways. In the inactivation pathway, AM isomerizes to hydrazone, which may be hydrolyzed to form monomethylhydrazine and formaldehyde. In the activation pathway, AM is oxidized, presumably by a microso-

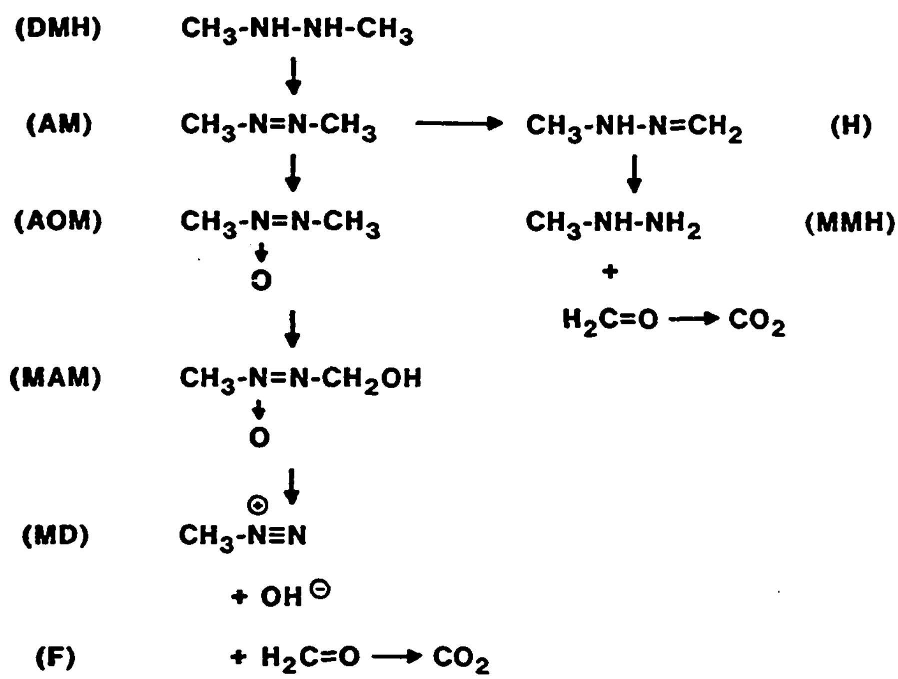

Chart 1. Postulated (12, 59) metabolic reactions leading to the activation and inactivation of DMH. Activation consists of a series of oxidations via AM and AOM to form MAM, which breaks down to formaldehyde (F), hydroxyl ion, and the active methylating species, methyldiazonium (MD). DMH is inactivated by oxidation to AM which isomerizes to hydrazone (H). This may be hydrolyzed to form monomethylhydrazine (MMH) and formaldehyde. The latter is oxidized to form CO_2.

mal *N*-oxygenase, to azoxymethane (AOM), which is hydroxylated to form methylazoxymethanol (MAM). Fiala (18) reported preliminary *in vitro* experiments demonstrating that MAM can be formed by a standard microsomal mixed function oxidase system. Homogenates or microsomal fractions from liver hydroxylated AM, but fractions from kidney or colon mucosa failed to do so. This suggested that the formation of MAM occurs in liver, but not in the target tissue. The breakdown of MAM to formaldehyde (presumably oxidized to CO_2), nitrogen, and methanol is believed to involve the highly reactive methyldiazonium ion, an ultimate carcinogen which forms a methyl carbonium ion. It is uncertain whether the breakdown of MAM is spontaneous or enzymatic. At nearly physiological conditions, MAM decomposed with a half-life of 8 hr (17). Schoental (63) proposed that MAM may undergo oxidation to methylazoxyformaldehyde by the action of an alcohol dehydrogenase. Zedeck *et al.* (87) supported this view, reporting that alcohol dehydrogenase activity was high in colon, duodenum, and cecum. Recent experiments demonstrated that pyrazole, an alcohol dehydrogenase inhibitor, blocked the oxidation of MAM (22). Fiala (18) speculated that noncovalent bonding of MAM occurred directly to bases of nucleic acids.

In the liver, MAM was believed to form a stable conjugate with glucuronic acid, which would be transported *via* the bile to the intestine, where it would be hydrolyzed by bacterial β-glucuronidase in colon to free MAM (17, 84). This would, in part, account for its tissue specificity. Reports that germ-free rats were less sensitive to the carcinogenicity of DMH than conventional rats (61) supported the view that intestinal microflora play an important role. However, more recent data (5, 23, 87) suggest that the carcinogen does not require biliary transport to the intestine in order to exert some of its effect. Tumors were found in sections of nonfunctional colon in colostomized rats after the s.c. administration of DMH or AOM, suggesting that the active metabolites may be transported to the intestine through the circulatory system. Rat colon mucosal cells are capable of activating carcinogens (16), and do possess a microsomal cytochrome *P*-450 enzyme system (15). Cultured human colon epithelial explants activated DMH to a metabolite which methylated the DNA (3).

Evidence that AM, AOM, MAM, and CO_2 are indeed metabolites of DMH has recently been provided (17-21). High pressure liquid chromatographic methods were developed to detect AM in the expired air, AOM in the bile, and unmetabolized DMH, AOM, and MAM in the urine. Evidence for metabolites such as hydrazone and monomethylhydrazine in the inactivation pathway has not been presented in the literature.

Alkylation of DNA by DMH

Recent experiments have shown that DMH, following activation, does indeed alkylate nucleic acids. Early alkylation experiments (28, 29) demonstrated the formation of 7-methylguanine (7-MeG) in liver, colon, small intestine, kidney, lung, and spleen of NMRI mice which were killed 6 or 12 hr after a 15 mg/kg s.c. injection of [^{14}C]DMH. Methylation (7-MeG) was also detected in liver, colon, and kidney nucleic acids from Wistar rats which received a 200 mg/kg s.c. injection of [^{14}C]DMH. Likhachev *et al.* (37) reported 7-MeG in the DNA of various rat tissues 3 hr after a 300 mg/kg s.c. injection of [^{3}H]DMH. Another product, *O*^{6}-methylguanine (*O*^{6}-MeG), was detected in small quantities of liver and colon DNA. The ratio of *O*^{6}-MeG to 7-MeG was four times higher in colon than liver. Rogers and Pegg (62) detected several methylated purines in DNA of rat liver, kidney, and colon 24 hr after a 4 mg/kg i.p. injection of [^{14}C]DMH. These included 7-MeG, *O*^{6}-MeG, 7-methyladenine, 3-methyladenine, 1-methyladenine, and possibly 3-methylguanine. Swenberg *et al.* (77) reported that *O*^{6}-MeG levels in the liver, colon, and ileum increased rapidly in the first 6 hr after s.c. administration of [^{14}C]DMH (20 mg/kg). Maximum alkylation occurred 6 to 12 hr after exposure. Loss of *O*^{6}-MeG between 12 and 72 hr was most rapid in liver and ileum, and least rapid in colon.

Significance of Alkylation

According to the multistage hypothesis, chemical carcinogenesis consists of three stages: initiation, promotion, and progression (4, 58). It is believed that initiation is an irreversible process representing a somatic mutational event which may be the result of promutagenic lesions in DNA, error-prone DNA repair, or other unknown mechanisms. The interaction of alkylating agents with DNA may result in such promutagenic lesions, leading to the initiation process. Thus, the alkylation of DNA may be an important initiating step in the induction of tumors by alkylating agents.

Sites of DNA alkylation

Various alkylated products have been isolated from DNA of various tissues after *in vivo* or *in vitro* exposure to labelled alkylating agents. The major product in all cases was 7-alkylguanine (25, 43, 62, 73, 74), probably because the *N*-7 position of guanine is highly nucleophilic (35). Other DNA sites that react with alkylating agents are the *N*-1, *N*-3, and *N*-7 of adenine, the *N*-3 and O^6 of guanine, the O^2, O^4, and *N*-3 of thymine, the O^2 and *N*-3 of cytosine, and phosphodiesters (70, 74). The extent of alkylation at the various DNA sites differs significantly depending on the alkylating agent.

