Open Archive TOULOUSE Archive Ouverte (OATAO)

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in : http://oatao.univ-toulouse.fr/
Eprints ID : 9099

Any correspondance concerning this service should be sent to the repository administrator: staff-oatao@inp-toulouse.fr.

Induction of Colonic Aberrant Crypts in Mice by Feeding Apparent N-Nitroso Compounds Derived From Hot Dogs

Michael E. Davis, Michal P. Lisowyj, Lin Zhou
Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska, USA
James L. Wisecarver and James M. Gulizia
Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, USA
Valerie K. Shostrom
College of Public Health, University of Nebraska Medical Center, Omaha, Nebraska, USA
Nathalie Naud and Denis E. Corpet
UMR Xenobiotiques INRA-ENVT, Aliments et Cancer, Ecole Nationale Veterinaire Toulouse, Toulouse, France
Sidney S. Mirvish
Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska, USA

Correspondence to Sidney S. Mirvish, Eppley Institute for Research in Cancer, University of Nebraska Medical Center (UNMC), Omaha, NE 68105, USA. E-mail: smirvish@unmc.edu

Abstract
Nitrite-preserved meats (e.g., hot dogs) may help cause colon cancer because they contain N-nitroso compounds. We tested whether purified hot-dog-derived total apparent N-nitroso compounds (ANC) could induce colonic aberrant crypts, which are putative precursors of colon cancer. We purified ANC precursors in hot dogs and nitrosated them to produce ANC. In preliminary tests, CF1 mice received 1 or 3 i.p. injections of 5mg azoxymethane (AOM)/kg. In Experiments 1 and 2, female A/J mice received ANC in diet. In Experiment 1, ANC dose initially dropped sharply because the ANC precursors had mostly decomposed but, later in Experiment 1 and throughout Experiment 2, ANC remained at 85 nmol/g diet. Mice were killed after 8 (AOM tests) or 17–34 (ANC tests) wk.Median numbers of aberrant crypts in the distal 2 cm of the colon for 1 and 3 AOMinjections, CF1 controls, ANC (Experiment 1), ANC (Experiment 2),and untreated A/J mice were 31, 74, 12, 20, 12, and 5–6, with $P <$ 0.01 for both ANC tests. Experiment 2 showed somewhat increased numbers of colonic mucin-depleted foci in the ANC-treated group. We conclude that hot-dog-derived ANC induced significant numbers of aberrant crypts in the mouse colon.

INTRODUCTION

Colon cancer has been linked to the consumption of red meat (e.g., beef, pork, and lamb) and, more strongly, of processed red meat (1–3). Processed meat generally signifies meat products that are preserved with sodium nitrite ($NaNO_2$), including hot dogs, bacon, ham, corned beef, and luncheon meat. Nitrite is added to impart specific flavors and a pink color, and to protect the consumer from botulism caused by Clostridium botulinum, which can multiply in meat products that are not refrigerated. In the United States, processed meat is prepared by adding 120 mg/kg of $NaNO_2$ and 500 mg/kg of sodium ascorbate or erythorbate (isoascorbate) to the meat. This reduces the formation of volatile nitrosamines and other compounds measured as total apparent N-nitroso compounds (ANC) (4).

We report here experiments testing whether feeding ANC derived from hot dogs could induce aberrant crypts (ABC) and mucin-depleted foci (MDF) in the mouse colon. There is evidence that the induction of ABC or ABC foci (ACF, a group of adjacent ABC) in the colons of rats and mice indicates the potential for inducing colonic tumors (5,6). Colonic carcinogens have induced ACF after only a few weeks of treatment (5,6).

Presumably, most ACF are derived from single clones of mutated cells. MDF are another type of premalignant lesion that develop much later than ABC and ACF, but may be more closely correlated than ACF with colon carcinogenesis in mice (7–10). We are examining the hypothesis that N-nitroso compounds (NOC) in processed meat are a cause of colon cancer. We focused on ANC derived by nitrosation of the ANC precursors (ANCP) in hot dogs (11,13). The ANC in foods and feces probably consist mainly of NOC (14–17). Fecal ANC output in humans rose threefold after subjects ate 420 g beef/day, were not affected when the subjects ate chicken or fish, which are not risk factors for colon cancer (18–20), and rose even more after the subjects ate processed meat (21). In 2001, we determined ANC in a number of foods (15).We also determined ANCP levels in foods by nitrosating the ANCP under mild conditions and determining the resulting ANC. The ANC method used here (12,13,15), which is similar to that used by the group of the late Sheila Bingham (18–22), involves liberating nitric oxide (NO) from the ANC with HBr in aqueous solutions and determining the resulting NO by thermal energy analysis. Red meat consumption by humans may have enhanced the formation in colonic DNA of O6-carboxymethylguanine, a likely premutagenic event (22). Processed meat products may be more potent than fresh red meat as a cause of colon cancer because ANC levels in processed meats are higher than those in fresh meat (15). Consumers of processed meat showed elevated ANC levels in the feces (and hence, probably, the colon) compared to consumers of fresh red meat (21). In 2003 we reported that fecal ANC output in mice increased 2.6–2.9 or 3.7–5.0 times when the diet contained 18% by weight of beef or 18% hot dogs, respectively (12).

