Fractionation of Dry Bean Extracts Which Increase Carbon Dioxide Egestion in Human Flatus

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A series of successive chemical and physical fractionations of cooked dry California Small White beans were made, and the activity of each fraction was measured, using human subjects, for its ability to increase the carbon dioxide component of flatus. The activity was found to be: extractable with 60% aqueous ethanol; dialyzable through a reconstituted cellulose membrane; soluble in 85% ethanol; and unabsorbed by a column packed with a strong cation exchange resin. This active column fraction was shown to contain the sugars, fructose, sucrose, raffinose, stachyose, and at least four polypeptides hydrolyzable into 22 amino acids. Raffinose and stachyose fed alone at levels found in California Small White beans did not increase the carbon dioxide level of the flatus.

For centuries cooked dry beans have made a significant contribution to the protein requirements of different peoples of the world. The annual consumption of dry beans in the United States is steadily decreasing. This is not only unfavorable for the American farmer but is undesirable in keeping with the concept of maintaining "balanced nutrition" by mixing protein from different food sources in the daily diet. But, deeply imbedded in our cultural knowledge is the fact that beans cause gas. For those people who are fortunate enough to eliminate the increased gas formed in the intestinal tract through respiration, this fact is only an amusing joke, but for a significant part of our population, eating beans in quantity leads to painful bloat with embarrassing intestinal rumblings and socially unacceptable egestion of noxious mixtures of gas.

It was proposed that an attempt be made to isolate and identify the factor(s) in cooked dry beans which causes flatulence and use the information gained to eliminate it through processing or genetic breeding. This would be done by a stepwise method of chemical and physical separations and after each operation, working with the fraction containing the major portion of the gas-forming activity as indicated by human feeding tests. Earlier investigation had shown that carbon dioxide was one of the major components of flatus from cooked dry beans. Its volume and percent composition measured in human flatus follows in identical pattern as the total flatus volume egested from a bean test meal (Murphy, 1963).

Steggerda *et al.* (1966) have tested a series of soybean fractions for their gas-forming properties using different techniques than those described in this paper.

MATERIALS AND METHODS

Dry beans (*Phaseolus vulgaris*), California Small White variety, were cooked for 2 hr in a steam kettle without prior soaking and lyophilized. The dry beans were ground in a Wiley Mill #1, Arthur H. Thomas Co., Philadelphia, Pa., with a 2-mm screen and stored at −10°C.

Fat Extraction. The dry bean powder was weighed in tared 60 × 180-mm extraction thimbles, Whatman Co., and placed in Soxhlet extractors containing diethyl ether and extractions were continued for 6 hr. The thimbles were then removed and dried to constant weight in a vacuum desiccator. The diethyl ether containing the fat-soluble fraction of the beans was reduced in volume by vacuum distillation at 40°C and stored at −10°C.

Alcohol Extraction. Sixteen-hundred grams of ether-extracted powder was added to a stirred 12-l. round-bottomed flask containing 6 l. of 60% aqueous ethanol. After the dry bean powder had been added, the temperature was maintained at 60°C for 4 hr. The alcohol extract was siphoned off by vacuum and this procedure was repeated for a total of twelve extractions. The alcohol extracts were reduced to a low volume and alcohol content in a rotary evaporator under vacuum. The extracts were combined, lyophilized, and weighed. Both the alcohol extract and residue were stored at −10°C. Extractions with 80 and 40% aqueous ethanol were made by this same procedure.

Dialysis. Four-hundred grams of the 60% ethanol extract was redissolved in 2 l. of distilled water and dialyzed in reconstituted cellulose casing, size 21, Visking Corp., Chicago, Ill., against distilled water. The alcoholic extract was circulated with a peristaltic pump in a continuous system connected with $^1/_4$-in., i.d., Tygon plastic tubing to 50 ft of cellulose casing coiled in a stainless steel tank containing 20 l. of distilled water. A 2-l. glass bottle was included in the system as a "surge tank" and as a point of initial introduction of the solution of alcoholic bean extract. The alcoholic extract was dialyzed for 4 hr against each of three changes of 20 l. of distilled water. The diffusates were combined, frozen, and lyophilized. The dialyzate was removed from the cellulose casing, frozen, and lyophilized. Both diffusate and dialyzate were weighed and stored at −10°C.

