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Metabolism of Acyclic and Cyclic *N*-Nitrosamines by Cultured Human Colon[1] (40294)

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N-Nitroso compounds are a major class of chemical carcinogens which are candidates to cause human cancers (1). *N*-Nitrosamines have been detected in ambient air over certain urban areas (2), in tobacco smoke (3), food and drinks (4, 5). Furthermore, they can be formed *in vivo* by the reaction of nitrite with nitrosable amines under acid conditions, such as in the stomach (6). They may also be formed by enteric bacteria e.g. *E. coli in situ* (7). *N*-Nitrosamines require metabolic activation to exert their mutagenic and carcinogenic activity (8–10). This requirement of activation could, in part, explain the organotrophic carcinogenicity of the *N*-nitrosamines (10) and furthermore could affect an individual's susceptibility to the carcinogenic action of *N*-nitrosamines.

Experimental systems to study carcinogenesis directly in human epithelia are being developed (11, 12). We have previously shown that cultured human colon can activate procarcinogens from several chemical classes; e.g. polycyclic aromatic hydrocarbons, dialkylhydrazine, N-nitrosamines, into metabolites which bind to cellular macromolecules (13). We now report metabolic studies of several aliphatic N-nitrosamines in cultured human colonic mucosa.

Materials and methods. Non-tumorous human colonic tissues were collected at the time of either surgery or "immediate" autopsy (14) from a total of 11 patients; 7 with and 4 without cancer of the colon. The tissues were immediately put in sterile containers on ice and immersed in L-15 medium within 15 min after removal from the patient and kept at 4° for 3 to 12 hr until cultured. The specimens were cut into squares (0.5 × 0.5 cm) and cultured as previously described (13).

After 24 hrs in culture, one of the following [^{14}C]labeled *N*-nitrosamines (New England Nuclear, Boston, MA) was added to the culture media to give a concentration of 100 μM: [^{14}C]Dimethylnitrosamine [35 mCi/mmole; prepared on NCI contract N01-CP-55677 and purified by the method of den Engelse *et al.* (15); *N*-[^{14}C-1-ethyl]diethylnitrosamine (14.5 mCi/mmole); *N*-[^{14}C-2,6]nitrosopiperidine (18.8 mCi/mmole); *N*-[^{14}C-2,5]nitrosopyrrolidine (16.2 mCi/mmole); *N*-[^{3}H-3,4]-nitrosopyrrolidine (5 mCi/mmole). *N,N'* [^{14}C(U)]dinitrosopiperazine (16.5 mCi/mole); *N*-[pyrrolidine-^{14}C-2] nitrosonornicotine (4.10 mCi/mmole).

Five explants per experimental variable in three sterile 60 mm plastic Petri dishes (Falcon Plastics, Oxnard, CA) were placed on a rack in a closed container (Nalgene plastic jar, 500 ml) which was modified with two ports for replacing air with 95% O_2–5% CO_2 (16). The containers were placed on a rocker platform and rocked approximately 10 cycles per minute for 24 hr. In order to remove ^{14}C-CO_2 formed by the metabolism of the *N*-nitrosamine the containers were flushed with N_2 for 5 min and the CO_2 absorbed in two tubes each containing 8 ml 0.2 *M* $Ba(OH)_2$. After removal of the explants, 1 ml 3M phosphoric acid (pH 3) was added to each culture dish to release CO_2 dissolved in the media. After 4 hr at 37°, the containers were then flushed with N_2 for another 5 min.

The tissue culture medium was transferred to a reaction flask (Kontes Glassware, Vineland, NJ) the sidearm of which contained a small vial with 0.5 ml 4N KOH, and oxidized by $HgCl_2$ (100 mg/ml) at 90° for 1 hr (15). The KOH-solution was added to the $Ba(OH)_2$-solution. The precipitate was collected on Whatman GF/C filters and washed with absolute ethanol until the count in the washing solution was negligible. Medium without explants of colon served as control. The precipitate and filter were suspended in

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3 ml water and 10 ml Aquasol liquid scintillation cocktail (New England Nuclear, Boston, MA) and counted.