We partially purified the ANCP in hot dogs and demonstrated that the derived NOC were directly mutagenic in the Ames test (23). One of the ANCP was identified as 1-deoxy-1-N-glucosyl glycine (Fig. 1 shows the structure of the corresponding nitrosamine) (23). Feeding sodium nitrite to mice also increased fecal ANC excretion; this process was inhibited by also feeding omeprazole or sodium ascorbate, indicating that most in vivo nitrosation by nitrite occurs in the acidic stomach (13).

Our main aim here was to determine whether ANC prepared from purified hot-dog-derived ANCP could induce ABC in the mouse colon. ANC in foods and biological materials can include, in addition to NOC, nitrosothiols (RSNO) and nitrosyl iron compounds (RFeNO), e.g., nitrosyl heme (17). Therefore, we determined the contribution of RSNO and RFeNO to the total ANC in the test material. The ABC tests were conducted on A/J mice, which are sensitive to ABC induction by azoxymethane. (AOM) (24). As positive controls, we first tested whether we could repeat the known induction of ABC with AOM (24,25).
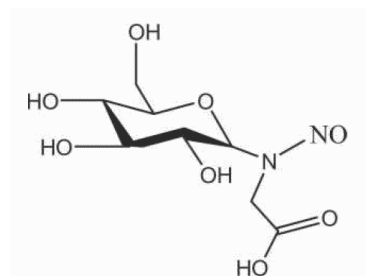

FIG. 1. N-Nitroso glucosyl glycine

METHODS

Safety
Because AOM is a potent, volatile carcinogen, its solutions were injected in a chemical hood, and the mice and their cages were kept in the hood for 3 days after each AOM injection. The hot-dog-derived ANC were handled with gloves.

Purification of ANCP from Hot Dogs
John Morrell hot dogs (600 g) containing chicken and pork were extracted with 2,700 ml water (23). The combined extract was roto-evaporated under vacuum at <40◦C to give 230 ml solution. The solution was adsorbed and then desorbed on silica gel and then cation exchange resin to give 300 ml ammonia extract, which contained most of the ANCP (23) and was stored at −30◦C. Purification was carried out 15 times during the feeding experiments.

Nitrosation of Purified ANCP and Addition of Resulting ANC to the Diet
Typically, a mixture of 111 ml ammonia extract containing 100 μmol ANCP and 18.7 ml each of 1.0 M NaNO2 and 1.0 M HCl was incubated for 1 h at 37◦C with gentle shaking, mixed with 18.7 ml of a saturated solution of sulfamic acid (SA) in water (SA reagent) and kept for 15 min. After a sample was put aside for ANC analysis, 1.0 M Na2CO3 was added to the bulk of the solution to bring the pH to 7. The solution (<150 ml) was mixed with 1.0 kg diet in an Osterizer food blender (Oster Corporation, Milwaukee, WI). Final ANC concentration was determined to be 85 nmol/g diet. The ANC-containing diet was stored at 4◦C for not more than 1 wk.

Induction of ABC

Diets
We mostly used semi-purified powdered diet from Harlan-Teklad (Madison, WI). Diets were stored at 4◦C. We now list the major or probably most relevant dietary constituents as g/kg diet (except for vitamin D) for the standard, high-fat, and high-fat stress diets (named TD-94045, TD-06463 and TD-96096, respectively, by Harlan Teklad): casein, 200, 230, and 240; cornstarch, 397, 230, and 150; maltodextrin, 132, 132, and 0; sucrose, 100, 100, and 311; corn oil or (for the standard diet) soybean oil, 70, 202, and 200; cellulose, 50, 50, and 60; mineral mix AIN-93G, 35, 40, and 16; vitamin D, 5,000, 1,150, and 120 international units/g. The mineral mix included calcium (as CaCO3), 5.1, 5.8, and 0.4 g/kg diet. The high-fat stress diet was introduced by Newmark et al. for studies on the mouse colon to mimic human Western-style diets; this diet caused hyperproliferation of epithelial cells in the colonic crypts (26).

Animal Tests
All mice were housed 5 per cage and were given tap water for drinking. Powdered diets were fed in stainless steel mouse feeders (Amcare, Bellmore, NY) (13). The mice were given group-specific ear notches and weighed monthly. Control groups received the same diets at the same times as the treated groups but without added ANC. Column 3 of Table 1 shows the number of mice in each group. In column 4 of Table 1, times were measured from the start of treatment. All mice were euthanized with CO2.