Biological importance

There has been much research and discussion of the biological importance of alkylation at these various positions. The extent of formation of the major product, 7-alkylguanine, does not correspond to the carcinogenicity of several alkylating agents (75, 76). For example, a single dose of ethyl methanesulphonate caused ten times as much ethylation of rat kidney DNA as *N*-ethyl-*N*-nitrosourea (ENU), but produced no tumors, whereas ENU did produce tumors. Base pairing of 7-MeG *in vitro* was similar to that of guanine (40). Loveless (39) first suggested that O^6-alkylguanine could lead to atypical base pairing. In cell-free experiments, O^6-methylguanine-containing templates for RNA polymerase misincorporated UMP and AMP (24), and O^6-methylated templates for DNA polymerase I (1) misincorporated dTMP into the product polymer. Phage mutagenesis has also been correlated with O^6-alkylation of guanine (36).

Studies have shown a relation between carcinogenicity and O^6-alkylguanine production. However, the initial degree of O^6-alkylation does not correlate with the carcinogenicity of certain compounds. For example, levels of O^6-MeG in rat liver DNA (a nontarget organ) exceeded that in kidney and colon DNA (the target tissue) even at 72 hr after a single carcinogenic dose of DMH (62, 77). The authors suggested that the sensitivity of the kidney and colon to carcinogenesis may be based on other factors such as cell turnover, alkylation of phosphodiesters, or formation of O^4-alkylthymine.

In another case (25), the initial degree of O^6-ethylation by ENU was higher in nontarget tissue (liver) than in target tissue (brain) in neonatal rats. However, the half-life of O^6-ethylguanine in brain DNA (220 hr) was much longer than in liver DNA (30 hr), and longer than other ethylated products. This persistence of O^6-ethylguanine in the DNA of replicating cells may explain the specific carcinogenic effect of ENU in the developing nervous system of the neonatal rat. Similar results were found with *N*-methyl-*N*-nitrosourea (MNU) (42). Brain DNA retained significantly more O^6-MeG than the liver and other tissues after five weekly applications. The authors suggested that rat brain is deficient in enzymes capable of excising O^6-MeG. In other work (8, 9), the induction of bladder and mammary cancer by MNU was correlated with the accumulation of O^6-MeG in the DNA of bladder and mammary tissue, respectively.

However, factors other than the amount of O^6-alkylguanine and its persistence or accumulation cannot be ruled out (62). Other alkylated sites in DNA may be biologically important based on *in vitro* experiments with polynucleotides and nucleosides. Misincorporation of UMP and AMP occurred when 3-methylcytidylic acid units were present in a DNA template for RNA polymerase (41). Experiments measuring codon-directed aminoacyl tRNA binding to ribosomes have indicated miscoding properties for O^2-ethylcytidine (72). Singer (70) suggested that alkylation at the O^2 of cytosine or thymine would weaken the glycosidic linkage causing depyrimidination which could lead to deletions. In O^2-alkylthymine, there is no proton at *N*-3 available for pairing with adenine, so normal pairing could not occur. Lawley (35) suggested that O^4-alkylthymine could mispair with guanine, 3- or 7-alkylguanine with thymine, and 3-alkyladenine with cytosine.

Sun and Singer (74) suggested that reaction of alkyl groups with phosphodiesters to form phosphotriesters could inhibit cation and histone binding, and could lead to changes in interaction with complementary polynucleotides. Singer has recently stated that there is no evidence that ethylphosphotriesters are mutagenic (71).

In summary, many of the known alkylated products could cause some structural changes in DNA. But when chemical agents with differing carcinogenic potency and alkylating ability are tested for miscoding or mispairing properties, those products that appear to be the most biologically significant are: O^6-alkylguanine (55, 56), *N*-3 and O^2-alkylcytosine (72), and O^2- and O^4-alkylthymine (55, 56, 71).

Inhibition of Colon Carcinogenesis

Inhibition of tumor induction

Several chemicals have recently been found to inhibit colon carcinogenesis in laboratory animals. Bracken fern, a human food delicacy and a bovine forage contaminant in certain parts of the world, induced intestinal tumors in all treated rats when administered in the diet (54). Dietary butylated hydroxyanosole, disulfiram, and calcium chloride decreased this incidence by 25-30%. It was suggested that calcium chloride may absorb or precipitate certain carcinogenic compounds in bracken fern. Butylated hydroxyanosole had a similar effect on DMH-induced tumors in mice (81). The mechanisms of inhibition by butylated hydroxyanosole and disulfiram are not clear. Both may act *via* an antioxidant function, and the latter is known to inhibit oxidative enzymes (21, 54).

Colon cancer induced by DMH or its metabolite, AOM, can be inhibited by some compounds including disulfiram. AOM-induced tumors were reduced by dietary disulfiram in Sprague-Dawley rats (51). No tumors were found in female CF_1 mice which received DMH injections after treatment with 5 mg disulfiram per gm of diet (81, 82). A related compound, sodium diethyldithiocarbamate, and two pesticides were also found to inhibit DMH. All three compounds have structural similarities to disulfiram, and contain a carbon disulfide moiety. Carbon disulfide itself inhibited DMH-induced colon tumors in mice (83).

Inhibition of DMH metabolism

Studies by Fiala *et al.* (18, 20, 21) showed that disulfiram, diethyldithiocarbamate, carbon disulfide, and bis(ethylxanthogen) inhibited the metabolic activation of [^{14}C]DMH in rats by significantly increasing the levels of exhaled [^{14}C]AM and decreasing the levels of exhaled $^{14}CO_2$. The levels of urinary AOM and MAM were significantly decreased. It was concluded that these compounds inhibit the *N*-oxidation of AM to AOM, and that the effective inhibiting agent is carbon disulfide or possibly carbonyl sulfide, both of which may be metabolites of the above parent compounds.

Inhibition of the alkylating ability of DMH

Disulfiram not only inhibits the metabolism of DMH but also the alkylation of DNA in various rat tissues by DMH (77). In disulfiram-treated rats, levels of 7-MeG in liver, colon, and ileum DNA were less than 1% of that found in rats treated with DMH alone. *O*^{6}-MeG was undetectable in disulfiram-treated rats. Methylation of DNA by DMH was also inhibited by aminoacetonitrile (57).

Selenium and Inhibition of Carcinogenesis

Another inhibitor of experimental carcinogenesis is selenium, usually in its inorganic form, sodium selenite or selenate (10, 26, 27, 31, 32, 45, 66, 68). Shamberger (68) reported that applications of sodium selenide significantly reduced the incidence of papillomas induced in ICR mice by 7,12-dimethylbenz[α]anthracene and various promoters. Dietary sodium selenite decreased the incidence of skin tumors in mice treated with benzo[α]pyrene. In Harr's experiments (27), rats which were fed a diet containing the carcinogen, *N*-2-fluorenyl acetamide, and 0, 0.1, 0.5, or 2.5 ppm sodium selenite had similar numbers of mammary adenocarcinomas and hepatomas, but the latency period increased with the dose of selenium. Griffin and Jacobs (26) showed that 6 ppm selenium (in the form of sodium selenite) in the drinking water or in the diet decreased the incidence of liver tumors induced in rats by the azo dye, 3'-methyl-4-dimethylaminoazobenzene. Selenium decreased the carcinogenicity and mutagenicity of 2-acetylaminofluorene and its derivatives, and altered the activity of enzymes involved in their activation (10, 33, 45).

Selenium also had inhibitory effects on colon carcinogenesis (31, 32). The colon tumor incidence in rats treated with DMH was reduced from 87% to 40% by 4 ppm selenium in the drinking water. The incidence in rats treated with MAM was not affected, but the total number of tumors was reduced from 73 to 42. All of the animals were sacrificed at the end of the 20-week treatment, so final tumor incidence remains unknown. Jacobs suggested that selenium may act in similar fashion to that proposed for disulfiram, by blocking the oxidation of AM and/or the hydroxylation of AOM. It could also interact directly with DMH metabolites. More investigation is needed to verify these mechanisms.