Precipitation with 85% Alcohol. Fifty grams of the diffusate was completely dissolved in 700 ml of 15% aqueous ethanol. Into this solution of diffusate was poured with vigorous stirring 3371 ml of 100% ethanol to bring the final alcohol concentration to 85% by volume. After standing overnight at 34°F, the supernatant was decanted. This procedure was repeated for a total of three precipitations. The supernatants were combined and reduced to a low volume and alcohol content, frozen, and lyophilized. The precipitates were dissolved in distilled water, frozen, and lyophilized. Both the dry supernatant and the dry precipitates were weighed and stored at −10°C.

Ion Exchange Column. A glass column, 10-cm × 200-cm was packed with 5 lb of Dowex, 50-4X, 50–100 mesh, cation exchange resin and recycled to the hydrogen form. One-

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hundred-fifty grams of the 85% ethanol supernatant was dissolved in 1500 ml of distilled water and placed on the column. The solution was washed into the column bed with 100-ml aliquots of distilled water. The nonexchanging or "through" fraction was eluted from the column with 30 l. of distilled water. The exchanged or "retained" fraction was eluted from the resin column with 20 l. of 1 *N* NH_4OH. Both through and retained fractions were reduced in volume in a rotary evaporator under vacuum, lyophilized, weighed, and stored at −10°C.

Flatus Measurement. The test subject used for flatus measurements in this study had no prior history of food allergy or gastrointestinal disorder. Flatus carbon dioxide measurements were made after nonflatulent and flatulent control meals were fed the test subject for comparison with carbon dioxide measurements made after feeding bean fractions. The low gas-forming control was the breakfast meal normally eaten by this subject, which excluded known gas-forming foods, and was the same meal throughout the testing of the bean fractions. It consisted of two fried eggs, bacon, toast, and coffee and was fed at 0800 hr, followed within 15 min with ingestion of the test bean fraction. Each of the sugars tested was weighed into gelatin capsules, size 000, and fed the test subject immediately after the non-gas-forming breakfast control meal. The gas-forming control, cooked California Small White beans, 100 g dry weight, was fed periodically throughout the 2 yr required for these studies to evaluate the flatus response of the test subject.

Flatus was collected rectally with a 6-in. plastic tube, female slip pipe, tapered $^1/_4$-in. to $^3/_8$-in. o.d. and perforated for the first 4 in. from its distal tip. It was connected by 50 cm of $^1/_4$-in i.d. thin-wall Tygon plastic tubing to a drying tube (Schwartz), 100 mm, filled with anhydrous magnesium perchlorate, Anhydrone, to absorb water vapor from the flatus. This drying tube was connected with 3 in. of plastic tubing to a second Schwartz drying tube filled with sodium hydroxide on asbestos (Ascarite, 20–30 mesh) for the quantitative absorption of carbon dioxide from the flatus. This second drying tube was connected by 3 in. of plastic tubing to a 4-in. straight drying tube filled with soda lime, 8 mesh, which was open to the atmosphere to vent the remainder of the unabsorbed flatus gases and to prevent back absorption of atmospheric moisture and carbon dioxide.

After ingesting the test meal the subject placed the flatus collection probe in his rectum, passed the plastic tube through a vent in his laboratory coat, connected it to the absorption tubes, and placed the system coiled up in a side coat pocket so as to stay ambulatory. Every 30 min for a period of 8 hr after ingestion of the test meal, the Ascarite carbon dioxide absorption tube was detached from the system and weighed to 0.0001 g on an analytical balance. The gain in weight of the Ascarite tube divided by the weight of carbon dioxide per cubic centimeter, 0.00197 g, gave the volume of carbon dioxide egested rectally in the flatus of the test subject during the 30-min measurement period. The volume of flatus carbon dioxide was calculated by the following relationship:

$$\text{ml } CO_2 = \frac{W_2 - W_1}{W_3} \simeq \frac{W_2 - W_1}{0.002}$$

where W_1 = weight of the Ascarite tube at the beginning of the 30-min measurement period; W_2 = weight of the Ascarite tube at the end of the 30-min measurement period; and, W_3 = weight of 1 ml of CO_2 at S.T.P. The same Ascarite absorption tube was used for the total measurement period.

The pH values were measured with a Beckman pH meter, Model 6, on 2% solutions of the bean fractions dissolved in ethanol–water (1:20).

