age (fig. C). Rare binucleate forms were seen (fig. D). In ^{3}H-thymidine-labeled cultured articular chondrocytes, both nuclei of binucleate cells had comparable numbers of grains (fig. E).

Discussion. The present results demonstrate a dichotomy between the replicative behavior of the 2 types of chondrocyte with respect to age in vivo. They provide no support for the occurrence of sizable numbers of binucleate chondrocytes to account for an apparently high DNA content in articular cartilage[7], but do not exclude polyploidy of mononuclear chondrocytes in the latter. Polyploidy and binucleation occur in mammalian tissues, particularly the liver. Polyploid and binucleate hepatocytes are capable of proliferating. Although there is a decline in the replicative activity of hepatocytes in aged animals, it is not the direct consequence of the polyploidy since the latter develops primarily in young individuals[11]. Auricular chondrocytes apparently do not divide in vivo but retain, despite their binucleation, a regenerative potential when cultured in vitro[4]. The mechanism for the binucleation of chondrocytes is not presently known. The apparent synchrony of DNA synthesis in the cultured chondrocytes illustrated in figure E does not in itself favor an acytokinetic mechanism over cell fusion. It has been demonstrated repeatedly for other cell types that fused homokaryons rapidly coordinate both DNA synthesis and mitosis[12]. Binucleation is a common finding in cultured articular chondrocytes[6, 13], as it is in many other animal cells in vitro. The phenomenon presumably is of stochastic origin, but its precise cause is unknown.

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Model systems demonstrating the volatile mutagenicity and carcinogenicity of sodium nitrite in rats[1, 2]

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Summary. Volatile mutagens derived from sodium nitrite buffered at various pH values or in the presence of human feces were detected using Ames *Salmonella* tester strain TA 1535 on petrie plates inverted over samples. Volatile mutagenicity increased as the pH decreased and was primarily a function of the nitrous acid produced from sodium nitrite and hydrogen ions. Sodium nitrite administered intracecally to 3 Wistar rats through surgically implanted cannula caused tumors (fibrosarcoma: ⅓ and squamous cell, ⅔). The possible role of nitrite-derived mutagens in GI cancer is discussed.

The discovery of the presence of low amounts of sodium nitrite in human feces[3] has initiated our investigation of the effect of sodium nitrite deliberately added to human feces on the mutagenicity of the volatile products[4, 5] and the chemical nature of the gases involved[5]. Additionally, we have preliminarily characterized some ether-soluble, Griess test-positive material produced on the incubation of feces with nitrite[6]. Volatile mutagens obtained by steam distillation of normal human feces have been shown to be carcinogenic N-nitroso compounds presumably derived in vivo from nitrite[7, 8]. However, a non-volatile fat soluble mutagen recently purified from human feces is not a N-nitroso derivative[9, 10].

The purpose of the following communication is to demonstrate that direct acting non-microbially produced volatile mutagens are evolved from sodium nitrite in the presence of hydrogen ions. We further describe an animal model designed to test the effect of sodium nitrite (or other pharmacological, biochemical or microbial agents) on the large bowel.

Materials and methods. Collection of human fecal samples. Stool samples from healthy volunteers were collected in Zip-Loc bags.

Assay for volatile mutagens. We employed an Ames assay system[11, 12] modified for detection of volatile mutagens as described earlier[3]. Plates containing *Salmonella typhimurium* strain TA 1535 (a base pair substitution mutant) were prepared according to Ames[11, 12] without liver microsomes. Nitrite was added to stool samples at a final concentration of 0.6 M or 0.2 M. Similarly, nitrite was incubated without feces. Buffers were 0.2 M imidazole HCl (pH 6.0–7.2) and 0.2 M citrate (pH 4.4–6.0). Systems were incubated with exposure of plates to the volatile mutagens for 21 h at 37 °C and then the exposed plates incubated a further 27 h at 37 °C before counting the revertant colonies as described before[4].