ABC Induction by AOM
AOM was obtained from the National Cancer Institute (NCI) Chemical Carcinogen Repository (Midwest Research Institute, Kansas City, MO), which reported that (a) the AOM showed a 1H-nuclear magnetic resonance (1H-NMR) spectrum in CDCl3 with 2 similar-sized

peaks at 3.25 and 4.15 ppm (this was confirmed here), and (b) on gas chromatography with flame ionization detection that 99% of the material occurred as a single peak. The AOM was stored at −30◦C. Freshly prepared AOM solutions in 10 ml/kg of normal saline were injected i.p. The mice were maintained on a semipurified (TD-94045) diet. All experiments on AOM were performed on female CF1 mice (Charles Rivers, Wilmington, MA) starting at 6 wk of age. In a preliminary study in which the mice were fed commercial diet, 10 mg/kg body weight of AOM from NCI was injected i.p. into 11 mice and survival was followed. Histology slides of the livers were stained with hematoxylin-eosin and examined. In the main experiment, the mice were fed the TD-94045 semi-purified diet. AOM (5 mg/kg in 10 ml saline/kg) was injected i.p. as a single dose into 5 mice or as 3 doses given weekly to 4 mice. Three untreated mice received the same diet at the same time as the treated mice. All these mice were euthanized 8 wk after the first or only AOM injection.

ABC Induction by Hot-Dog-Derived ANC
These tests were conducted on female A/J mice (Jackson Laboratories, Bar Harbor, ME). They were fed hot-dog-derived ANC in the diet continuously from 4 wk of age.

Experiment 1 on ABC Induction by ANC. To increase the susceptibility to ABC induction, (a) the initial diet was the high-fat TD-06463 diet (27) and (b) from week 8 of the experiment the diet was switched to the high-fat stress TD-96096 diet containing 20% corn oil and reduced levels of calcium and vitamin D (26). ANC was added to both these diets. A large batch of ANCP-containing ammonia extract was prepared as described in the section entitled "Purification of ANCP from hot dogs." The entire batch of ANCP was thawed every 1–2 wk at <40◦C, and samples were nitrosated and mixed with the diet as described in the section entitled "Nitrosation of purified ANCP and addition of resulting ANC to the diet." Diet was added to the feeders every second day and was fed continuously to female A/J mice from 4 wk of age. The same batch of the stock ANCP solution was used throughout weeks 1–11 of Exp. 1. At about 2-wk intervals the entire ANCP solution was thawed in water at 40◦C, and samples were nitrosated and added to the diet as described in the section entitled "Nitrosation of purified ANCP and addition of resulting ANC to the diet." The apparent initial dose of 85 nmol/g diet, as calculated from the initial ANCP analysis, was doubled every 1–3 wk until it reached 3,600 nmol/g at week 12, with no obvious ill effect. ANCP solutions were concentrated by reduced-pressure roto-evaporation at 40◦C before the nitrosation, so that the added water did not exceed 15% of the diet. On week 13, a fresh ANCP solution was prepared and stored in 20-ml test tubes at −80◦C. From then on, only 1–2 tubes at a time were thawed and then nitrosated. The mice were killed after treatment for 17–19 wk (Table 1).

Experiment 2 on ABC Induction by ANC. The mice were maintained on the high-fat stress diet as described in the section entitled "Diets." throughout the experiment. ANCP solutions were stored, nitrosated, and dispensed as described for the treatment starting at week 12 of Experiment 1. The mice were killed after 38–39 wk (Table 1).

Diagnosis of ABC
After sacrifice, the distal 2 cm of each colon was removed, washed in phosphate-buffered saline, stripped of adhering connective tissue, slit lengthwise, rinsed in saline, and fixed overnight in phosphate-buffered 10% formalin. The 2-cm colon sections were pinned lumen side up onto sheets of pink base-plate dental wax (Electron Microscopy Sciences, Fort Washington, PA). The colons were stained for 4 min with a filtered saturated solution of 0.2% methylene blue (Aldrich) in water, rinsed in saline, placed on microscope slides lumen side up, covered with overslips, and examined at 400x magnification using a Motic B5 microscope

fitted with an integrated digital camera (Motic Instruments, Richmond, BC, Canada). ABC were identified mainly by their large, irregular, slit-like lumens (27–29) and were counted and photographed. For all the treated mice and 8 of the 12 control mice in Experiment 2, we also recorded the number of foci and of ABC/focus. These procedures were carried out primarily by Michael E. Davis and Michal P. Lisowyj, who were not blinded about the treatment, in consultation with each other and with Sidney S. Mirvish. Some colons and all the photographs were also examined by a pathologist (James M. Gulizia or James L. Wisecarver).

Diagnosis of MDF
After the colons were assayed for ABC, a number of randomly selected colons were examined for the presence of MDF after they were stained by the high-iron diamine Alcian blue procedure (7). A single investigator (Nathalie Naud), who was blinded about the mouse treatment, evaluated the number of MDF per colon and the number of crypts per MDF. The criteria for MDF were as follows: Focus with at least 3 crypts with little or no apparent mucin, crypts with distorted lumens, and lesions elevated above the mucosa level (7). Each MDF was photographed and pictures were reviewed by an independent observer (Denis E. Corpet).

Analyses for ANC and ANCP

ANC in Aqueous Solutions
Amixture of 425 µl aqueous ANC solution, 25 µl of 2NHCl and 50µl SA reagent was kept for up to 4 h in ice and 100µl samples were subjected to thermal energy analysis (12,13,15,23). Outputs of the analyses were integrated with a model 202 computer program from Peak Simple (Torrance, CA).