The mucosa was scraped from the explant, and DNA and protein isolated by the phenol extraction procedure. DNA was purified on a CsCl-gradient and the binding level measured as previously described (17). Binding to protein was also assayed (13). One explant from each variable was fixed in 3% glutaraldehyde buffered with 0.1 *M* s-collidine (pH 7.4) and prepared for light microscopy (18).

DNA, isolated from a total of 54 explants (pooled from three cases), was hydrolysed with 0.1 *M* HCl at 70° for 1 hr and bases were isolated by high-pressure liquid chromatography (Column: Durrum DC 1-A; 15 × 0.21 cm; Durrum Chemicals, Sunnyvale, CA; Solvent: 0.1 *M* ammonium formate, pH 4.5; Flow rate: 0.6 ml/min). Markers for N-7 and O-6 methylguanine were added to the hydrolyzed DNA; the elution was monitored at 254 m*M* and 0.4 ml fractions were collected. The radioactivity was measured by liquid scintillation methods. The material eluting in the void volume (90% of the radioactivity) was treated with conc. perchloric acid at 100° for 1 hr and methanol removed by vacuum-distillation and the radioactivity was determined.

Results. Formation of ^{14}C-CO_2 after incubation of *N*-nitrosamines with human colon indicates that cultured human colonic mucosa is able to metabolize both acyclic *N*-nitrosamines (Table I), such as dimethylnitrosamine (DMN) and diethylnitrosamine (DEN), and cyclic *N*-nitrosamines (Table II). Variation in the ability to metabolize cyclic *N*-nitrosamine was observed among individuals. Under these test conditions only *N*-nitrosopyrrolidine (NPy) was metabolized by all cases studied, *N*,*N*′-dinitrosopiperazine (DNP) by five cases and *N*-nitrosopiperidine only by one case. No ^{14}C-CO_2 was formed from *N*-nitrosonornicotine possibly due to the chemical structure (the C-14 labeled atom

TABLE I. METABOLISM OF *N*,*N*-DIALKYLNITROSAMINES BY CULTURED HUMAN COLON.[a]

	Dimethylnitrosamine			Diethylnitrosamine		
Case	DNA[b]	Protein[b]	CO_2-formation[c]	DNA[b]	Protein[b]	CO_2-formation[c]
62	570	106	6920	N.D.[d]	22	6632
66	36	59	1381	N.D.	26	N.D.
83	12	29	566	N.D.	55	93
87	23	49	823	26	11	217
92	50	178	1040	N.D.	14	N.D.
99	29	133	849	N.D.	18	N.D.

[a] Colonic explants were cultured in chemically defined media for 24 hrs and the [^{14}C]labelled *N*-nitrosamines were added at a concentration of 100 μM to groups of five explants for 24 hr.
[b] *p*moles nitrosamine bound per mg of either DNA or protein, single determination.
[c] *p*moles ^{14}C-CO_2 formed per mg DNA.
[d] N.D. = not detectable.

TABLE II. METABOLISM OF CYCLIC *N*-NITROSAMINES BY CULTURED HUMAN COLON.[a]

	N-nitrosopyrrolidine			*N*-nitrosonornicotine			*N*-nitrosopiperidine			*N*-nitrosopiperazine		
Case	DNA	Protein	CO_2-formation[c]	DNA	Protein	CO_2-formation[c]	DNA	Protein	CO_2-formation[c]	DNA[b]	Protein	CO_2-formation[c]
62	55	56	2410	N.D.[e]	15	N.D.	N.D.	23	188	N.D.	185	9531
66	21	49	4276	N.D.	17	N.D.	N.D.	N.D.	N.D.	N.D.	216	N.D.
83	13	125	1190	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	15	596
87	103	51	478	22	N.D.	N.D.	N.D.	N.D.	N.D.	15	169	520
92	22	80	479	N.D.	7	N.D.	N.D.	21	N.D.	N.D.	206	1344
99	12	147	1056				N.D.	40	N.D.	N.D.	227	591
105[d]	71	5910										
111[d]	86	18,220										
114[d]	99	6776										