Surgery. Young male Wistar rats weighing approximately 200–250 g were cannulized. The pre-sterilized cannula consisted of a hollow, flatheaded, stainless-steel bolt approximately 1.5 cm long, (o.d. = 2.0 mm, i.d. = 1.0 mm and 2 washers and 2 nuts; see fig. 1). A vertical incision approximately 3 cm long was made in the lower abdomen of the halothane-anesthetized animal and the cecum located. A purse-string stitch was performed and the head of the cannula plus the small stainless steel washer (approximately 8 mm in diameter) was inserted into the cecum, approximately 2.5 cm from the ileal cecal junction. The purse string was firmly closed. A second larger washer (teflon) 13 mm in diameter was placed on the cannula, which was passed through the abdominal wall via a small stab

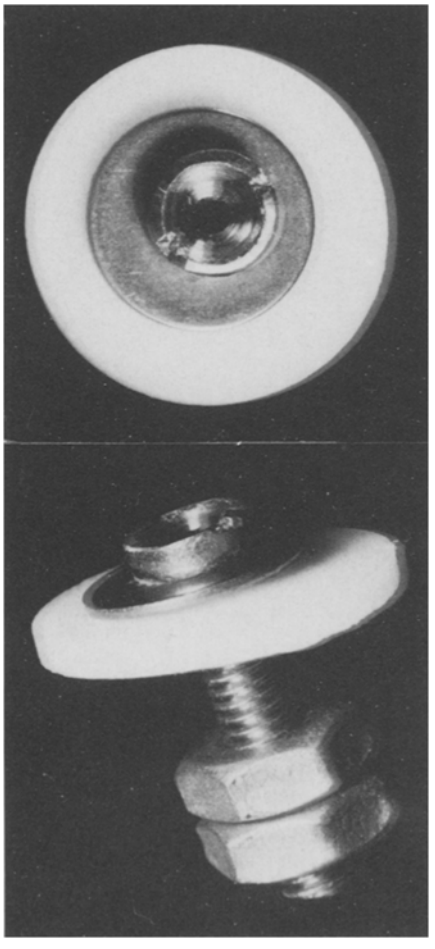

Figure 1. A cannula consisted of (a) a stainless steel screw which was drilled hollow (b) a small stainless washer, (c) a larger teflon washer and (d) 2 stainless steel nuts. The head and the small washer were inserted intracecally. The cannula is viewed from side and end.

wound. The 2 stainless steel nuts were tightened so they were flush with the outer end of the cannula and the abdominal incision was then closed.

Intracecal administration of sodium nitrite. The animals were allowed to heal (approximately 3–5 weeks) and were injected via the cannula with either a) 1 ml of 0.2 M $NaNO_2$ 3 times weekly for 4 weeks (3 rats) or b) 1 ml of saline solution 3 times weekly for 4 weeks (3 rats). The rats were allowed to live with no further treatment for 600 days or longer when apparently healthy and asymptomatic, but sacrificed earlier when there was evidence of tumor or severe sickness. All animals were kept in cages with plastic bottoms and fed Purina Rat Chow *ad libitum*.

Autopsy of the rats. The autopsies on all 6 rats (3 study group and 3 controls) were performed immediately after they were sacrificed in a CO_2 chamber. The large bowel including the cecostomy site, rectum and anal canal were removed, opened along the antimesenteric axis, pinned flat on a piece of cork and put in 10% buffered isotonic formalin. After 24–48 h of fixation, 'Swiss-roll' preparations of the entire bowel, rectum and anal canal were made, put through routine histotechnical processing, and cut into 5 µm thick sections at intervals of 2–3 mm. The sections were stained with hematoxylin and eosin. Multiple sections were also taken from the tumors arising in the skin at the cecostomy sites in the study group.

Results. Effect of pH and feces on volatile mutagenicity of sodium nitrite. When the volatile mutagenicity was plotted against the pH of the same fecal-nitrite suspension, a significant negative correlation was found both at 0.2 M and 0.6 M nitrite (fig. 2). Correlation coefficient values were estimated at -0.722 and -0.825 respectively. Moreover, by buffering the sodium nitrite at various pH values, in the absence of feces, we showed increasing mutagenicity with decreasing pH values, not only a higher nitrite concentrations, but concentrations in the mM range (fig. 2). Mutagenicity could be measured in the pH range of 3.8–6.8, with lower concentrations of sodium nitrite required to give the same number of reversions per plate at lower pH values within this range. In table 1, the nitrous acid concentrations (derived from sodium nitrite and calculated from the Henderson-Hassleback equation[13]) are represented with the amount of nitrite required to give 300 reversions per plate at various pH values (fig. 3). At the 5 highest nitrite concentrations tested, the average concentration of nitrous acid calculated for those systems yielding 300 reversions per plate, was $8.4 \times 10^{-4} \pm 4.2 \times 10^{-4}$ M. Thus, the data represented in figure 2 and table 1 suggest that the concentration of unionized nitrous acid is important in determining the mutagenicity in the gaseous phase.