The carbohydrates in the through fraction were identified by chromatography (Block, 1952b) of known sugars and aliquots of a solution of the bean fraction on Whatman No. 1 chromatographic paper in the solvent system, *n*-butanol–pyridine–H_2O (6:4:3, v/v). After development and drying the sugars were made visible with an ammoniacal silver nitrate spray.

Electrophoresis (Smithies, 1955) of the through fraction was done on starch gel in a citric acid buffer, pH 8.2. The peptide bands were made visible with Amido-Swartz 10B dissolved in methanol–water–acetic acid (50:50:10, v/v).

The identification of the amino acids after hydrolysis of the through fraction by heating with 6 *N* HCl was done by two-dimensional chromatography (Block, 1952a) and an automatic amino acid analyzer (Spinco Model 120) (Stein and Moore, 1951). The through and retained fractions from the cation exchange column were hydrolyzed and then chromatographed with an authentic sample of *S*-methylcysteine. Two-dimensional paper chromatograms were developed in the solvent systems methanol–pyridine–water and collidine–lutidine–water, and the amino acids were compared by their colors produced by ninhydrin and platinum iodide sprays by the method of Rinderknecht (1958).

A Lieberman-Buschard test, Gilman (1950), for saponin compounds was made on the through and retained fractions by adding to a solution of the fraction on a spot plate, ten drops of concentrated H_2SO_4 and five drops of acetic anhydride.

RESULTS

In Figure 1 can be seen the increases in volumes of egested flatus after a test meal of cooked dry beans as compared to the flatus volume measured after a control meal. The changes in volume of the carbon dioxide component of flatus correspond to changes in the total volume of flatus.

Figure 2 represents the successive steps in fractionation of the whole bean in an attempt to isolate the carbon dioxide producing factor(s) in California Small White beans. The

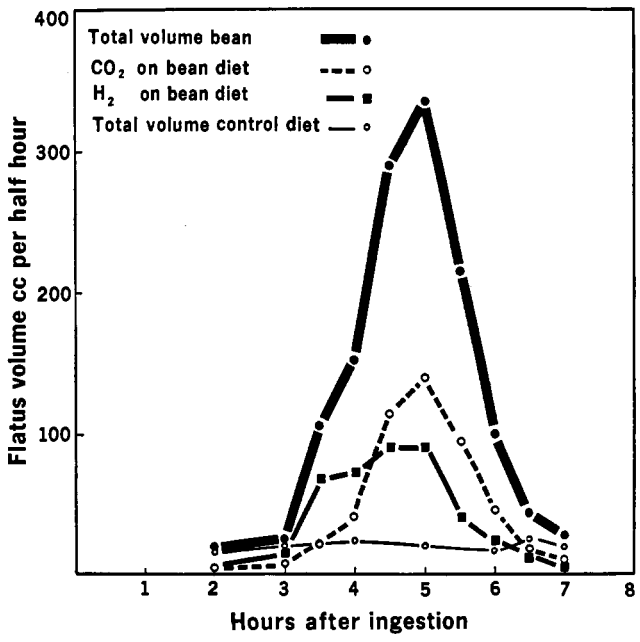

Figure 1. Flatus composition and volume

Table I. Percent Fractionation and Chemical Composition

Fraction	Fractionation, %	Whole[a] bean, %	pH	Nitrogen, %	Total[c] sugar	Sulfur, %
Cooked whole bean	...		6.2			
Ether extract	1.5	1.5	5.5	0.27	0.2	...
residue	98.5	98.5	...	...	...	...
80% Ethanol extract[c]	7.3	7.2	...	3.36	34.8	...
residue	92.7	91.3	...	2.85	3.7	...
60% Ethanol extract[b]	28.5	28.1	6.2	4.32	28.3	0.93
residue	71.5	70.4	6.5	3.71	0.2	1.46
60% Ethanol extract[b]	34.7	34.2	...	4.63	17.6	0.54
residue	65.3	64.3	...	3.54	<0.1	0.17
40% Ethanol extract[c]	24.8	24.4	...	4.49	13.4	...
residue	75.2	74.1	...	4.08	0.3	...
Diffusate	51.7	14.5	5.3	2.93	31	0.75
Dialysate	48.3	13.6	6.8	3.89	4.8	0.42
85% Ethanol supernatant	61	8.8	6.4	3.37	41.0	0.97
precipitate	39	5.7	5.5	2.11	4.2	1.43
Dowex 50 Column Through	70.2	6.2	...	1.6	90.9	0.06
Retained	29.8	2.6	...	11.3	1.7	2.75

[a] Average values adjusted for mechanical loses. Twelve extractions. [b] Six extractions.