ANCP in Ammonia Extracts
A mixture of 100 µl ammonia extract (section 2) or concentrates thereof, 650 µl water, 50 µl of 2 N HCl, and 100 µl of 1 M $NaNO_2$ was incubated for 1 h at 37∘C, mixed with 100 µl SA reagent, kept for 5 min, diluted 10 or 100 times in water containing 10% SA reagent, and analyzed for ANC as in section entitled "Analysis for ANC in aqueous solutions." Typical analyses of 2 batches of ammonia extract showed 294 and 520 µmol ANC/ml extract.

ANC in Diet
A mixture of 1.5 g diet and 15 ml water was vortexed, stored for 18 h at room temperature and vortexed again. One ml of the mixture was centrifuged for 10 min at 7,000 *g,* and the supernatant was analyzed for ANC as in section entitled "Analysis for ANC in aqueous solutions." When a diet containing 76 nmol ANC/g was stored for 8 days at 4∘C, its ANC content dropped by 14%.

ANC in Feces
After the mice in Experiment 2 were fed ANC for 4 wk, feces were collected for 24 h individually from 3 treated and 3 untreated mice, dried to constant weight (13), and weighed [ANC are stable under these conditions (12)]. Each fecal sample (weighing 200–400 mg) was soaked for 30 min in 10 ml water, vortexed, blended for 2 min in a Potter-Elvejhem homogenizer, and centrifuged. Amixture of supernatant (850 µl), 2 N HCl (50 µl), and SA reagent (100 µl) was kept for 15 min, diluted 10 or 100 times with water containing 10% SA reagent, and analyzed for ANC as in section entitled "Analysis for ANC in aqueous solutions."

RSNO and RFeNO Compounds in the Hot-Dog-Derived

Purified ANC

RSNO and RFeNO were defined as the disappearance of ANC on treatment of aqueous ANC solutions with reagents that destroy RSNO or RFeNO, respectively (17). To 700 μl ANC solution we added 100 μl of 50 mM mercuric chloride (for RSNO), 100 μl of 50 mMpotassium ferricyanide (for RFeNO), or 100 μl water (for total ANC). We incubated the mixtures for 30min at roomtemperature, added 100μl SAreagent and 100μl of 0.1 N HCl to each tube, kept the tubes at room temperature for at least 15 min and then analyzed 100 μl of each mixture for ANC as in section entitled "Analysis for ANC in aqueous solutions."

Statistics

The PC SAS v. 9.2 program was used for all analyses. The ABC data for all experiments are summarized in Table 1 using the medians and interquartile ranges (IQ) from 25% to 75% of the results. Because the data failed the test for normality, comparisons of mean values were not appropriate. Hence, all ABC results in Tables 1 and 2 were analyzed by the nonparametric Wilcoxon rank order test and were recorded as median and IQ values. Because the number of foci per colon in the untreated group for 3 and 4 ABC/focus (Table 2) had values of just 0 or 1, these data were recategorized as present or absent for foci/2 cm of colon and were analyzed by Fisher's exact test.

RESULTS

Contribution of RSNO and RFeNO to Total ANC Produced From Hot-Dog-Derived ANCP

RSNO and FeNO concentrations were calculated for two samples of nitrosated purified hot-dog-derived ANCP. Each set of results included those for total ANC and for ANC after addition of mercuric chloride (HgCl2), a test for RSNO, or of potassium ferricyanide [K3Fe(CN)6], a test for nitrosyl iron compounds [RFeNO] (17). Percent loss of the total ANC indicated the contributions of RSNO and FeNO to the total ANC. Results for the 2 samples indicated that the concentration of RSNO was 6% and 14% (mean = 10%), and that of RFeNO was 1% and 3% (mean = 2%) of the total ANC.

Induction of ABC by AOM

We initially injected 10 mg/kg of AOM from NCI into 11 mice. The dose was the same as that used by Papanikolaou et al. (24), who reported that this dose of AOM (obtained from Sigma) was not lethal for mice. However, 10 of the treated mice died within 2 days. On autopsy they showed severe centrilobular liver necrosis, which was the probable cause of death. Similar findings were already reported in 1974 (30). Acute toxicity tests of AOM from NCI and from Sigma showed similar mortality, which was similar whether the mice were injected with AOM i.p. or s.c. We suggest that the AOM used before (24) was impure, so that the actual dose was lower than was reported (purity checks were not reported). In contrast, tests on the purity of the AOM used here (that from NCI) gave satisfactory results (see **Methods**). It appears that both our sources now supply pure AOM. Accordingly, the effect of AOM (from NCI) was tested here on ABC induction using only 5 mg AOM/kg body weight/dose. All the mice survived this treatment. This experiment was conducted on 3 untreated mice (killed at the same age as the treated mice), 4 mice given 1 AOM injection and 5 mice given 3 AOM injections. The numbers of ABC/2 cm of colon, listed in the order shown in the previous sentence, were expressed as mean and (in parentheses) intraquartile range. These numbers were 12 (9–21), 31 (26–39), and 74 (70–79).