Effect of intracecal sodium nitrite on rats. In our pilot study, 3/3 rats receiving intracecal sodium nitrite developed grossly apparent tumors while 3/3 rats receiving saline were tumor-free (table 2). Gross autopsy examination of the large bowel of all rats revealed no evidence of tumor there. In the 3 rats comprising the study group, firm tumors ranging from 1.5 to 4 cm in diameter were found at the cecostomy site. These had infiltrated the abdominal skin but there was no distant metastases. The rest of the organs were grossly normal. Sections of the tumor in the study group revealed 2 well differentiated keratinizing squamous cell carcinomas and 1 low grade, well differentiated fibrosarcoma. These had infiltrated abdominal wall muscle and soft tissues. Sections of large bowel and rectum from all 6 animals were studied microscopically for evidence of dysplasia, hyperplasia and neoplastic change. The mucosal epithelium was entirely normal in all animals. On the other hand, none of the 3 saline-treated animals had any evidence of cancer either on gross or microscopic examination. One of the control animals developed at least partial G.I. blockage (table 2). The pH value for rat cecal contents (control rats) was estimated at 6.2+0.2.

Discussion. The role of sodium nitrite in the etiology of bowel cancer is controversial[14]. Only very low levels occur in normal human feces[3]. Circumstantial evidence suggests that nitrite derived mutagens (volatile[7,8] fat soluble[4] or water soluble) can cause tumors in rats when introduced intracecally, but the tumors are not colonic in origin. A somewhat greater mutagenicity on the average, could be obtained from nitrite in the presence of feces than nitrite in the presence of buffers (without a pH change), implying that fecal material may somehow contribute to mutagen formation. Data in table 1 suggests that nitrous acid formation is a requirement for volatile mutagen production. Thus in spite of a possible role of intestinal bacteria in mutagenesis, the presence of a high H^+ is most important in this regard. Data in table 2 tend to negate the intracecal introduction of nitrite as a useful model for colon cancer: None of the tumors were colonic in origin and the potent fecal

Table 1. Calculated nitrous acid concentrations at various pH values at nitrite concentrations giving a mutagenicity of 300 reversions per plate

Nitrite (M)	pH Value	Calculated (nitrous acid)
0.6	6.00	1.4×10^{-3} M
0.2	5.85	8.3×10^{-4} M
0.06	5.85	3.9×10^{-4} M
0.02	5.00	4.7×10^{-4} M
0.006	4.05	1.1×10^{-3} M

Average (nitrous acid) = $8.4 \times 10^{-4} \pm 4.2 \times 10^{-4}$.