[a] Colonic explants were cultured in chemically defined media for 24 hr and the [^{14}C]labelled *N*-nitrosamines were added at a concentration of 100 μmoles to groups of five explants for 24 hr.
[b] dpm per 100 μg of either DNA or protein; single determination.
[c] pmoles ^{14}C-CO_2 formed per mg DNA.
[d] Incubated.
[e] N.D. = none detectable.

had only one C-H bond)—but nonlabeled CO_2 could have been formed from other carbon–atoms in the pyrrolidine ring. Only DMN and NPy consistently formed alkylating moieties which reacted with cellular DNA in all cases. DMN, DEN, NPy, and DNP bound to protein; when compared to the other *N*-nitrosamines high binding levels of DNP to cellular protein were observed. The binding data in Table II is given as either dpm per 100 μg DNA or dpm per 100 μg protein as the exact chemical structure of the adducts formed between the *N*-nitrosamines and the macromolecules are unknown at the present. A positive correlation ($r = 1.00$) was found between alkylation of DNA by DMN and CO_2-formation, while NPy did not show any correlation ($r = 0.24, p > 0.1$). No correlation between DMN and NPy binding to protein and CO_2-formation was found ($r = 0.14$, $p > 0.1$, and $r = 0.41$, $p > 0.1$, respectively). DMN alkylated DNA in both N-7 and O-6 position of guanine (Table III). However, most of the radioactivity was associated with material in the initial peak. Treatment of this material with conc. perchloric acid released ^{14}C-MeOH (40% of radioactivity). The morphology of the explants, as monitored by high resolution light microscopy, showed good preservation in all the reported cases.

Discussion. *N*-Nitroso compounds induce cancer in many animal species (10) and have been implicated in causing human cancers (1). *N*-Nitrosamines rarely induce colonic cancer in experimental animals. However, *N*-nitrosamides such as *N*-methylnitrosourea (19) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (20), caused colo-rectal carcinoma in rodents when applied intrarectally.

Procarcinogens require metabolic activation to exert their carcinogenic effect (21). Procarcinogens, implicated in colon carcinogenesis, could be activated enzymatically in: (a) organs other than the colon and reach the target tissue via the blood circulation (22); (b) the intestinal lumen by deconjugation of metabolites by the microflora (23, 24); and (c) the intestinal mucosa by various enzymes e.g. the mixed-function oxidases (24). We have previously shown that both human and rat colonic mucosa in culture can activate procarcinogens into metabolites that bind to DNA; explants of human colon can metabolize DMN, 1,2-dimethylhydrazine and benzo-[*a*]pyrene (BP) (13, 25). This observation suggests the importance of the third pathway described above.

TABLE III. METHYLATION OF HUMAN COLONIC DNA BY [^{14}C]DMN.

Base	dpm[a]
O^6-MeGua	20 (2)
N^7-MeGua	38 (13)
Guanine	57 (4)
Initial peak	1175 (84)

[a] Numbers in parentheses, percentage of the total number of dpm added to the column.