Table II. Carbon Dioxide Activity of Bean Fractions

Fraction	Total CO_2, ml (4–7 hr)	% of Fractionation activity	Wt (g) test meal	Specific activity, $cm^3 \cdot CO_2/g$	Flatulence index CO_2 test/ CO_2 control
Control	26.9	...	...	...	1
Whole bean	257	...	100	2.57	9.6
Ether extract	10.5	...	1.5	6.56	0.38
Whole bean	278.9	...	100	2.79	10.4
80% Ethanol extract	176.4	45	7.3	24.16	6.6
residue	218.8	55	91.4	2.38	8.1
Whole bean	296.7	...	100	2.97	11.0
50% Ethanol extract	167.7	80	30	5.59	6.2
residue	42.0	20	70	2.1	1.6
60% Ethanol extract	129.8	93.5	28	4.66	4.8
residue	9.0	6.5	70	0.13	0.33
50% Ethanol extract	354.7	89.8	24.8	14.3	13.2
residue	40.1	10.2	75.2	0.53	1.5
Diffusate	259.9	81.9	18.5	14.0	9.7
Dialysate	57.2	18.1	11.2	5.1	2.1
Whole bean	264.8	...	100	2.65	9.8
60% Ethanol supernatant	570.8	80.4	13.8	41.5	21.2
precipitate	138.5	19.6	7.8	17.8	5.1
Whole bean	251.5	...	100	2.51	9.3
Dowex 50 Through	305.7	88.4	7.2	42.5	11.4
Retained	48.3	11.6	2.5	19.3	1.8

activity is not extractable with diethyl ether, can be extracted with 60% aqueous ethanol, dialyzes through a reconstituted cellulose membrane, is soluble in 85% ethanol, and is not retained on a strong cation exchange resin. The figures in brackets are the percentage of the original whole bean represented by each fraction.

In Table I are the yields and chemical compositions of the bean fractions. In the left-hand column are the average percentage figures representing the degree of separation obtained in the specific operation tested. In the next column to the right these fractionation percentages are adjusted to represent the fraction percent of the whole bean. In the final fractionation step on the ion exchange column the chemical compositions of the fractions indicate that the nitrogen and sulfur content is higher in the retained fraction while total sugar is higher in the through fraction. The percent total sugar increases as the fractionation of the bean proceeds stepwise.

In Table II are the total volumes of carbon dioxide absorbed from the flatus for a 3-hr period 4 to 7 hr after each bean

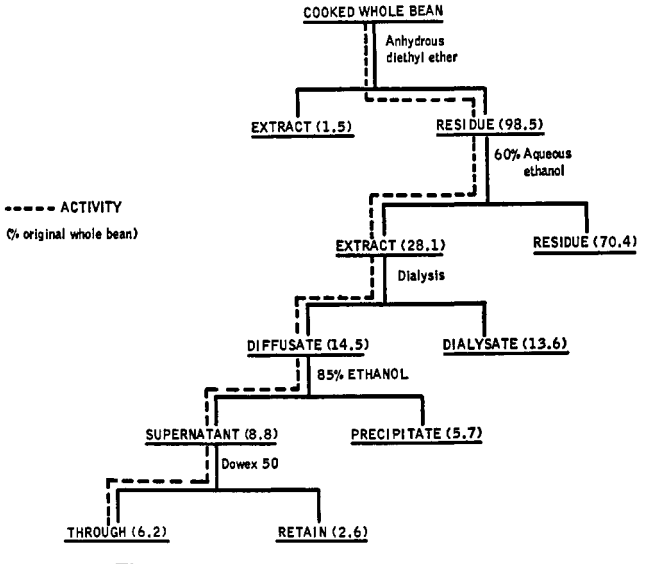

Figure 2. Flow chart of flatulence activity

Table III. Carbon Dioxide Activity of Bean Sugars

Wt (g) test meal	Total C_2O, ml (4–7 hr)	Flatulence index CO_2 test/CO_2 control
Beans[a] 100	345	35.4
Control	9.75	1
Fructose 2	11.2	1.2
Raffinose 2	14.4	1.5
Stachyose 1	38.5	3.9
2	20.4	2.1
4	56.1	5.8