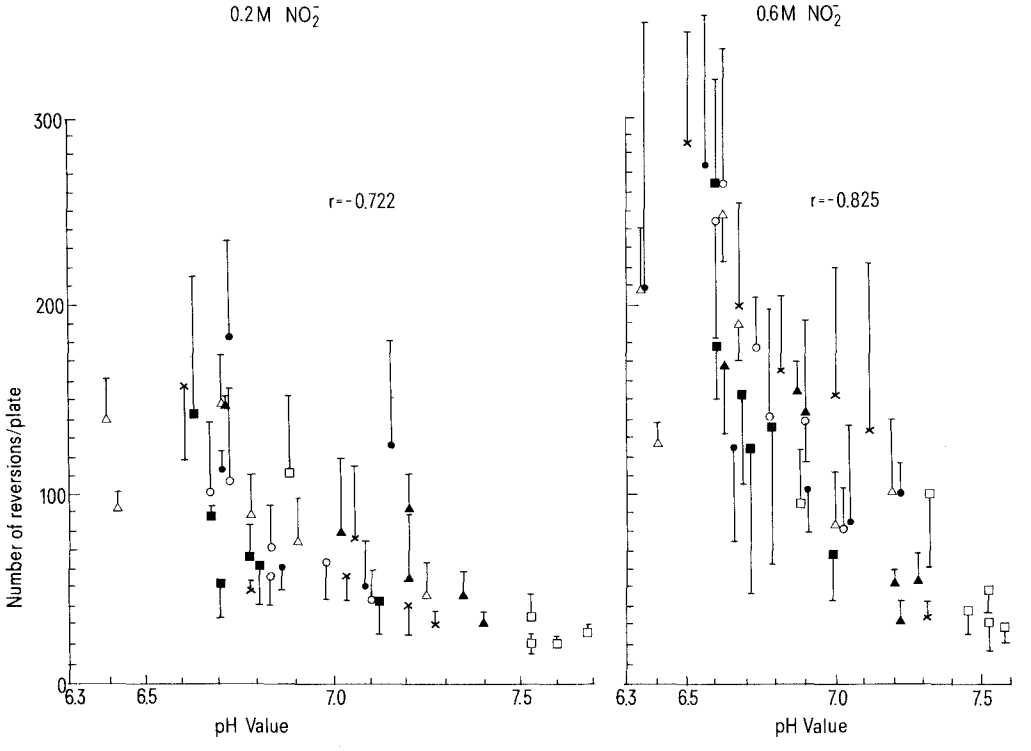

Figure 2. The mutagenicity of the volatile phase at a) (nitrite) = 0.2 M and b) (nitrite) = 0.6 M plotted against fecal pH value. Each symbol represents 1 subject studied on several occasions.

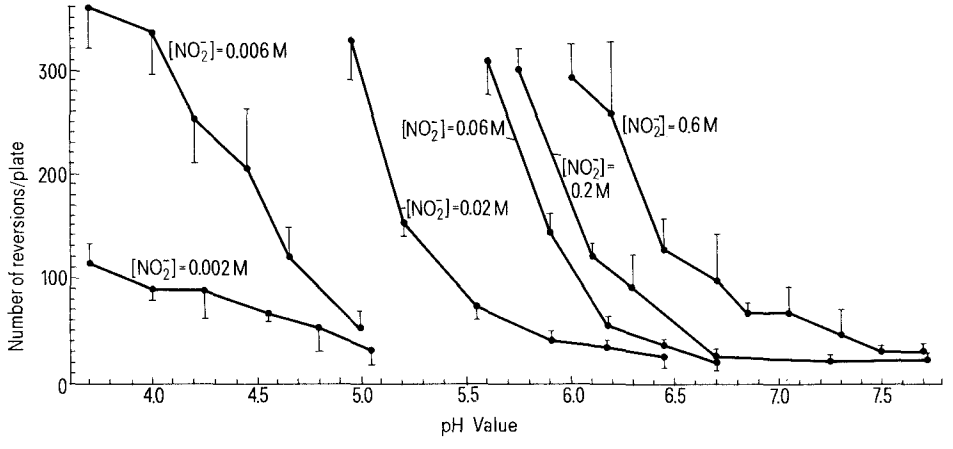

Figure 3. The mutagenicity of the volatile phase at various nitrite concentrations, over a wide pH range in the absence of feces.

mutagen described by other investigators is not nitrite-derived[9,10].

However, the in vitro data presented here as well as that described earlier[2,3] may be relevent to the etiology of stomach cancer. Human stomach nitrous acid concentrations from oral ingestion of dietary nitrite (or nitrate) have been estimated at 0.0057 mM, 0.034 mM and 0.58 mM in control subjects, Billroth I and Billroth II patients, respectively[15]. Moreover, higher concentrations of nitrosamines were measured in the last 2 groups[15] in which a higher risk of stomach cancer is known[16]. While the nature of the volatile mutagens is not known, mass spectroscopy data on the gases evolved suggest that the nitrogen oxides NO_2 and NO may both be present in the gaseous phase and NO is mutagenic to *S. typhimurium*[17].

Table 2. Long term effect of intracecal nitrite administration vs saline administration of male Wistar rats

Rat No.	1.0 ml 0.2 M sodium nitrite 3 times/week for 4 weeks	1.0 ml saline 3 times/week for 4 weeks	Number of 'cecal' tumors present	Approx. diameter of tumor	Days between commencement of treatment and sacrifice	Cellular origin of tumor
1	+		1	3 cm	445	fibro blast
2	+		1	4.0 cm	624	squamous
3	+		1	1.5 cm	686	fibro blast
4		+	0		400*	
5		+	0		630	
6		+	0		686	

* Rat No. 4 showed some signs of G.I. blockage on sacrifice.