TABLE 1
ABC and MDF in the distal 2 cm of the colons of mice that were treated with hot-dog-derived ANC

Experiment no.	Treatment	No. of mice	Median time (wk) from start of treatment to death (IQ range)	Median no. of ABC/2 cm colon (IQ range)	$P<$	MDF incidence[a]
1	ANC in diet	11	19 (14–43)	20 (14–28)	0.01^b	1/10
	Untreated	6	17.5 (16–19)	5 (3–6)		0/6
2	ANC in diet	23	38 (24–42)	12 (9–17)	0.01^b	2/13
	Untreated	12	39 (24–42)	6.5 (5.5–9.0)		1/7

ABC indicates aberrant crypts; MDF, mucin-depleted foci; ANC, apparent N-nitroso compounds; IQ, intraquartile.

a- Nunber of colons with MDF/total number of colons examined for MDF.
b- P value for comparison with the untreated group in the same experiment.

Induction of ABC by ANC (Exps. 1 and 2 of Table 1)

The ANC-treated mice did not show drops in body weight or other obvious ill effects. In both Experiments 1 and 2,mean body weights of both the treated and control groups were initially (at 4 wk of age) 12–13 g and rose to 17–18 g at 5 wk, 26–27 g at 12 wk, 27 g at 17 w, and 29–30 g at 21 and 24 wk (times were measured from the start of treatment).

Experiment 1
During week 1–5, we administered an apparent dose of 85 nmol ANC/g diet to 11mice.As the mice appeared unaffected by the ANC, the apparent ANC dose was repeatedly doubled until, at week 12, it reached 3,600 nmol/g diet. However, analysis at week 12 showed that the diet and feces actually contained only 3.1 and 2.3 nmol ANC/g, respectively. These low ANC levels probably occurred because most of the stock ANCP had decomposed when the single large batch of ANCP solution was repeatedly thawed and refrozen. Accordingly, from week 12, the ANCP solution was stored in a number of tubes and only 1–3 tubes at a time were thawed, nitrosated, and mixed with the diet. No toxic effects were observed apart from the development of ABC. The ABC showed enlarged and irregular crypt lumens and appeared similar to those induced here by AOM and to those described in previous reports on colonic ABC induction in mice (5,24). The second of these references shows photographs of typical murine ABC, which appeared similar to those seen here. The median number of ABC/2 cm colon was 4-fold higher, with $P < 0.01$, in the ANC-treated groups than in the untreated controls (Table 1). No tumors were observed.
We determined ANC in freshly prepared samples of diet containing ANC added at a level of 100 nmol/g diet (prepared by the procedure used in Experiment 2). This indicated an observed initial ANC level of 85 nmol/g diet. ANC levels dropped to 57 and 42 nmol/g for diet samples that were stored for 30 days at −30◦C or at room temperature, respectively. Because of this relatively slow decline in ANC content, the much larger decline in ANC content in Experiment 1 is attributed mainly to decomposition of the ANCP during its repeated thawing and refreezing.

Experiment 2
The treated mice were fed 85 nmol ANC/g diet. The ANC-containing diet was prepared and then stored at −30◦C by the method adopted after week 12 in Experiment 1. Because of our test of ANC stability in the diet (see Experiment 1), this diet was presumed to maintain a constant ANC level. At week 4, the 24-h feces from 3 ANC-treated and 3 untreated mice showed 32 ± 11 and 0.7 ± 0.2 nmol ANC/g feces (mean $\pm$ *SD*), respectively.
The ABC appeared similar in Experiments 1 and 2 and in the test of AOM. As in Exp. 1, the number of ABC/2 cm colon was significantly higher ($P<0.01$) in the ANC-treated group than in the untreated controls (Table 1). In Experiment 2, more than 11 ABC/colon were observed in 13 of 23 mice receiving ANC, but in only 1 of 12 untreated controls. No tumors were detected. In this experiment we also recorded the number of foci containing 1, 2, 3, or 4 ANC/focus (Table 2).

Induction of MDF
MDF were detected in a total of 6 mice, including 2 mice given 3 AOM injections of 5 mg/kg (see **Results**) and 4 mice (3 treated and 1 untreated) in the ANC experiment (Table 1).MDF were not observed in the 4 mice given a single AOM injection. None of these results showed significant differences.

TABLE 2
Median numbers and (in parentheses) interquartile ranges of foci containing 1, 2, 3, or 4 ABC in the distal 2 cm of the colons from Exp. 2*a*

Type of focus	No. of foci in 23 ANC-treated mice	No. of foci in 8 untreated mice	*P* value for number of foci/colon in treated mice compared to number in untreated mice
With 1 ABC/focus	4 (1–5)	4 (1–5)	0.83
With 2 ABC/focus	2 (0–2)	1 (0.5–2.5)	0.74
With 3 ABC/focus	1 (0–2)	0 (0.0–0.5)	0.10
With 4 ABC/focus[b]	0 (0–1)	0	0.64
For all foci	7 (5–8)	5 (3.5-6.5)	0.21

ABC indicates aberrant crypts; ANC, apparent N-nitroso compounds.
a- Colons examined here were taken from all 23 ANC-treated mice and from 8 of the 12 untreated mice, both from Experiment 2. Interquartile ranges are shown in parentheses.
b- We saw no foci with more than 4 ABC.

