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RELATIONSHIP OF BEAN SUBSTRATES AND CERTAIN INTESTINAL BACTERIA TO GAS PRODUCTION IN THE DOG

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The mechanism of intestinal gas production has been a subject of much speculation. Danhof et al.[1] and others[2] argue that, in view of the high carbon dioxide concentrations often found in the flatus, the secretion in the intestine, particularly the pancreatic secretions, might be a major factor. Others have suggested that either swallowed air[3] or the diffusion of gases from the blood into the intestinal lumen[4] are responsible factors. Although all of these should be considered as contributing factors, gas production following ingestion of various dietary components, especially bean products, remains a matter of concern to the normal public. The majority of available information dating from the early review of Kantor[5] in 1918 to more recent work[6-8] suggests that the association between bean diets and gas production is in some way related to bacterial fermentation within the lumen of the intestine and colon. Little attempt has been made to find whether gas production is related to one or more specific types of microflora in the gastrointestinal tract. In recent literature[9-12] dealing with the composition and function of the normal intestinal flora of man and animals no reference was made to their influence on gas production in the presence of various food products.

That there may be specific types of organisms in the gastrointestinal tract that can change markedly both the quantity and the quality of the gas produced in the presence of certain specific foods is suggested by the recent work of Steggerda and Dimmick,[13, 14] who recorded differences in flatus production and composition in man with different kinds and quantities of bean diets. They observed that the average concentration of carbon dioxide in the collected flatus changed from 11% on a controlled non-gas producing diet to 51% when large quantities of pork and beans were consumed. In other experiments in which homogenates of navy beans were injected into isolated segments of the duodenum, jejunum, ileum, and colon of intact anesthetized dogs,[15] a significant increase in gas production, with high concentrations of CO_2 and H_2, was noted in all of the segments. The high H_2 in the dog is said to be related to the lack of methane-producing organisms in the intestinal flora. It was observed that these responses could be dramatically inhibited by pretreating the animals with a combination of antibiotics and bacteriostatic agents.

As a result of these experiments two deductions were made concerning the possible mechanism of flatulence production when bean products were ingested. (1) Bacteria are probably involved in the process since there is an indirect correlation between the amount of gas formed and the inhibition of microbial growth upon the addition of antibiotic and bacteriostatic agents. (2) The high CO_2 production suggests that the type of bacteria involved is probably the clostridia group of gram-positive, spore-forming anaerobes normally inhabiting the intestine and colon of both man and animals.[16]

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The purpose of the present study is to demonstrate by indirect evidence that the above deductions are logical and tenable. It is shown that the high rate of gas production and its CO_2 and H_2 composition previously observed in man and in segments of the dog's intestine and colon with gas-producing food substrates can be reproduced by culturing anaerobic clostridia strains of bacteria isolated from the intestine and colon loops under the same environmental conditions, in vitro. It is also shown that the anaerobic bacterial count can be increased in the presence of bean products or inhibited by antibotics and bacteriostatic agents both in vivo and in vitro.

Materials and Methods

Female mongrel dogs, dewormed 2 weeks earlier were fasted for 24 hr before the experiment. The dogs were anesthetized with 30 mg per kg of pentobarbital sodium, and four isolated intestinal segments were prepared, the duodenal, jejunal, and ileal segments being 50 cm long, and that of the colon, 40 cm. To obtain a bacterial culture from each segment the surface was cleaned with Zephiran chloride (4:750), and a sterile swab inserted through a 1-cm incision on the antimesenteric border near the upper end of each segment (fig. 1, *step 1*). These swabs were transferred to a sterile anaerobic thioglycollate medium and held at 80 C for 20 min to kill the aerobic and anaerobic vegetative bacteria (fig. 1, *step 2*). The tubes were then transferred to a 37 C water bath and incubated for 24 hr (fig. 1, *step 3*).

To test the in vitro anaerobic cultures on gas-producing substrates, pairs of 30-cc syringes were lubricated with oil and autoclaved, and were half filled with 15 cc of an homogenized substrate made from either pork and beans or a dog food containing a relatively high concentration of soybean meal (fig. 1, *step 4*). These were prepared by homogenizing 1-lb cans of commercial pork and beans or dog food with 100 cc of demineralized water. In the presentation of the data the pork and bean product is referred to as navy bean homogenate and the commercial dog food as soybean homogenate.