[a] Canned baked beans, S&W, 1-lb can, 100 g dry weight.

fraction was ingested. In the second column are the percentages representing the extent to which the carbon dioxide activity was fractioned by each separation step. The third column gives the weight of the bean fraction fed. This weight was calculated to be the weight of that fraction present in 100 g of the original beans. The "specific activities" are calculated for each fraction based on cubic centimeters of carbon dioxide produced per gram of fraction fed. The "specific activity" is not a true value since the mechanism of flatulence is still unknown and hydrogen, the other major gaseous "reaction" product, was not measured. Therefore, it is offered as another means of expressing activity to be used as a guide in judging the degree of flatulence. The "specific activity" increases from 2.57 g for whole bean to 42.5 g for the through fraction. The "flatulence index" is the ratio of the volume of carbon dioxide collected on the test meal to the volume of carbon dioxide on the control meal.

In Table III are a series of carbon dioxide measurements for a series of sugars. The gas-forming control used in this series of measurements was canned baked beans and since this was different from the cooked dry white beans used as a control in the case of the bean fractions, a new nonflatulent control value, 9.75 cm^3, was measured on the subject in this series. The volumes of carbon dioxide collected after each sugar are relatively near bland control level compared to the carbon dioxide volumes egested by this subject after a test meal of canned baked beans.

By chromatography with known sugars, stachyose, raffinose, sucrose, and fructose were shown to be present in the through fraction of the cation exchange column. Only stachyose and raffinose were present in significant amounts in the alcoholic extract fraction. Therefore most of the sucrose and fructose are probably artifacts produced in the fractionation.

Electrophoresis of the through fraction and staining showed four distinct peptide bands. After hydrolysis of the through fraction, chromatography showed 22 different amino acids present in the mixture.

When authentic *S*-methylcysteine was added to the hydrolyzed mixture of the through fraction and chromatographed in two dimensions in methanol–pyridine–water and collidine–lutidine–water a new spot appeared, R_f 0.62:0.35. It was ninhydrin positive on one 2D chromatogram and platinum iodide positive on another which had been developed in an identical manner. This spot was absent from the chromatogram of the hydrolyzed mixture of the through fractions without adding *S*-methylcysteine.

The Lieberman-Buschard test for saponin compounds by producing a pink-red color gave no change in the case of the through fraction and a questionable pink color with the retained fraction.

DISCUSSION

Following a bland diet, the flatus of a healthy person contains 1–5% carbon dioxide. It can arise in the intestinal tract as a product of microbial metabolism, from the carbonates of food and intestinal secretions, and by diffusion into the lumen from the blood. Its concentration in the gut is normally kept below blood level by continuous dilution with swallowed air and elimination as a component of flatus. However, after a flatulent food such as cooked dry beans, carbon dioxide together with hydrogen suddenly become the major components of flatus. High concentrations of carbon dioxide in the flatus have been shown to correlate with the consumption of cooked dry beans (Murphy, 1963; Steggerda and Dimmick, 1966). In our laboratory carbon dioxide is frequently measured as 60–70% of the total flatus volume during a period of maximum flatus egestion following a test bean meal. Carbon dioxide absorption on Ascarite is the basis of the direct combustion method for carbon in steel and carbon–hydrogen determination in organic compounds. This is the principle of the simple apparatus worn coiled up in a laboratory coat pocket of the ambulatory test subject in our measurements of carbon dioxide in egested flatus.

In Figure 1, the level of total flatus remains the same as with the control diet for 3 hr after ingestion of the bean test meal. It increases to a maximum volume at 5 hr and returns to control level by 7 hr after the bean meal. Both carbon dioxide and hydrogen follow a similar pattern, with maximum component volumes at 5 hr with this subject. No comparable period of increase in total volume of flatus egestion occurs after a bland control test meal. Thus, if the carbon dioxide component of the flatus is measured quantitatively during this 3-hr period for a series of test foods, their degree of flatulence is proportional to the total volume of carbon dioxide collected after each is ingested.

In the fractionation scheme, Figure 2, it was intended to isolate the flatulence activity, step by step, each time working on that fraction which proved to contain the major part of the activity, as shown by flatus carbon dioxide measurements after ingestion of the fraction. Each step was designed to make a specific chemical separation, such that after several successful fractionations had reduced the complexity, the chemical compound(s) in the active fraction could be associated with or identified as the cause of human flatulence from cooked dry beans. The beans were cooked as a starting material to eliminate heat labile compounds.