The purpose of this work was to examine the possible mechanisms of selenium inhibition of DMH carcinogenesis. It is possible that selenium affects the metabolism of DMH and the alkylation of DNA by a mechanism similar to that of disulfiram, as suggested by Jacobs. Assuming that methylation of DNA by DMH is an important factor in tumor initiation, and that O^6-MeG is a promutagenic lesion in DNA, the effect of selenium treatment on levels of O^6-MeG and 7-MeG was studied. To determine whether selenium affects the metabolism of DMH, the amount of [^{14}C]AM and $^{14}CO_2$ in the exhaled air from rats treated with [^{14}C]DMH was measured. These two metabolic products were chosen as indicators of [^{14}C]DMH metabolism because both have been used as indicators of metabolic inhibition (20, 21), and both are the major metabolites found in the exhaled air (18, 19, 21).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (CD) rats weighing 50-90 g were obtained from Charles River Breeding Laboratories, Inc., Portage, MI. They were provided Purina Lab Chow (Ralston Purina Co., St. Louis, MO) and deionized water alone, or deionized water containing sodium selenite *ad libitum*. Rats weighed 120 g or more when given $[^{14}C]$DMH.

Chemicals

The specific activity of 1,2-di$[^{14}C]$methylhydrazine·2HCl (New England Nuclear, Boston, MA) was decreased from 10 mCi/mmol to 5.02 and 0.552 mCi/mmol by the addition of nonradioactive 1,2-dimethylhydrazine dihydrochloride (Aldrich Chemical Co., Milwaukee, WI). Trisodium EDTA was added to a final concentration of 15 μg/ml, and the pH was adjusted to 6.5 with 1 N NaOH. Selenium in the form of sodium selenite (Na_2SeO_3, Pfaltz and Bauer, Stamford, CT) was freshly prepared twice weekly in deionized water at concentrations of 2, 4, 6, or 8 ppm of the element (Se).

Treatment

Pilot alkylation experiments were done using 1-2 rats per treatment. The animals were provided with drinking water containing 0 or 2 ppm Se for 2 weeks, 4 ppm for 2 weeks, or 8 ppm for 2 or 4 weeks before a single 20 mg/kg s.c. injection of [^{14}C]DMH (5.02 mCi/mmol, 2.32 mg/ml). These rats were also used in metabolism experiments and were killed by decapitation 12 or 72 hr after the [^{14}C]DMH injection. Rats which were used in alkylation experiments or in metabolism experiments alone received [^{14}C]DMH at a specific activity of 5.02 or 0.552 mCi/mmol, respectively.

Metabolism experiments were done using 2-4 rats per group to establish a Se dose that might affect metabolism of [^{14}C]DMH, but would not cause liver toxicity or a severe decrease in body weight gain. Rats were provided drinking water with 4 ppm Se for 2, 4, 6, or 8 weeks, or 6 ppm for 6 weeks before a single 20 mg/kg s.c. injection of [^{14}C]DMH (0.552 mCi/mmol, 2.57 mg/ml). A control group received no Se, but received the same dose of [^{14}C]DMH. The rats were killed by decapitation 12 hr after the injection. Based on the results, a treatment of 4 ppm Se for 4 weeks was selected for a 12 hr metabolism and alkylation experiment with 4 rats.

[^{14}C]DMH Metabolism Studies

Measurement of expired $^{14}CO_2$ and azo[^{14}C]methane (AM), collected according to the method of Fiala (18-20), was used as an indicator of [^{14}C]DMH metabolism. The rats were fasted overnight prior to the [^{14}C]DMH injection, and were then placed in glass metabolism chambers for 12 hr with access to food and water *ad libitum*. Dried air was drawn through at a rate of 250-350 ml/min. During the first 6 hr, air leaving the chamber was drawn through a series of three gas washer bottles. The first trapped [^{14}C]AM and contained 100 ml absolute ethanol cooled to -70° in a dry-ice-absolute ethanol bath. The second trapped $^{14}CO_2$ and contained 150 ml 1 N NaOH, and the third contained 150 ml 1 N H_2SO_4 to trap any remaining [^{14}C]AM. The contents of the first bottle were sampled (1 ml aliquots) and changed every hour for the first 6 hr, then this bottle was removed from the series. The second and third bottles were sampled every hour for the first 6 hr and at 8, 10, and 12 hr. The contents of the second bottle were changed at 4 and 8 hr, and the contents of the third bottle were not changed during the 12 hr experiment. Aliquots of 1 ml were placed in scintillation vials with 4 ml water and 10 ml Aqueous Counting Scintillant (ACS, Amersham Corp., Arlington Heights, IL), were shaken, and counted at a counting efficiency of 80% using the external standard ratio method of quench correction. Expired $^{14}CO_2$ and [^{14}C]AM were expressed as cumulative percent of total dose of [^{14}C]DMH for each sampling time. The group means and standard errors of cumulative percents were calculated and plotted against time. Using a three-compartment model to fit the data, the rates of expiration of [^{14}C]AM and $^{14}CO_2$, and the rate of metabolism of [^{14}C]AM were estimated for individual rats and each group of rats (47). These estimates were then compared by analysis of variance (Duncan's multiple range test).

Tissue Collection

The rats were killed by decapitation 12 hr after [^{14}C]DMH injection. Kidneys, livers, and 12-14 cm samples of duodenum, ileum, and colon were excised, immediately frozen in liquid nitrogen, and stored at -70°. Contents of the intestinal lumen were rinsed out prior to freezing.

DNA Isolation

The selected tissue DNA was purified by a modification of the Marmur method (44, 86). Frozen tissue was weighed (1-2 g), thawed, and homogenized in a Braun-Potter homogenizer (Sargent-Welch Scientific Co., Skokie, IL) in 0.15 M NaCl (10 ml/g tissue) at 4°. Sodium lauryl sulfate was added to the homogenate (final concentration 1%), and the mixture was incubated for 30 min at 37° with moderate shaking. A volume of 5 M NaCl equal to ¼ the volume of the mixture was added. Chloroform:isoamyl alcohol (24:1) equal to ½ the total aqueous volume was added, and this mixture was shaken at 120 oscillations per min for 30 min at 25°. The mixture was centrifuged for 5 min at 10,000 rpm, at 4°. The aqueous supernatant was removed and extracted again with ½ volume of chloroform:isoamyl alcohol twice as before. DNA was precipitated from the final supernatant with cold 2-

ethoxyethanol equal to twice the supernatant volume. The precipitate was air dried on filter paper and dissolved in a solution consisting of 3 ml cold distilled water, 0.15 ml saturated aqueous sodium acetate, and 0.4 ml of 2 mg/ml RNAse (Ribonuclease A, Type 1A, Sigma; heated at 80° for 10 min). The resulting solution was stored 18-20 hr at 4°. The DNA was precipitated with 7.1 ml of cold 2-ethoxyethanol, washed twice with 6 ml cold ethanol, once with 6 ml cold ethyl ether, dried on filter paper, and stored at -20° in capped vials.