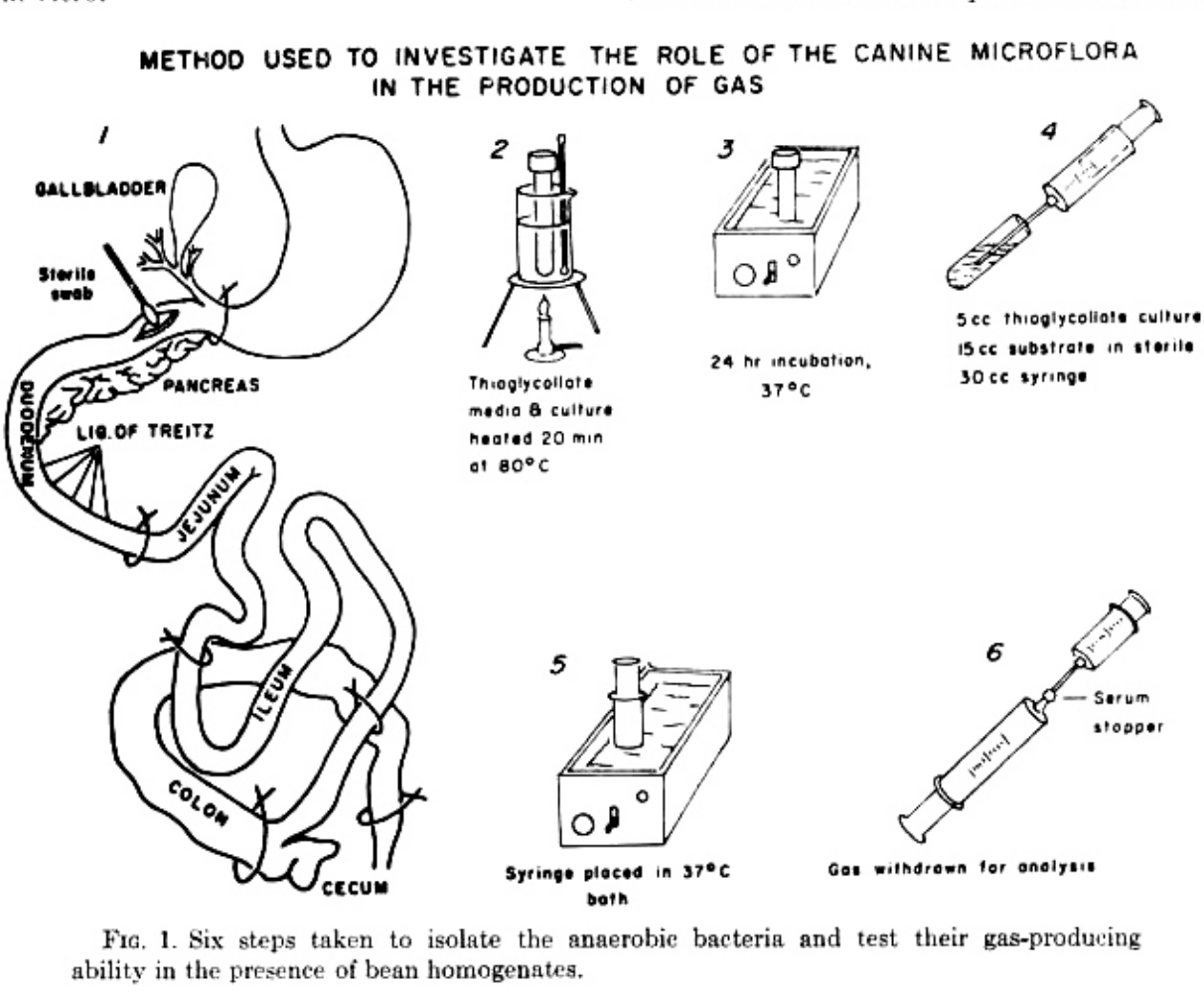

FIG. 1. Six steps taken to isolate the anaerobic bacteria and test their gas-producing ability in the presence of bean homogenates.