A 50-fold inter-individual variation was found in the binding of DMN to human colon DNA, lower than the 100-fold variation observed in the binding levels of BP to DNA in cultured human colon (26) and the 75-fold variation in the binding levels of BP to DNA in cultured human bronchus (27). Several factors for this variation were considered. The *intra*-individual variation due to the methodology was minimal, i.e., coefficient of variation 0.1 (13). The viability of the tissue as monitored by high-resolution microscopy was good in all the reported cases; however, changes in cellular physiology could, in part, account for some of the observed differences. There is a positive correlation between the level of radioactivity associated with DNA and CO_2-formation. Alkylation took place at both the O-6 and N-7 position giving a ratio of 0.5. However, this radioactivity only accounted for a small part of the total radioactivity. Treatment of the material in the initial peak with strong acid, released about 40% of the radioactivity in form of methanol, indicating that the major alkylation site could either be the phosphate groups or the oxygens in thymidine and/or cytosine. This finding however requires further investigtion. Incorporation of ^{14}C from ^{14}C-CO_2 in the purine ring of the nucleic acids by *de novo* synthesis could also account for some of the radioactivity associated with DNA (13). Human liver slices (28) and human bronchus (29, 30) are also able to metabolize DMN into CO_2 and alkylating species which reacted with DNA. DMN has been shown mainly to alkylate the O-6 and N-7 positions of guanine in DNA (31); the ratio of methylation of O-6 to N-7 being nearly 1.1 in cultured human bronchus (30), while a lower ratio was found in animal experiments (32).

The ability of the colon to metabolize the different N-nitrosamines varies among individuals. While colon from all investigated cases could metabolize DMN, only two cases could metabolize DEN into metabolites which reacted with DNA. Since the [^{14}C]-atom is located at the two-position of the ethyl group the alkylating moiety can be deduced as being an ethyl group. NPy was also metabolized by colon from all the cases. Binding of both ^{3}H- and [^{14}C]NPy suggests that an adduct(s) is formed between a metabolite of NPy and DNA. Opening of the ring in NPy indicated by CO_2-formation suggests that several possibilities for alkylating species exist. Lack of correlation between alkylation of DNA by NPy and CO_2-formation could also implicate a more complex pattern of metabolism. It has been suggested that two of the reaction-products between NPy and nucleic acids are 7-(2-carboxy)ethylguanine and/or 7-methylguanine (33). However, a recent observation indicates that the alkylation species could be 3-formyl-1-propanediazohydroxide (34). The molecular structure of the DNA adduct in human colon is under investigation. Formation of ^{14}C-CO_2 *in vivo* by rats injected with either 2,5-[^{14}C]NPy or 3,4-[^{14}C]NPy shows that ring oxidation occurs at both two and three positions (33). DNP had a high binding level to protein, while binding to DNA was only observed in one case. This observation of a high level of protein binding is similar to our results from cultured human bronchus (16).

N-nitrosamines may reach the colonic mucosal epithelial cells by several routes, where they could be metabolically activated. DMN has been detected in the blood of people ingesting both spinach and bacon; spinach is recognized as a rich source of nitrate/nitrite (35). *N*-nitrosamines have also been detected in the feces of human subjects, whose diet did not contain any detectable *N*-nitrosamines indicating that the compounds were formed *in situ* (36).

The etiology of human colonic cancer is a complex problem. No exogenous chemical compounds have been so far proven to cause this carcinoma in the human. Our observations, that human colonic mucosa can activate several types of procarcinogens (*e.g.* BP, 7,12-dimethylbenz[*a*]anthracene, 1,2-dimethylhydrazine and aliphatic *N*-nitrosamines) into forms that bind to DNA, suggests that the colon should be added to the list of organs which are likely to be susceptible to the carcinogenic action of these compounds.

Summary. Cultured human colon mucosa was found to metabolize both acyclic and cyclic *N*-nitrosamines as measured by ^{14}C-CO_2 formation and reaction of the activated moieties with cellular macromolecules. Dimethylnitrosamine and N-nitrosopyrrolidine were metabolized by explants from all patients studied. A positive correlation between binding of dimethylnitrosamine to DNA and CO_2-formation was observed. DMN alkylated DNA in both O-6 and N-7 position of guanine. However, most of the radioactivity was associated with an acid labile compound. High binding levels of *N*,*N*′-dinitrosopiperazine to protein without concomitant binding to DNA were detected. Inter-individual variation in both binding level to DNA and ability to metabolize the different N-nitrosamines was observed.

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