Purine Chromatography

DNA was hydrolyzed in 2.0 ml of 0.1 N HCl at 37° for 20-24 hr. Aliquots of 20 μl of a 3.0 mg/ml solution (0.1 N HCl) of each of the nonradioactive markers, 3- and 7-methyladenine, 7-MeG, and O^6-MeG, were added. Two tenths ml ammonium formate (0.5 M) was added to this solution, and the pH was adjusted to 4.8 with 1 N NaOH. Purine bases were separated on a Sephadex G-10 column, 0.9 x 100 cm, using 0.05 M ammonium formate, pH 6.4, as eluant. Column flow rate was maintained at 15 ml/hr using a Minipuls 2 peristaltic pump (Gilson Medical Electronics, Middleton, WI). The absorbance was monitored at 254 nm using a Type 6 Dual Beam UV-Visible Optical Unit (ISCO, Lincoln, NE) in conjunction with a UA-5 (ISCO) chart recorder and a Digitec HT-6150 digital printer (United Systems Corp., Dayton, OH), which printed the eluate absorbance, elapsed time, and cumulative absorbance (calculated by equipment designed and constructed at The Upjohn Co.) at 5 min intervals. Five ml fractions were collected every 20 min, and each was mixed with 10 ml ACS, and counted at 82% counting efficiency. Total dpm of 7-MeG, O^6-MeG, and incorporated [^{14}C] in guanine and adenine were calculated. The amounts of guanine and adenine were determined by measurement of the absorbance at 254 nm of relevant fractions, based on extinction coefficients of 10,870 and 12,450 liters/mole·cm for guanine and adenine, respectively. The concentrations of 7-MeG and O^6-MeG in DNA were expressed as fractions of total guanine or adenine, assuming that the specific activity of the methylated purines was half that of the injected [^{14}C]DMH. This assumption is based on the transfer of one labelled $-^{14}CH_3$ from [^{14}C]DMH (which contains two isotopic carbon atoms) to the purine. A Student's t test was used to compare the means of 7-MeG, O^6-MeG, and labelled guanine and adenine for rats treated with 0 and 4 ppm Se for 4 weeks.

RESULTS

Pilot Alkylation Experiments

Pilot alkylation data of Table 1 shows considerable variation between individual rats. The data are not sufficient for statistical tests. Qualitatively, the results indicate that a treatment of 8 ppm Se for 2 weeks had no effect on alkylation of liver and colon DNA 12 hr after [^{14}C]DMH injection. A treatment of 8 ppm Se for 4 weeks decreased levels of O^6-MeG and 7-MeG in colon and liver DNA, but the data are variable. This may have been due to individual animal variation in liver toxicity, and a failure of two chromatographs to detect O^6-MeG. There was no indication that the treatment of 8 ppm Se for 2 weeks had any effect on removal of 7-MeG or O^6-MeG from the DNA of tissues listed in Table 1. Se doses of 8 ppm caused a decrease in body weight gain, icterus, and congestion and mottling of the liver. Toxicity at this level, therefore, agrees with that reported by others (53, 67).

[^{14}C]DMH Metabolism

Charts 2-5 show the mean and S.E. of exhaled [^{14}C]AM for each group. Charts 6-8 show the mean and S.E. of exhaled $^{14}CO_2$ for each group. Although some of the data show a large degree of variation between individual rats (Charts 3, 5, 6), there is a dose-response effect, both for increasing length of treatment

Table 1

Alkylation of DNA 12 and 72 hr after single s.c. injection of [^{14}C]DMH

Rats were treated with 0 or 8 ppm Se in the drinking water for 2 or 4 weeks before injection of [^{14}C]DMH, 20 mg/kg, 5.02 mCi/mmol.

Tissue	Selenium treatment ppm	Selenium treatment wks	Alkylation (methylguanine/guanine x 10^6) 12 hr 7-MeG	12 hr O^6-MeG	72 hr 7-MeG	72 hr O^6-MeG
Liver	0		3177	363	959	133
			3347	413	1663	126
	8	2	2842	290	1489	148
			3618	400	1558	139
	8	4	2040	230		
			1027	77		
Colon	0		266	33.3	71	12.3
			261	0	61	13.5
	8	2	269	28.7	101	12.1
			241	26.7	96	10.2
	8	4	219	12.1		
			207	0		
Duodenum	0		267	56.7	24.4	0
			109	4.9	21.1	0
	8	2	407	65.8	18.0	0
			139	0	16.6	0
	8	4	89	7.2		
			161	20.7		
Kidney	0		133	10.6	57	4.2
			279	27.5	75	2.1
	8	2	138	9.8	67	4.2
			233	0	112	7.3
	8	4	205	19.1		
			147	10.9		

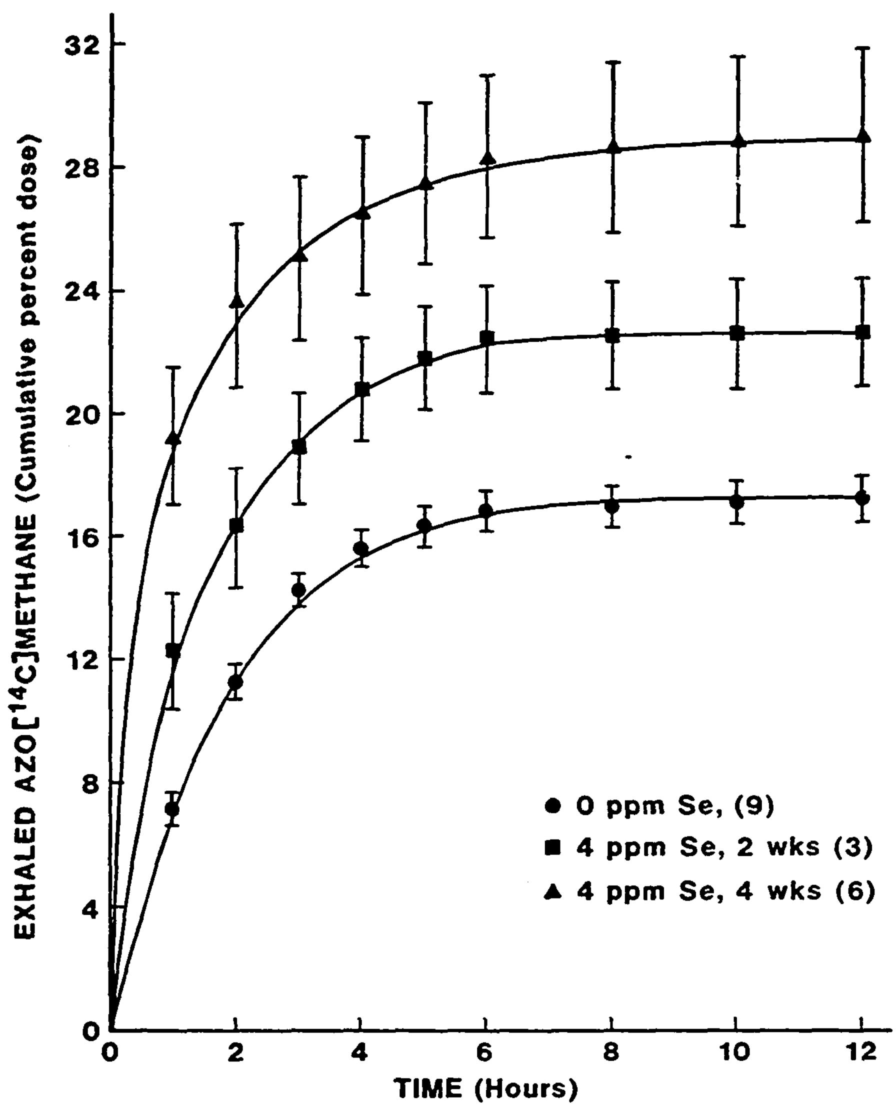

Chart 2. Exhalation of [^{14}C]AM from rats treated with 0 or 4 ppm Se for 2 or 4 weeks before s.c. injection of [^{14}C]DMH, 20 mg/kg. *Bars*, S.E.; *numbers in parentheses*, number of rats.