To these substrates in separate syringes, 5 cc of the stock thioglycollate anaerobic bacterial culture were added, and sterile serum stoppers were placed over the tips. The contents were mixed by rolling the syringe between the palms of the hands; the syringe was then immersed in a 37 C water bath with the plunger directed upward (fig. 1, *step 5*). The initial time of gas production from each substrate was noted and the volume of gas produced per hour was measured for 4 hr. Gas samples, obtained by inserting a sterile 20-gauge needle attached to a 20-cc oiled syringe through the serum stopper, were analyzed for CO_2, O_2, N_2, and H_2 with a Fisher-Hamilton gas partitioner (fig. 1, *step 6*).

To correlate the effect of inserting bean products into the intestinal loops, in vivo, with the anaerobic flora activity cultured in vitro, the following procedure was used. Each isolated intestinal segment was fitted with two 3-inch cannulae, made from ½-inch flexible Tygon tubing. One cannula was inserted and ligated in position through the 1-cm incision previously made in the upper end of each segment, and the other through an incision at the opposite end. With this preparation bean slurries could be introduced through the upper cannula and any air or intestinal contents in the segment expelled through the lower cannula. Fifty cubic centimeters of the previously described homogenate were injected into each segment, after which all of the cannulae outlets were closed and the segments were returned to the abdominal cavity, which was also closed. After 3 hr the food samples were removed from each segment and cultures were taken from the mucosal surface in the manner previously described. In the presentation of the data, the culture taken prior to the introduction of the homogenate is referred to as the initial culture and the culture taken 3 hr after the homogenate had been introduced as the terminal culture.

Next, to note whether inhibition of gas production could be produced in the anaerobic bacteria cultures previously removed from the isolated segments, experiments were performed in which either 50 or 100 mg of a combination of Neomycin and sulfathalidine were added to the culture medium just before a 5-cc sample of the culture medium was added to the 15-cc sample of the bean substrate. Then the mixture was incubated at 37 C as described above. The cultures in these experiments were taken from the mucosa after each segment had been exposed to a navy bean homogenate for 3 hr, and are referred to as terminal cultures.

To record whether the gas-producing activity of the anaerobic bacteria could be inhibited in vivo as well as in vitro, a number of animals were pretreated with a bacteriostat, Vioform. A dose of 250 or 500 mg of the drug was given orally four times a day for 2 days prior to the experiment. These cultures, as in the Neomycin and sulfathalidine experiments, were taken after the mucosa of the segments had been exposed to the bean homogenate for 3 hr.

To test the effectiveness of cultures previously exposed to soybean substrates and their sensitivity to combinations of Neomycin and sulfathalidine, the different intestinal and colon segments were injected with 50 cc of a homogenate containing soybean meal. After 3 hr, isolated cultures were prepared and tested for in vitro gas production as previously described. Cultures without antibiotics were compared with those containing antibiotic combinations of 50 mg of Neomycin and 50 mg of sulfathalidine.

Furthermore, to demonstrate whether the cultures originally taken from the mucosal surface and developed in vitro were mainly of the anaerobic clostridia type the following procedure was followed. Cultures from the mucosa of the same ileum and colon segments were taken before (initial) and after (terminal) soybean homogenates had been in contact with the segments for 6 hr. The cultures were first transferred to a thioglycollate medium and heated at 80 C for 20 min. Then, after 10-fold serial dilution, a sample was plated out on a thioglycollate agar medium, incubated for 24 hr, and then counted for anaerobic clostridia spore-forming colonies. The counting was done with a Quebec colony counter with illuminated grid and magnifying glass attachments.

Results

All experimental results relating to gas production under various conditions are summarized in plate 1. As noted in section 1, the anaerobic (initial) cultures taken from the four different intestinal segments, produced gas when mixed with a navy bean substrate. All results represent a mean of 5 different animal experiments. Section 1 also shows the comparative in vitro gas production of the anaerobic (terminal) bacteria after exposure to a navy bean substrate in the intestinal segments for 3 hr. The initial cultures from the small intestinal segments produced considerably less gas than the terminal cultures. The initial cultures produced on an average hourly basis: duodenum, 1.8 cc; jejunum, 2.0 cc; ileum, 4.3 cc; and colon, 11.9 cc of gas. Terminal cultures, taken after the anaerobic bacteria had grown on a navy bean homogenate for 3 hr, showed an average hourly gas production of: 9.2 cc for duodenum; 4.5 cc for jejunum; 9.7 cc for ileum; and 10.8 cc for colon. The gaseous composition of the collected samples was approximately 40% CO_2, 53% H_2, 6% N_2, and 2% O_2. The percentage composition of the gases produced did not vary from segment to segment, whether the cultures were taken before or after the navy bean homogenate had been present in the segment for 3 hr.