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Effects of ouabain and furosemide on saliva secretion induced by sympathomimetic agents in isolated, perfused rat submandibular glands

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22 June 1983

Summary. The presence of 10^{-3} M ouabain or furosemide in the perfusate inhibited saliva secretion induced by either isoproterenol (10^{-5} M) or phenylephrine (10^{-5} M) from isolated rat submandibular glands and caused characteristic alterations in the electrolyte composition of saliva.

Recent studies in this laboratory have demonstrated that an isolated, perfused preparation of the rat submandibular gland secretes saliva in response to either parasympathomimetic[1,2] or sympathomimetic[3] agents. Both the volume and electrolyte composition of the secretion elicited by each of these agonists were similar, furthermore, to those observed following stimulation of the gland in situ[1,3,4]. It was also found that the presence of ouabain in the perfusion solution caused a 96% reduction in the volume of saliva secreted in response to acetylcholine and altered salivary Na^+ and K^+ concentrations[2]. Furosemide, on the other hand, caused a 75% reduction in saliva volumes and altered salivary Cl^- concentrations[2]. These findings were interpreted as indicative of the presence of both an ouabain-sensitive Na^+, K^+ ATPase and a furosemide-sensitive NaCl cotransport system in the salivary cells, which contribute to acetylcholine-induced fluid secretion in the rat submandibular gland[2]. The purpose of the present study was to compare the effects of these 2 transport inhibitors on saliva secretion induced in the isolated, perfused gland preparation by α and β-adrenergic receptor agonists, in order to establish whether the same ion transport systems also participate in the formation of saliva elicited by adrenergic agents.

Methods. Adult, male rats of the Sprague-Dawley strain were fed a standard diet and water ad libitum and were anesthetized with Na pentobarbital (6–8 mg/100 g b.wt, i.p.). The procedure for the isolation and perfusion of the submandibular gland was carried out as previously described[1-3]. The glands were prepared with the aid of a Leitz binocular dissecting microscope. After cannulation of the main excretory duct with a short plastic cannula (Clay Adams polyethylene tubing PE10), the arterial branch to the gland was identified and the main trunk of the external mandibular artery and its branches to other neck structures were identified and dissected. All branches except the glandular branch were cut between tight double ligatures and the main arterial trunk was cannulated with a plastic cannula (Clay Adams polyethylene tubing PE50), which was advanced to within a few mm of the glandular branch. Loose ligatures previously placed proximal to the glandular branch were then securely tied and the vessel divided. The gland was removed and placed in a perfusion bath kept at 37 °C. The gland was perfused at a rate of 3 ml min^{-1} with a Krebs Ringer bicarbonate solution (KRB) of the composition previously indicated[1-3]. The perfusate was infused by means of a peristaltic pump and was kept at 37 °C and continuously exposed to a 95% O_2–5% CO_2 mixture. Secretion was induced by administering either isoproterenol sulfate or phenylephrine hydrochloride by way of a 3-way valve connected to the perfusion line and infused at a rate of 0.3 min^{-1} from a Harvard Instruments Co. constant infusion pump, to obtain a final concentration of 10^{-5} M. Either ouabain (10^{-3} M) or furosemide (10^{-3} M) were added to the perfusate in parallel experiments. Saliva samples were collected in all experiments from the main duct cannula in pre-weighted microsample tubes. The volume of each sample was estimated gravimetrically and analyses for Na^+, K^+, Cl^- and Ca^{++} were performed as previously noted[1-3]. Electrolyte concentrations were expressed in relation to rates of flow (mg min^{-1} g^{-1}) as conventionally done in this type of study[1,3-5]. The latter were calculated from the sample volumes, the wet weight of the gland and the time of collection. The doses of secretagogue and of inhibitors which were used are those previously determined to cause maximum effects[1-3].

Results. The effects of 10^{-3} M ouabain or furosemide on the volume of saliva secreted in response to either isoproterenol or phenylephrine are summarized in the table. These results indicate that ouabain reduced salivary fluid secretion 79% and 95%, respectively, when isoproterenol and phenylephrine were used to elicit salivation. The corresponding values for furosemide were 69% and 87%, respectively. Both inhibitors significantly inhibited, therefore, saliva secretion induced from the isolated gland preparation by stimulation of α- or β-adrenergic receptors, but the effect of ouabain was generally larger. As expected, the maximum rate of flow observed with the 2 secre-