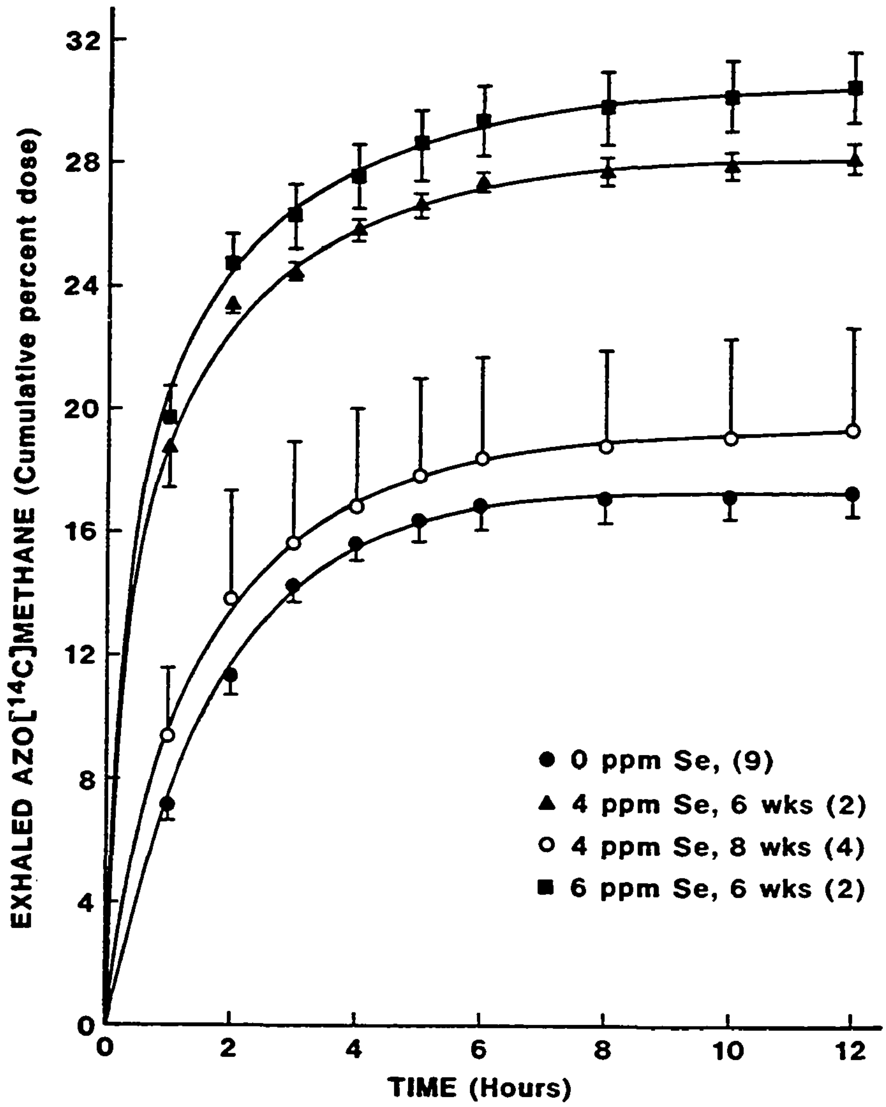

Chart 3. Exhalation of [^{14}C]AM from rats treated with 0 or 4 ppm Se for 6 or 8 weeks, or 6 ppm for 6 weeks before s.c. injection of [^{14}C]DMH, 20 mg/kg. *Bars,* S.E.; *numbers in parentheses,* number of rats.

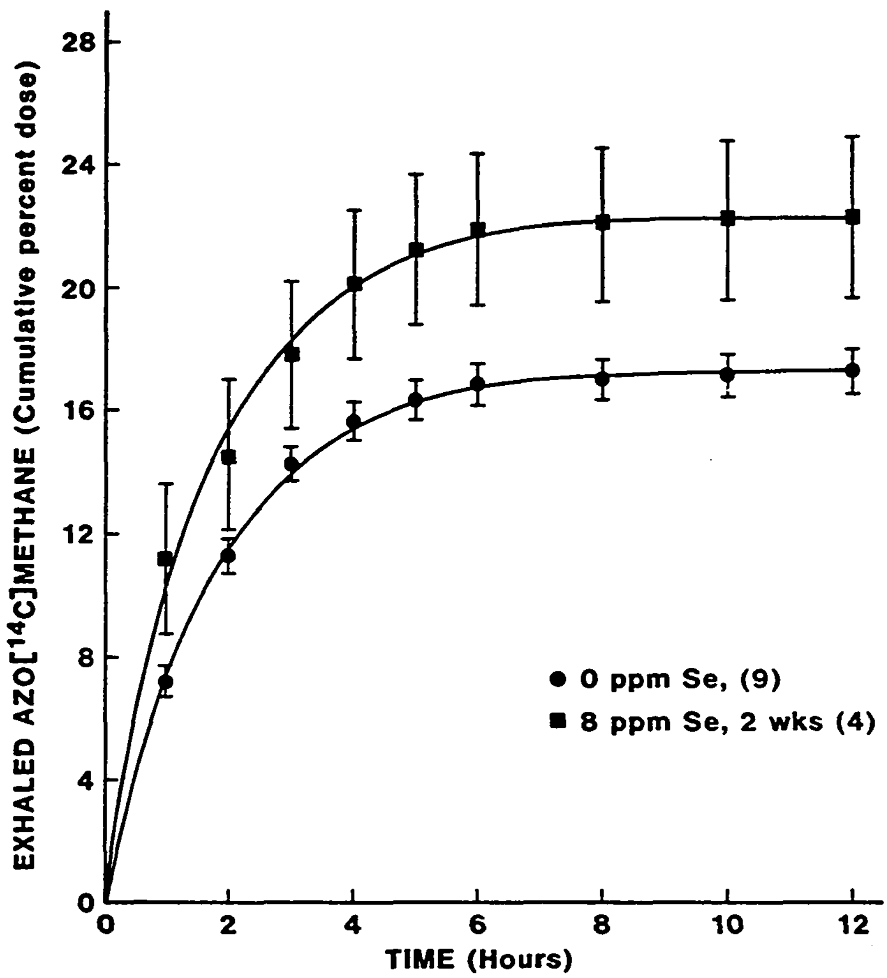

Chart 4. Exhalation of [^{14}C]AM from rats treated with 0 or 8 ppm Se for 2 weeks before s.c. injection of [^{14}C] DMH, 20 mg/kg. *Bars,* S.E.; *numbers in parentheses,* number of rats.

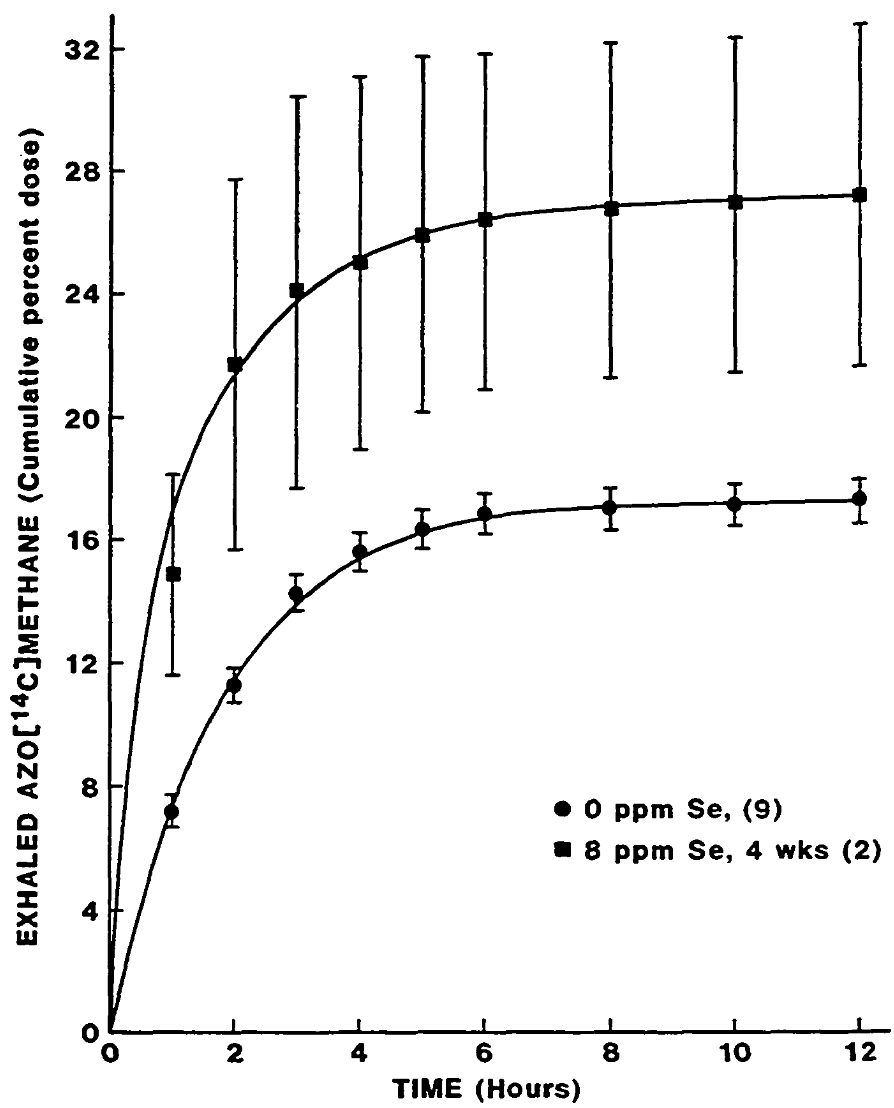

Chart 5. Exhalation of [^{14}C]AM from rats treated with 0 or 8 ppm Se for 4 weeks before s.c. injection of [^{14}C] DMH, 20 mg/kg. *Bars,* S.E.; *numbers in parentheses,* number of rats.