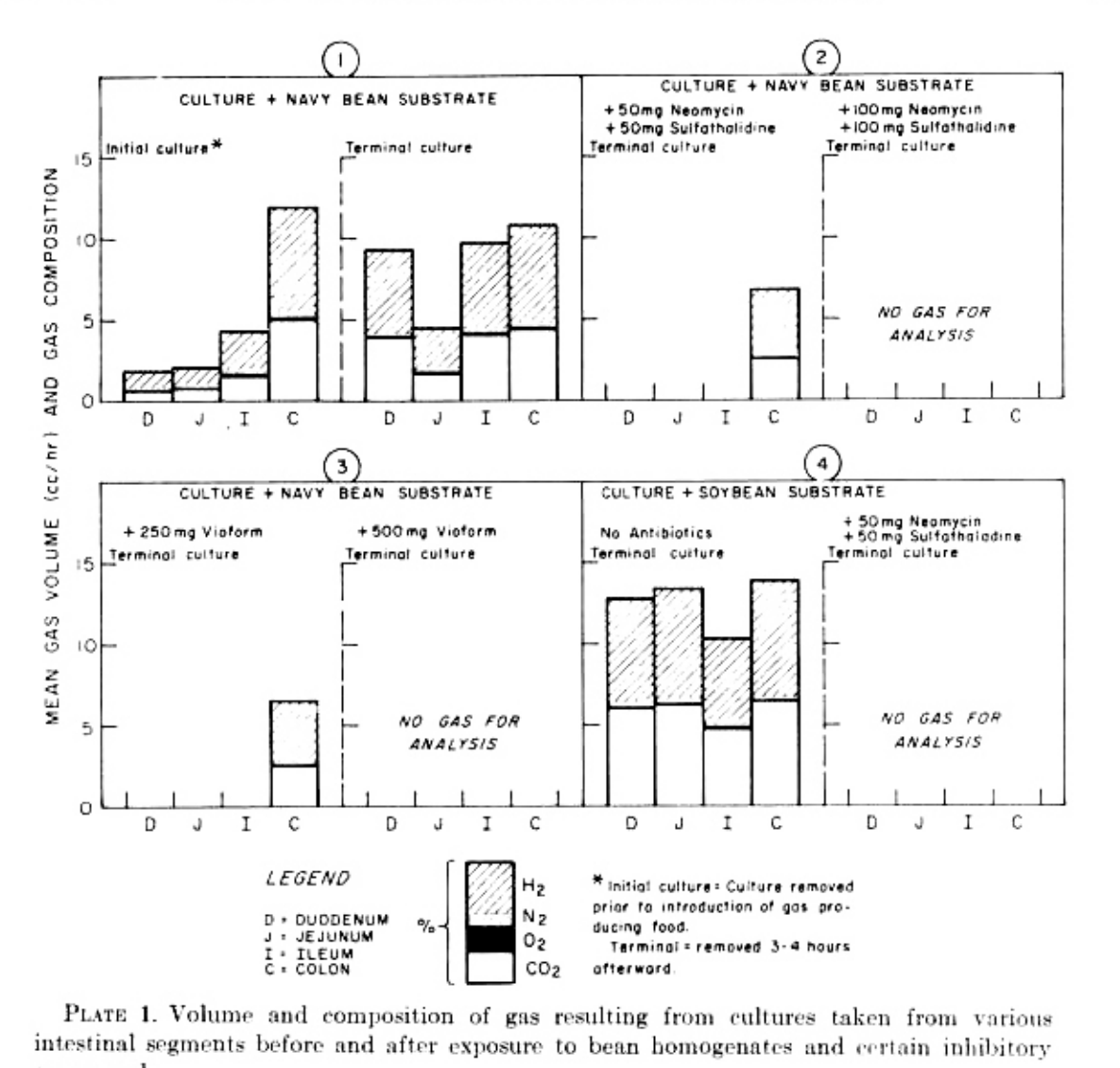

PLATE 1. Volume and composition of gas resulting from cultures taken from various intestinal segments before and after exposure to bean homogenates and certain inhibitory compounds.