DISCUSSION

The tests on AOM were performed on CF1 mice, whereas those on the hot-dog-derived ANC used A/J mice. The reason for this difference is that detecting and counting aberrant crypts was a new technique for us, so we did the test on AOM first using outbred mice to learn the techniques and to compare the number of ABC induced by the ANC with those induced by AOM. The AOM results suggest that at least one previous report on AOM induction of ABC [24] underestimated AOM activity due to the use of impure AOM. Because our AOM and ANC experiments were carried out on different mouse strains and at different times, we did not include the AOM results in Table 1 but described them only in the text of **Results**. Despite the different mouse strains used, we compared the relative potencies of the ANC and AOM, as this at least indicates that ANC activity was much lower than that of the standard colon carcinogen, AOM.

Our results support the view that ANC prepared from purified ANCP are mostly NOC, with about 10% contamination by nitrosothiols and little or no contamination by nitrosyl iron compounds. In Experiment 2 the fecal ANC level for the mice fed ANC was 46 times that for untreated mice. This confirms our finding (12,13) that feeding ANC to mice increases their fecal ANC output.

Feeding the hot-dog-derived ANC significantly increased the median number of ABC by factors of 4.0 (Experiment 1) and 1.85 (Experiment 2), with $P < 0.01$ for the differences from the controls in both experiments (Table 1). In Experiment 2, we fed 85 nmol ANC/g diet, 15 times the mean ANC level of 5.5 nmol/g hot dog (15). Experiment 1 was meant to be only a preliminary test but is presented here because it showed a much larger effect on ABC induction than did Experiment 2 (Table 1). The untreated controls in Experiments 1 and 2 showed median values of 5–6ABC/2 cmof colon (Table 1). Earlier studies (5,25) did not report ABC or ACF numbers for their untreated controls. Although the workers in our laboratory who initially diagnosed the ABC were not blinded, their diagnoses were checked by others, and the same workers also found increased numbers of ABC after the treatments with AOM, a known colon carcinogen (see **Results**).

We did not find significantly increased numbers of MDF in the ANC-treated mice, though the results suggest that a larger testwith higher ANC doses would have given positive results. In previous tests where ACF and MDF gave contradictory results, both colonic MDF and tumors, but not ACF, were suppressed by feeding various synbiotics (7). Induction of colonic MDF and tumors, but not that of ACF, was promoted by cholic acid (8). MDF had a mutation profile closer than ACF to that in colonic adenocarcinoma (9). Nevertheless, we mostly studied ABC here because they have been used in more than 1,000 published studies (5), given doses of colon carcinogens induce 10–30 times more ACF than MDF (8,9) and ACF develop far earlier than do MDF.

Potency for ABC induction by the ANC in Experiment 1 was compared with that for the ANC in Experiment 2 based on the median ABC values shown in Table 1. For this purpose, we assumed that (a) mean body weight was 25 g (close to the observed values), (b) the mice ate 5 g diet/day, and (c) the number of induced ABC was proportional to AN dose. In Experiment 1, mean ANC intake during the first 12 wk was roughly estimated to be 43 nmol/g diet (the mean of the initial dose of 85 nmol/g diet and the final dose, taken to be 0). ANC intake during the last 7 wk of Experiment 1 was 85 nmol/g diet.Hence the estimated totalANC intake/mouse in Experiment 1 was 38.9 μmol, which is the sum of (a) 18.1 μmol ANC (=43

nmol ANC/g diet × 5 g diet/day × 84 days/1,000) and (b) 20.8 μmol ANC (=85 nmol/g diet × 5 g/day × 49 days/1,000). This dose induced 15 ABC/2 cm colon (20 in the treated group minus 5 in the control group). Therefore, in Experiment 1, one ABC was induced by 38.9/15 = 2.6 μmol ANC.

In Experiment 2, dietary ANC was maintained at 85 nmol ANC/g diet throughout the experiment. Total ANC dose/week was then 113 μmol ANC/mouse (=85 nmol ANC/g diet × 5 g diet/day × 38 wk × 7 days/wk/1,000). This dose induced 5.5 ABC/colon (12 in the treated group, 6.5 in the control group). Therefore, in Experiment 2, one ABC was induced by 113 μmol/5.5 ABC = 21 μmol ANC. Similar calculations for our test of AOM given as 3 injections indicated that one ABC/2 cm colon was induced by 0.082 μmol AOM/mouse.