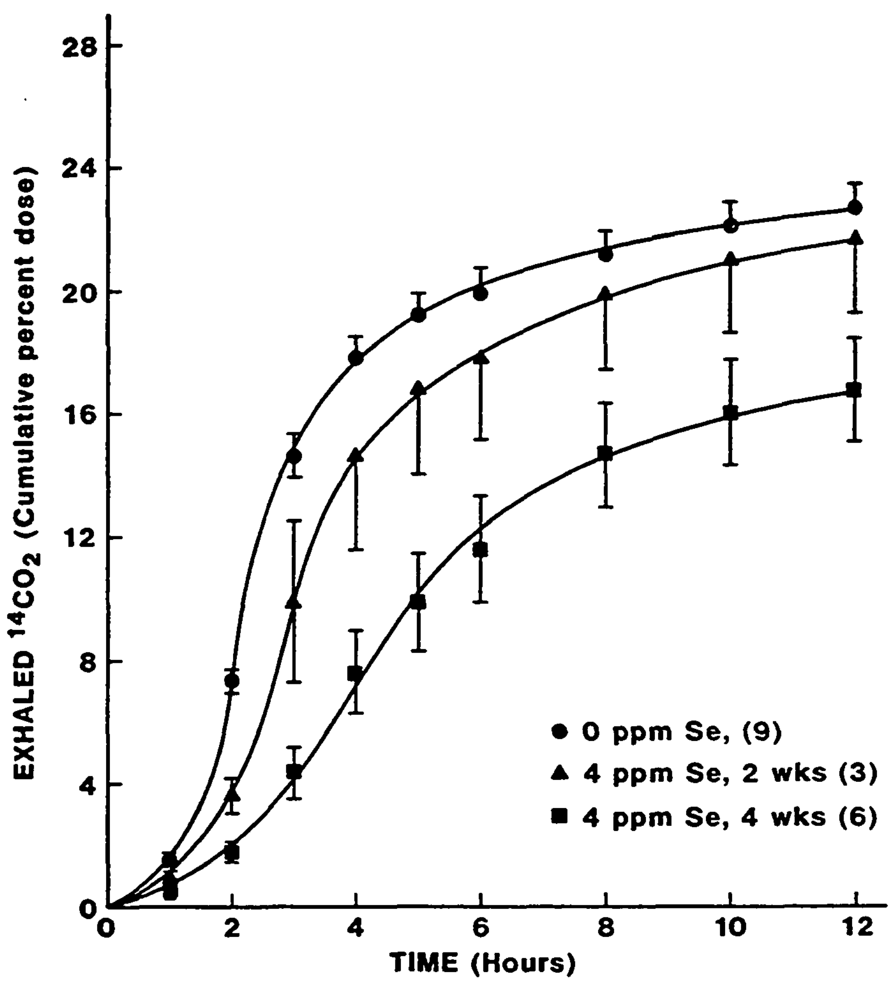

Chart 6. Exhalation of $^{14}CO_2$ from rats treated with 0 or 4 ppm Se for 2 or 4 weeks before s.c. injection of [^{14}C]DMH, 20 mg/kg. *Bars,* S.E.; *numbers in parentheses,* number of rats.

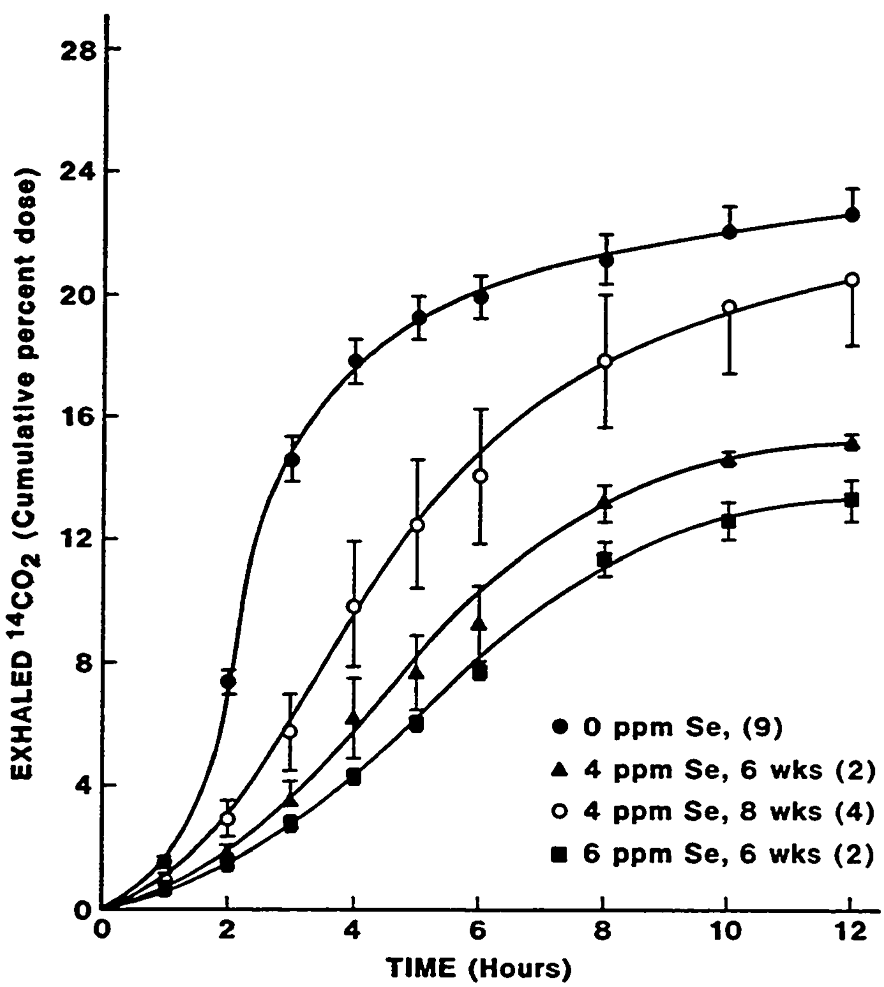

Chart 7. Exhalation of $^{14}CO_2$ from rats treated with 0 or 4 ppm Se for 6 or 8 weeks, or 6 ppm Se for 6 weeks before s.c. injection of [^{14}C]DMH, 20 mg/kg. *Bars*, S.E.; *numbers in parentheses*, number of rats.