Although the gaseous volume produced by the anaerobic bacteria from the different segments is expressed as an average in cubic centimeters per hour in the bar graph, there was a time lag of approximately 1 hr after the addition of the anaerobic culture to the navy bean substrate before gas production began. It was observed that gas production continued for considerably longer periods of time (12 hr or more) than the 4-hr test or collection period in these experiments. The data obtained, however, give specific information as to the relationship between anaerobic bacteria and gas-producing substrates in vitro. The gas production from the initial and terminal colon cultures was approximately the same.

TABLE 1. *Effects of soybean homogenates in the ileum and colon segments of the dog on the count of heat-resistant spore-forming anaerobic bacteria*

Segment of intestine	Media	Expected type of organism present	Colony count after 24-hr incubation: Before soybean injection (initial)	Colony count after 24-hr incubation: 6 hr after soybean injection (terminal)
Ileum	Thioglycolate	Anaerobic (spore formers)	40	398,000
Colon	Thioglycolate	Anaerobic (spore formers)	20	2,312

Section 2 (plate 1) shows the in vitro inhibiting effects of mixtures of Neomycin and sulfathalidine, and section 3 (plate 1) the in vivo effect of Vioform on the gas-producing ability of anaerobic bacteria. When the smaller doses (50 mg) of the drugs were used the gas production of the microorganisms was inhibited completely in cultures taken from the small intestine, but not in those taken from the colon. This was true both in vitro and in vivo. In experiments in which the doses of the antibiotics or bacteriostatic agent were doubled, complete inhibition of gas production was observed in both the small intestine and the colon incubated with navy bean homogenates for 3 hr.

Section 4 (plate 1) shows the reaction of the anaerobic bacteria on commercial dog food containing high concentrations of soybean meal. Bacterial cultures taken from the segments exposed to soybean meal homogenates for 3 hr and then cultured in vitro on a sterile soybean substrate were very active gas producers. The average volumes of gas per hour were: duodenum, 12.7 cc; jejunum, 13.5 cc; ileum, 10.2 cc; and colon, 14.0 cc. These data show that the soybean substrate produced more gas than the navy bean substrate. Although CO_2 concentration was on an average slightly higher, the distribution of the various gases present was not significantly different.

This series of experiments also shows that the soybean gas production was completely inhibited by 50 mg of Neomycin and sulfathalidine, whereas 100 mg of each drug were required to produce the same degree of inhibition with the navy bean experiments. These differences may be due to differences in the chemical composition of the two bean products.

The results shown in table 1 support the hypothesis that the difference in gas production in the above experiments could be associated with increased numbers of anaerobic organisms produced in the presence of soybean homogenates. In these experiments a comparison of the count of what is thought to be true spore-forming anaerobic organisms was made before and after ileum and colon segments were exposed to soybean homogenates for 6 hr.

Although the data in table 1 must be considered only preliminary, the marked difference in the numbers of colonies in the ileum and colon segments after exposure to soybean homogenates leaves little doubt that tremendous changes in the intestinal flora can occur at a very rapid rate upon consumption of gas-producing foods.

Discussion

The above results lend indirect support to the hypothesis that, when bean products are introduced into the gastrointestinal tract, the resulting increase in gas production and its high concentrations of carbon dioxide and hydrogen may be caused by the interaction of some constituent in the bean and certain anaerobic bacteria inhabiting the small and large intestine. That the reaction may be associated with the carbohydrates in the bean was suggested by Anderson[17] as early as 1924, when he demonstrated that the primary gases evolved in the course of anaerobic cultivation on various carbohydrate media were high percentages of carbon dioxide and hydrogen and low percentages of nitrogen and oxygen.