We will define relative potency for ABC induction as 1/dose in μmol/mouse that induced one ABC/2 cm colon. Therefore, if our assumptions are correct and we ignore the likely different sensitivities of the 2 mouse strains used in these tests, relative potencies for AOM in Experiment 1, ANC in Experiment 1, and ANC in Experiment 2 were 1/0.082, 1/2.6 and 1/21, i.e., 12.2, 0.38, and 0.048, respectively. Hence relative potency of the ANC for inducing ABC was 3.1% in Experiment 1 and 0.4% in Experiment 2 of that for AOM, and potency of the ANC in Experiment 1 was 8 times that for the ANC in Experiment 2. The ANC did not significantly increase the number of ACF (Table 2), the measure adopted most frequently by others to evaluate such studies (5). Accordingly, it appears that counting individual ABC is a more sensitive assay than counting ACF. We conclude that ANC activity for inducing ABC was clearly demonstrated but was relatively weak.

The falling ANC level in the diet during weeks 1–12 of Experiment 1 is attributed to a decline in the ANCP content of the single large batch of ANCP solution used there, which was repeatedly thawed and refrozen. The ANCP appeared more unstable than the derived ANC. To explain the 8 times higher potency of the ANC in Experiment 1 compared to that in Experiment 2, we suggest that the ANCP fraction yielding more active ANC decomposed more slowly than that yielding less active ANC. Alternatively, the ANCP in the commercial hot dogs used in Experiment 1 might have yielded a higher proportion of active ANC than did the ANCP used in Experiment 2.

We will now compare the ANC dose consumed by the mice with human exposure to ANC in hot dogs. A 70-kg person eating two 60-g hot dog/day containing 5.5 nmol ANC/g hot dogs (15) would consume 5.5 nmol ANC/g hot dog × 120 g = 660 nmol ANC/day or 660/70 = 9.4 nmol/kg body weight/day. From the calculations presented in paragraphs 6 and 7 of **Discussion**, we estimate that the mice in Experiments 1 and 2 consumed mean ABC dose that were, respectively, 11 and 34 μmol/kg body weight/day. According to these estimates, human exposure/kg body weight/day was about 0.01% in Experiment 1 and 0.03% in Experiment 2 of the exposure/kg/day in the mice.

Our results complement those in a study with similar end-points using cured meat instead of ANC as the test material (31). Rats were pretreated with the colon carcinogen 1,2-dimethylhydrazine, fed diets containing various meat products, and then scored for colonic ACF and MDF. Cured meat significantly increased the number of ACF/colon. Among several meat products tested, only a cooked, nitrite-treated and oxidized high-heme meat increased both fecal ANC excretion and MDF induction in the colon.

In conclusion, when an ANC fraction, prepared from purified ANCP in hot dogs, was fed to mice, it induced colonic ABC and showed a tendency to increase the induction of MDF. The effect on ABC induction is considered weak because it showed a molar potency that was only 0.4–3.1% of that for AOM. Also, ABC are only putative precursors of colon cancer. Nevertheless, our findings support the view, first proposed for fresh red meat by Rowland et al. in 1991 (32), that ANC produced from nitrite in processed meat are a cause of colon cancer. This is the first time that a purified fraction of processed meat has induced ABC. The results presented here support the view that we should consider reducing our intake of processed meat and/or reducing their content of biologically active ANC.

REFERENCES

1. Ward MH, Cross AJ, Divan H, Kulldorff M, Nowell-Kadlubar S, et al.: Processed meat intake, CYP2A6 activity and risk of colorectal adenoma. *Carcinogenesis* **28,** 1210–1216, 2007.

2. Santarelli RL, Pierre F, and Corpet DE: Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. *Nutr Cancer* **60,** 131–144, 2008.

3. Chao A, Thun MJ, Connell CJ, McCullough ML, Jacobs EJ, et al.: Meat consumption and risk of colorectal cancer. *J Am Med Assn* **293,** 172–182, 2005.

4. Mirvish SS: Methods for the determination of N-nitroso compounds in foods and biological fluids. Chapter 18. Food Contaminants and Residue Analysis. In: *Comprehensive Analytical Chemistry*, Vol. 51, Y. Pico (ed.). Amsterdam, The Netherlands: Elsevier, 2008, pp. 653–684.

5. Corpet DE and Tache S: Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. *Nutr Cancer* **43,** 1–21, 2002.

6. McLellan EA and Bird RP: Specificity study to evaluate induction of aberrant crypts in murine colons. *Cancer Res* **48,** 6183–6186, 1988.

7. CaderniG, FemiaAP,GianniniA, FavuzzaA, Luceri C, et al.: Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. *Cancer Res* **63,** 2388–2392, 2003.

8. Femia AP, Dolara P, and Caderni G: Mucin-depleted foci (MDF) in the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon carcinogenesis. *Carcinogenesis* **25,** 277–281, 2004.

9. Femia AP, Dolara P, Giannini A, Salvadori M, Biggeri A, et al.: Frequent mutation of Apc gene in rat colon tumors and mucin-depleted foci, preneoplastic lesions in experimental colon carcinogenesis. *Cancer Res* **67,** 445–449, 2007.

10. Pierre FH, Santarelli RL, Allam O, Tache S, Naud N, et al.: Freeze-dried ham promotes azoxymethane-induced mucin-depleted foci and aberrant crypt foci in rat colon. *Nutr Cancer* **62,** 567–573, 2010.