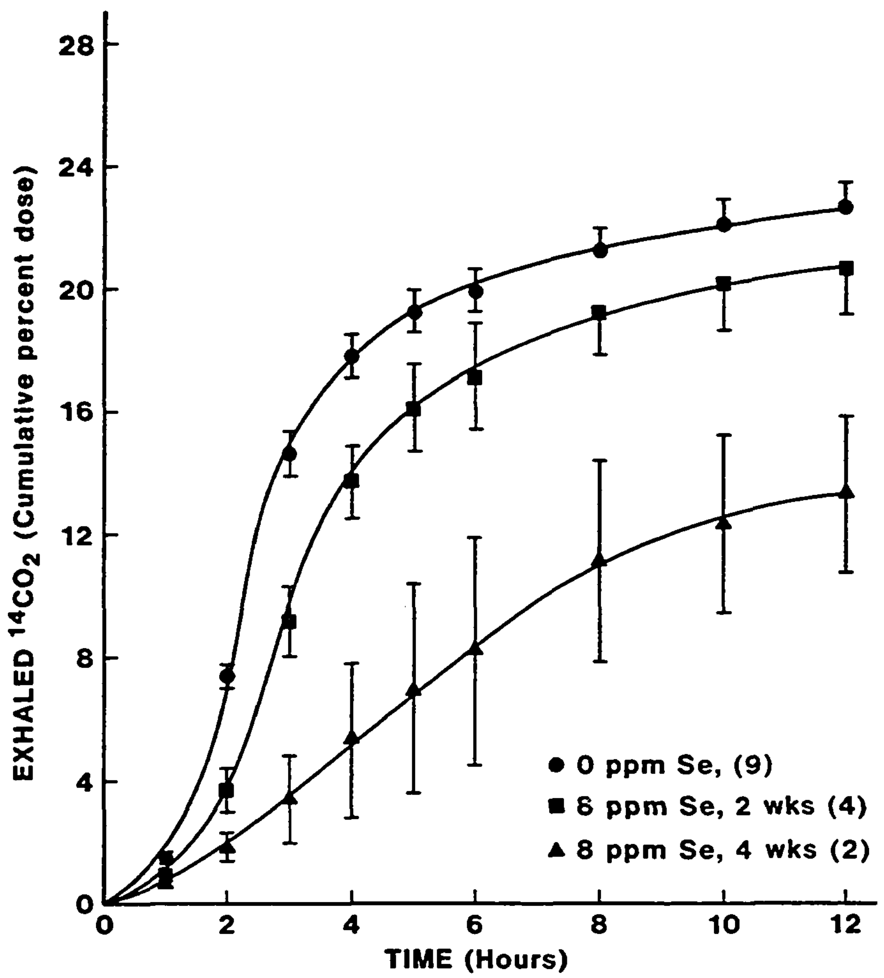

Chart 8. Exhalation of $^{14}CO_2$ from rats treated with 0 or 8 ppm Se for 2 or 4 weeks before s.c. injection of [^{14}C]DMH, 20 mg/kg. *Bars*, S.E.; *numbers in parentheses*, number of rats.

and increasing dose. Total expired [^{14}C]AM increased as length of treatment with 4 ppm Se increased from 2 to 4 weeks (Chart 2). This was accompanied by a decrease in total expired $^{14}CO_2$ from 2 to 6 weeks (Charts 6, 7). Total expired [^{14}C]AM increased as the Se dose increased from 4 ppm to 6 ppm (both at 6 weeks, Chart 3), and this was accompanied by a corresponding decrease in expired $^{14}CO_2$ (Chart 7). The mean exhaled [^{14}C]AM from rats treated with 4 ppm Se for 8 weeks was similar to control levels (Chart 3). This was a result of individual animal variation which is shown by the large standard error for that group.

Chart 9 is a three-compartment model which gives very good fits of the averaged [^{14}C]AM and $^{14}CO_2$ data. The model is a simplification of the proposed metabolism of DMH (Chart 1), and thus is only an approximation. The rates R10, R12, R23, and R30 for individual rats were estimated (47), and the group means were compared for statistically significant differences by Duncan's multiple range test. According to this model, treatments of 4 ppm Se for 4 or 6 weeks, and 6 ppm for 6 weeks showed significantly greater rates of [^{14}C]AM expired (R10), and a significantly smaller ratio of the rate of [^{14}C]AM metabolism to the summed rates of [^{14}C]AM metabolized and expired, that is, R12/R12+R10. All groups of Se-treated rats expired $^{14}CO_2$ at a significantly slower rate than control rats.

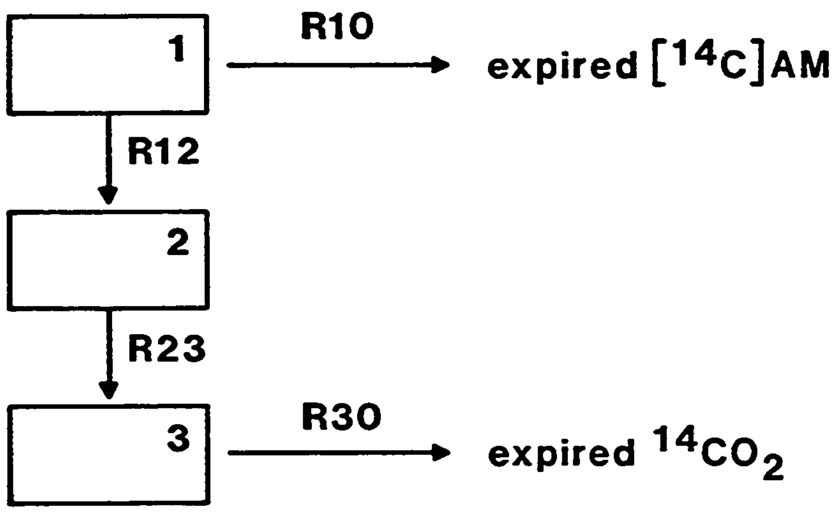

Chart 9. Model for [^{14}C]AM and $^{14}CO_2$ data. At time zero, all of the AM is in Compartment 1. Some of the AM is expired at a rate arbitrarily designated R10. The remainder is metabolized to an intermediate (Compartment 2) at a rate R12. After another metabolism step at a rate R23, the CO_2 is expired at a rate R30.

Alkylation

Table 2 shows the incorporation of [^{14}C] into guanine and adenine, and relative amounts of 7-methylguanine and O^6-methylguanine in DNA of five tissues from rats treated with 0 or 4 ppm Se for 4 weeks, and killed 12 hr after the [^{14}C]DMH injection. Alkylation was 10-15 times greater in the liver than in the colon, kidney, or duodenum. Alkylation of ileum DNA was minimal. The following statistically significant differences were found in the Se-treated rats. Incorporation of [^{14}C] into guanine and adenine of colon DNA was 69% and 72% lower, respectively, than control colon DNA (Chart 10). This may be the result of a decrease in the amount of DNA synthesis in colon mucosal cells. Incorporation into guanine of duodenum was 44% higher. Liver DNA had 20% less 7-methylguanine and 27% less O^6-methylguanine. Colon DNA had 40% more O^6-methylguanine. Ileum DNA had 49% more 7-methylguanine.

Incorporation of [^{14}C] into guanine of liver was 20% greater in rats treated with Se, suggesting an increase in cell turnover due to cell loss associated with the toxic effects of Se.

Table 2

Incorporation of [^{14}C] and alkylation of DNA 12 hr after single s.c. injection of [^{14}C]DMH

Rats were treated with 0 or 4 ppm selenium in the drinking water for 4 wks before injection of [^{14}C]DMH, 20 mg/kg, 5.02 mCi/mmol. Each value is the mean of 4 rats except where noted.

	Selenium treatment		Incorporation (dpm/μmol)				Alkylation (methylguanine/guanine x 10^6)			
Tissue	ppm	wks	Guanine	% change	Adenine	% change	7-MeG	% change	O^6-MeG	% change
Liver (4)[a]	0		181 ± 19[b]		130 ± 25		3137 ± 212		371 ± 26	
(4)	4	4	217 ± 75	+20	131 ± 27	0	2511 ± 143[c]	-20	270 ± 24[c]	-27
Colon (3)	0		2336 ± 146		1883 ± 193		233 ± 21		27.8 ± 3.2	
(4)	4	4	729 ± 129[d]	-69	523 ± 106[c]	-72	334 ± 28	+43	39.0 ± 3.0[e]	+40
Duodenum (3)	0		2134 ± 325		2817 ± 220		150 ± 32		13.0 ± 4.3	
(4)	4	4	3082 ± 195[f]	+44	2874 ± 143	+2	169 ± 15	+13	19.4 ± 1.7	+49
Kidney (3)	0		162 ± 33		120 ± 25		182 ± 49		16.2 ± 8.0	
(4)	4	4	130 ± 14	-20	84 ± 10	-30	243 ± 5.9	+61	22.4 ± 0.8	+38
Ileum (4)	0		2966 ± 306		2736 ± 113		15.6 ± 1.2		0	
(4)	4	4	2645 ± 404	-11	2978 ± 972	-9	23.3 ± 0.6[f]	+49	0	0

[a]Numbers in parentheses, number of rats

[b]Mean ± S.E.