This possibility is supported by the observations of Steggerda et al.[18] that, when various fractions of soybean meals were consumed by human subjects, the low molecular weight carbohydrate fractions were especially potent in their gas-producing ability as compared with the fat, protein, and complex polysaccharide fractions. It is possible that there are a number of different anaerobic bacteria present in the intestine and colon that could contribute to the observed gas production but, considering the manner in which the cultures were grown and heat-treated, and the observed characteristic gas production (i.e., both amount and composition), it is believed that the organisms responsible are the same as or closely related to the gram-positive *Clostridium perfringens* type. In preliminary experiments pure cultures of *C. perfringens* exposed to bean homogenates produced gas similar in volume and composition to that observed in the above experiments. Page et al.,[19] in a number of reactions between glucose and *C. perfringens,* noted, along with a large number of different enzyme systems, a glycolytic enzyme system that is similar to the Embden-Meyerhof-Parnas pathway. Also, that the reaction involves some type of organism present in the intestine is supported by the inhibition of the reaction antibiotic and bacteriostatic agents.

The combination of Neomycin and sulfathalidine was selected because of the marked inhibition in the bacteria flora observed by Waksman[20] in patients treated with these agents; the bacteriostatic compound Vioform was used because it was found in earlier experiments to be the active principle in a compound, Mexaform, which caused an inhibition of gas production in human subjects on diets high in pork and beans.[21] The manner in which this inhibiting effect is produced is not well understood. Waksman et al.[22] observed that Neomycin does not specifically inhibit the true *Clostridium* spore-forming type of bacteria. They found, however, that coliform bacteria and possibly the vegetative bacteria, one of the suggested sources of the spore formers, are inhibited by the antibiotic. Sulfathalidine is known to produce marked decreases in the coliform and clostridial counts, and Spaulding et al.[23] suggested that certain advantages may accrue from the combined use of streptomycin and sulfathalidine. Eisman et al.[24] suggested that Vioform may act via the *Escherichia coli* which are said to increase markedly in number and thereby reduce the effectiveness of other bacteria present.

Kakade and Borchers[25] reported on the effects of feeding rats raw navy beans with and without added amounts of procaine penicillin and streptomycin; they found a reduction in gas production under certain conditions. That the clostridia anaerobic organisms normally reside in the gastrointestinal tract of the dog was shown by Bornside and Cohn,[26] who reported that the count for clostridia in 85% to 90% of their dogs ranged from 10^2 to 10^8 per g of the intestinal or colon contents. The *C. perfringens* that these investigators isolated from the stomach, ileum, and rectum of the dog was a gram-positive spore former.

We were surprised that cultures taken from intestinal segments that had been exposed to either navy or soybean homogenates for only 3 hr produced consistently higher amounts of gas than cultures taken before the administration of the homogenates (plate 1, section 1). That this can be caused by a stimulation of growth and number of the organisms is indicated by the results presented in table 1.

An increase of this type in the intestinal flora has previously been observed by Goldsmith,[27] who reported on the effects of dietary changes, and Cohn and Bronside,[28] who observed that intestinal strangulation can cause the bacterial composition to increase 4- or 5-fold within 6 hr. They reported that the clostridia organisms can show marked increases in count in relatively short time intervals.

The increase of gas produced by the colon segment was not so great as that observed in the small intestinal segments after the injection of navy bean homogenates (plate 1, section 1). Although the cause for this difference is not known, a similar difference between colon and ileum was found after soybean homogenate injections (table 1).

This investigation suggests that there are specific intestinal anaerobic bacteria associated with the formation of intestinal gases in the presence of bean products. The behavior of the anaerobic bacteria is very similar to that of the gram-positive, spore-forming clostridia perfringens group which usually inhabit the gastro intestinal tract of man and dogs. The in vitro and in vivo responses to bacteria-inhibiting agents were similar; thus they support the relationship of the in vitro experiments to conditions existing in the intact intestine and colon. Moreover, the increased number of anaerobic spore-forming organisms in the intestine following the injection of bean products is reflected in the in vitro cultures and gas production. This investigative procedure is easily performed and lends itself effectively to the screening of food fractions for gas-producing properties.