11. Mirvish SS: Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. *Cancer Lett* **93,** 17–48, 1995 (Erratum appeared in *Cancer Lett* **97,** 271, 1995).

12. Mirvish SS, Haorah J, Zhou L, Hartman M, Morris CR, et al.: N-nitroso compounds in the gastrointestinal tract of rats and in the feces of mice with induced colitis or fed hot dogs or beef. *Carcinogenesis* **24,** 595–603, 2003.

13. Mirvish SS, Davis ME, Lisowyj MP, and Gaikwad NW: Effect of feeding nitrite, ascorbate, hemin and omeprazole on excretion of fecal total apparent N-nitroso compounds in mice. *Chem Res Toxicol* **21,** 2344–2351, 2008.

14. Pignatelli B, Chen CS, Thuillier P, and Bartsch H: Group-selective determination of total N-nitroso compounds in nitrate-containing human urine samples. *Analyst* **114,** 1103–1108, 1989.

15. Haorah J, Zhou L, Wang X, Xu G, and Mirvish SS: Determination of total N-nitroso compounds and their precursors in frankfurters, fresh meat, dried salted fish, sauces, tobacco and tobacco smoke particulates. *J Agric Food Chem* **49,** 6068–6078, 2001.

16. Wang J, ChanWG, Haut SA, Krauss MR, Izac RR, et al.: Determination of total N-nitroso compounds by chemical denitrosation using CuCl. *J Agric Food Chem* **53,** 4686–4691, 2005.

17. Kuhnle GG, Story GW, Reda T, Mani AR, Moore KP, et al.: Diet-induced endogenous formation of nitroso compounds in the GI tract. *Free Radic Biol Med* **43,** 1040–1047, 2007.

18. Bingham SA, Pignatelli B, Pollock JRA, Ellul A,Malaveille C, et al.: Does increased endogenous formation of *N*-nitroso compounds in the human colon explain the association between red meat and colon cancer. *Carcinogenesis* **17,** 515–523, 1996.

19. Bingham S: High-meat diets and cancer risk. *Proc Nutri Soc* **58,** 243–248, 1998.

20. Hughes R, Cross AJ, Pollock JRA, and Bingham S: Dose-dependent effect of dietary meat on endogenous colonic *N*-nitrosation. *Carcinogenesis* **22,** 199–202, 2001.

21. Joosen AM, Kuhnle GG, Aspinall SM, Barrow TM, Lecommandeur E, et al.: Effect of processed and red meat on endogenous nitrosation and DNA damage. *Carcinogenesis* **30,** 1402–1407, 2009.

22. Lewin MH, Bailey N, Bandaletova T, Bowman R, Cross AJ, et al. Red meat enhances the colonic formation of the DNA adduct O6-carboxymethyl-guanine: implications for colorectal cancer risk. *Cancer Res* **66,** 1859–1865, 2006.

23. Zhou L, Haorah J, Perini F, Carmella SG, Shibamoto T, et al.: Partial purification from hot dogs of N-nitroso compound precursors and their mutagenicity after nitrosation. *J Agric Food Chem* **54,** 5679–5687, 2006.

24. Papanikolaou A, Wang QS, Papanikolaou D, Whiteley HE, and Rosenberg DW: Sequential and morphological analyses of aberrant crypt foci formation in mice of differing susceptibility to azoxymethane-induced colon carcinogenesis. *Carcinogenesis*, **21,** 1567–1572, 2000.

25. Corpet DE and Pierre F: How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats,mice andmen. *Eur J Cancer* **41,** 1911–1922, 2005.

26. Newmark HL, Lipkin M, and Maheshwari N: Colonic hyperproliferation induced in rats and mice by nutritional-stress diets containing four components of a human Western-style diet (series 2). *Am J Clin Nutr* **54,** 209S–214S, 1991.

27. McLellan EA and Bird RP: Aberrant crypts: potential preneoplastic lesions in the murine colon. *Cancer Res* **48,** 6187–6192, 1988.

28. McLellan EA, Medline A, and Bird RP: Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer Res* **51,** 5270–5274, 1991.

29. Rosenberg DW and Liu Y: Induction of aberrant crypts in murine colon with varying sensitivity to colon carcinogenesis. *Cancer Lett* **92,** 209–214, 1995.

30. Ward JM, Yamamoto RS, Benjamin T, Brown CA, and Weisburger JH: Experimentally induced cancer of the colon in rats and mice. *J Am Vet Med Assoc* **164,** 729–732, 1974.

31. Santarelli RL, Vendeuvre JL, Naud N, Tache S, Gueraud F: et al.: Meat processing and colon carcinogenesis: cooked, nitrite-treated, and oxidized high-heme cured meat promotes mucin-depleted foci in rats. *Cancer Prev Res (Phila Pa)* **3,** 852–864, 2010.

32. Rowland IR,Granli T, BockmanOC,Key PE, andMassey RC: Endogenous N-nitrosation in man assessed by measurement of apparent total N-nitroso compounds in feces. *Carcinogenesis* **12,** 1395–1401, 1991.