[c]$p < 0.025$.

[d]$p < 0.005$

[e]$p < 0.05$.

[f]$p < 0.01$.

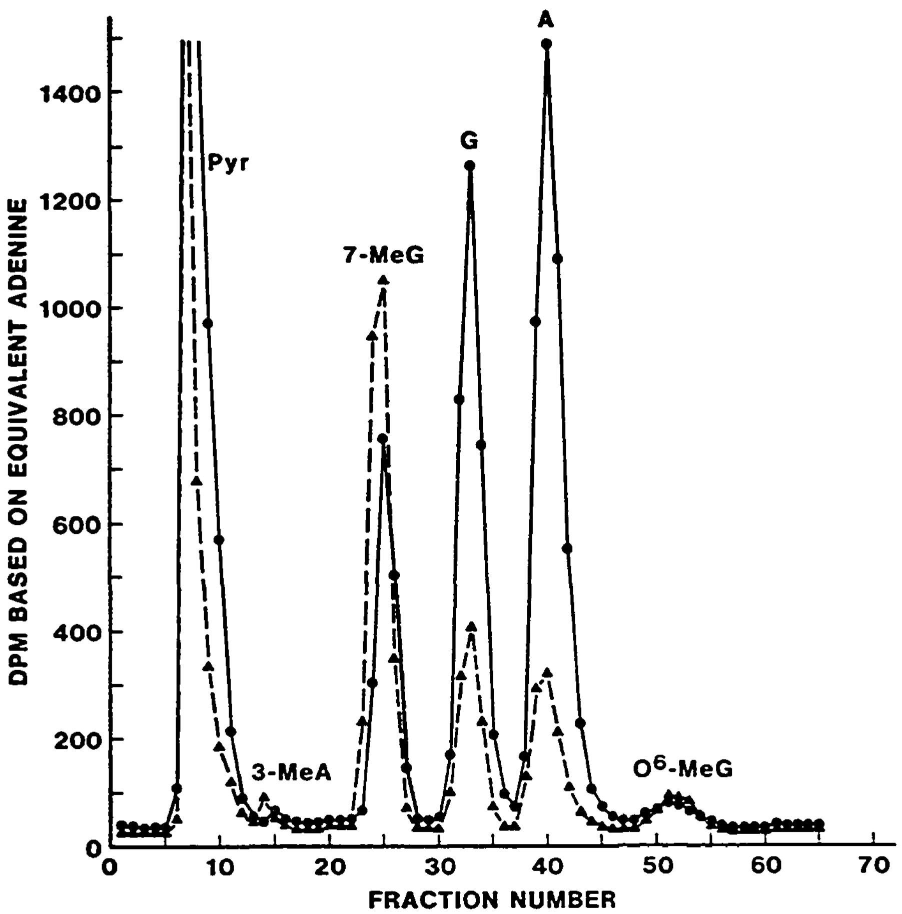

Chart 10. Sample Sephadex G-10 chromatographs of acid hydrolysates of colon DNA from a control rat (•) and a rat pretreated with 4 ppm Se for 4 weeks (▲). Each rat received a single s.c. injection of [^{14}C]DMH, 20 mg/kg, 5.02 mCi/mmol, and was killed 12 hr later. DNA was hydrolyzed in 0.1 N HCl for 20 hr at 37°. Ammonium formate (0.2 ml, 0.05 M) was added, and the pH was adjusted to 4.8 with 1 N NaOH. *Pyr*, pyrimidine nucleotides; 3-MeA, 3-methyladenine; 7-MeG, 7-methylguanine; *G*, guanine; *A*; adenine; O^6-MeG, O^6-methylguanine.

DISCUSSION

Selenium treatment significantly increased the rate of expired [^{14}C]AM, and decreased the rate of expired $^{14}CO_2$. This effect indirectly supports the hypothesis that Se interferes with the metabolic activation of DMH. The cellular and molecular nature of the Se effect on metabolism is not clear. Jacobs *et al.* (33) postulated that selenium may replace oxygen and sulfur to form organoselenium amino acid analogs and thus alter a cellular component critical to metabolic activation. It is possible that the effect of Se at doses above 2 ppm is a result of general liver toxicity. Histologic lesions of toxic hepatitis were observed in rats treated chronically with 2.5 ppm Se (27). Cirrhosis and decrease in liver size were observed in selenate-treated rats (53). If most of the metabolism of DMH occurs in the liver, a generalized toxicity may impair the activity of microsomal drug-metabolizing enzymes. Alternatively, Se may act in a nontoxic manner by inhibiting only certain enzymes involved in the activation pathway. Se has recently been shown to alter the metabolism of the carcinogens, benzo[α]pyrene and 2-acetylaminofluorene (45, 60).

An inhibition of the metabolic activation of DMH by Se should lead to a decreased production of the active methylating species, and, therefore, a decrease in alkylation in Se-treated rats. This was the case for liver DNA, but alkylation in the colon was higher. Thus, the target tissue DNA contained higher levels of O^6-MeG in Se-treated rats. Such an effect is not found with disulfiram and aminoacetonitrile, which inhibit alkylation of DNA in both liver and colon (57, 77). This discrepancy suggests that Se inhibits DMH metabolism primarily in the liver, causing a systemic increase in unmetabolized DMH, which was subsequently metabolized by other organs. Increased expiration of AM supports a slower removal of AM through hepatic metabolism. A buildup of AM could thus slow the rate of the nonenzymatic conversion of DMH to AM, causing an increase in the systemic levels of DMH. This, in turn, could lead to greater exposure of the kidneys and colon to DMH and its metabolites. Since colon mucosa is capable of activating DMH (3, 23), and contains enzymes capable of metabolizing other carcinogens (15, 16), the circulating DMH and AM would be activated and thus alkylate the DNA, which could account for the increased alkylation in the colons of Se-treated rats.

The decreased incorporation of [^{14}C]DMH-derived radioactivity into guanine and adenine of colon DNA from Se-treated rats (Table 2) is similar to the effect of disulfiram on incorporation (77). Incorporation of radioactivity into guanine and adenine is mainly due to the formation of [^{14}C]formaldehyde which rapidly enters the C_1 pool (7, 77). Formaldehyde is produced at the end of both the activation and detoxification pathways (12, 17). It is possible that Se inhibits both pathways, and thus causes a decrease in [^{14}C] incorporation. The radioactivity would then be exhaled as $[^{14}C]$AM. However, Se is known to inhibit mitosis (38). A reduction of DNA synthesis in the colon would lead to a decreased incorporation of $[^{14}C]$ into adenine and guanine. Tissues with little or no cell turnover (liver) have very low incorporation (Table 2). The increased levels of alkylation in the colon, and the known rapid cell turnover and associated DNA replication are conditions conducive to carcinogenesis according to the somatic mutation theory of cancer (62, 77). Thus the anti-carcinogenic effect of Se may be the result of reducing the rate of DNA synthesis, and, hence the chance for pre-neoplastic somatic mutations to occur. More research is necessary to determine the effect of Se on cell turnover in the colon.

Further studies on the effects of Se on DMH carcinogenesis are also necessary to determine if the decrease in colon tumor incidence in Se-treated rats sacrificed at 20 weeks (32) is due to an increase in the latency period of tumor induction. Se may or may not decrease the tumor incidence of rats allowed to live 20-30 weeks after a 20-week treatment with DMH.

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