Summary

1. In vivo and in vitro tests indicated that certain anaerobic bacteria in the small and large intestine are closely related to gas production in the presence of navy bean and soybean products.
2. The characteristic high carbon dioxide and hydrogen content of the gas produced in the in vitro experiments gives support to the assumption that the gas production is closely related to fermentation by the clostridia type of bacteria.
3. Gas production in the presence of bean homogenates was completely inhibited with antibiotic and bacteriostatic agents.
4. After injection of gas-producing bean products into intestinal loops the numbers of anaerobic spore-forming organisms present on mucosal samples of the ileum and colon were markedly increased.
5. The methods used in this study can be used effectively for detecting the presence of gas-producing factors in foods.

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epithelium, thickening of the lamina propria, and accumulation of neutrophils and cellular debris in crypts. Other tissues were not examined microscopically.

Microbiologic Findings

A 10% suspension of food samples derived from cages of affected animals were diluted in a reduced broth[6] and plated on solid medium.[7] The plates were incubated anaerobically at 37°C for 18 – 24 hours and characteristic black clostridial colonies enumerated. Clostridia (less than 10 organisms/g) were observed in only three of 20 samples of the partially consumed canned rations examined and were not observed in any of the liquid rations.

Gastric contents from 25 animals were processed for viral agents by mixing approximately 1 ml material into 9 ml brain-heart infusion broth containing 750 units/ml penicillin, 750 mcg/ml streptomycin, and 20 units/ml mycostatin. The specimens were placed on ice, transported to another laboratory[8] where they were centrifuged (1,000 gravities 20 minutes). The supernatant and 10- and 100-fold dilutions of the supernatant in Hanks balanced salt solution were processed for virus isolation on human fibroblast (AW995), squirrel monkey ($LL\text{-}MK_4$) cell lines, baboon kidney, and marmoset lung primary cell cultures. Viral organisms were not detected.

Additionally, the gastric contents were gram stained, a portion placed in tetrathionate pre-enrichment broth, and a portion streaked onto two blood agar plates, one plate of differential medium,[9] and one egg yolk agar plate. One blood agar plate and the egg yolk agar plate were incubated anaerobically[10] at 37°C overnight. Tetrathionate broth and the other media were incubated aerobically at 37°C overnight. After overnight incubation, tetrathionate broth was streaked onto differential medium for overnight incubation at 37°C. Colonies on egg yolk agar plates surrounded by diffuse opalescence were subcultured on lactose egg yolk-half antitoxin agar plates for further anaerobic incubation. *Clostridium perfringens* Type A was recovered from gastric contents of 25 animals. Approximately 10^5 sporeforming organisms/ml gastric material were recovered from five samples subjected to enumeration studies.

[6]Reinforced Clostridial Broth®. Consolidated Laboratories, Chicago, IL.
[7]SPS®. Difco Laboratories, Detroit, MI.
[8]Southwest Foundation For Research & Education, San Antonio, TX.
[9]XLD®. Baltimore Biological Laboratories, Baltimore, MD.
[10]GasPak® System. Baltimore Biological Laboratories, Baltimore, MD.

Discussion

Under ordinary circumstances, animals such as marmosets which consume only small portions of food at a time are less likely to develop acute gastric dilatation than are animals which may be prone to overconsume. However, factors which alter the usual activities or relative composition of the animal's gastric flora may also play an important role in acute gastric dilatation. Thus, situations which contribute to the development of a gastric flora which thrives on carbohydrates and develops unusually large numbers of aerogenic organisms would likely predispose the individual to acute gastric dilatation.

Clostridium perfringens Type A was recovered from gastric contents of the 25 animals subjected to necropsy. Clostridia could not be consistently recovered from the food, yet high numbers of organisms (approximately 10^5 organisms/ml) were observed in gastric contents of the animals subjected to enumeration studies. These findings suggest that the organisms proliferated in the intestinal tracts of affected animals. The origin of the observed clostridia is not known. Clostridia constitute a minor portion of the normal flora of many species of monkeys, and under certain circumstances, these organisms could proliferate to high levels (4,5). The role of *C perfringens* as a primary etiological agent in acute gastric dilatation is suggested by the finding of clostridia as the only gas-producing bacteria recoverable in gastric contents (1). The present outbreak possibly began when an antimicrobial regimen directed toward controlling a potential shigella epizootic selectively altered the gastric flora so as to predispose to proliferation of *C perfringens.*

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