An aqueous alcohol extraction was made, Table II, to extract the carbohydrate fraction of the bean. As the percent of alcohol decreased from 80 to 40%, the sugar content of the extract fraction decreased and the weight and gelatinous colloidal nature of the alcohol extract increased. However, as the ratio of alcohol decreased, the effectiveness of the separation of the flatulence activity increased (Table II). To extract as much sugar as possible and to make filtration easier, while at the same time effectively fractionating the activity, 60% aqueous ethanol was used to make succeeding extractions; but it was necessary to go from 6 to 12 extractions to equal the effectiveness of 40% alcohol in separating the carbon dioxide activity.

About 50% by weight of the 60% ethanol extract dialyzed through a reconstituted cellulose membrane and contained the major portion of the carbohydrate (Table I). When 18.5 g of the diffusate was fed to a human subject it produced almost as much carbon dioxide in the flatus as a 100-g test meal of the original whole bean (Table II). This meant that the molecular diameter of the factor(s) in cooked dry

beans causing the increase in the carbon dioxide component of flatus during flatulence was equal to or smaller than 4.8 mμ, the average pore size of the membrane. The maximum molecular weight of the factor(s) was therefore 5000–6000, falling well below the large class of bean proteins which are commonly considered as above 10,000 in molecular weight. This does not eliminate the free amino acids, peptides, or complexes containing amino acids in peptide bonds whose molecular weights are low enough to diffuse.

After noting that organic solvents produced a precipitate when added to a water solution of the diffusate, precipitation by the addition of absolute ethanol produced a supernatant containing 61% of the weight, 41% sugar, and 80% of the activity as compared to the precipitate. Precipitation with acetone at this point also fractionated the diffusate in a ratio 36:64 by weight, but produced a supernatant with 45% of the carbon dioxide activity and a precipitate with 55% of the activity.

Seventy percent by weight of the ethanol supernatant passed through a chromatography column containing a strong cation exchange resin. Six-and-two-tenths grams of this through fraction produced 11.4 times as much carbon dioxide in the flatus as the control meal when fed to the test subject. It contained 88% of the carbon dioxide activity by comparison to the fraction retained by the resin.

Two commonly found high molecular weight oligosaccharides in legume seeds are raffinose and stachyose, a mono- and digalactosido-sucrose, respectively (Shallenberger, 1967). Because the human does not possess an α-galactosidase in the gastrointestinal tract and the sucrase or invertase that is present cannot hydrolyze substituted sucrose, man is unable to utilize the galactosido-sucrose series of sugars. Thus, the oligosaccharides escape digestion and absorption and are available for fermentation into carbon dioxide and hydrogen by the microorganisms of the gut. In the stepwise fractionation of the bean, the carbohydrate content had been concentrating in the fraction which always proved to have the majority of the carbon dioxide activity; and then stachyose and raffinose were identified in the active "through" fraction. These two oligosaccharides became prime candidates for the cause of the increase in carbon dioxide and hydrogen volume in dry bean flatulence. When these sugars were fed with a nonflatulent control meal at levels which exceed the level found in California Small White beans (raffinose 0.65%, stachyose 3.0%) (Lee *et al.*, 1970) there was no significant increase in carbon dioxide volume measured 4 to 7 hr after ingestion of the test meal as compared to the control meal (Table III). This agrees with the findings of Calloway and Murphy (1968) in which stachyose was fed to human subjects with a bland formula diet resulting in a significant rise in the hydrogen level of the breath but no increase in the level of egested flatus. Thus, these bean sugars make a major contribution to the hydrogen component of the breath and flatus during bean flatulence and the major factor(s) responsible for the increase in carbon dioxide volume remains unidentified.

Another possible candidate for the flatulence factor(s) has been γ-glutamyl-*S*-methylcysteine, since it was isolated in a significant level from California Small White beans by Rinderknecht (1958). Since many sulfur-containing compounds are potent carbonic anhydrase inhibitors, it was thought that this bean polypeptide might interfere with the normal rate of active transport of carbon dioxide through the gut wall and into the blood, thus raising the carbon dioxide level in the flatus after a bean meal. Schwimmer (1969) has offered a possible mechanism by suggesting that this polypeptide might contribute methyl mercaptan as a metabolic byproduct which he demonstrated as an inhibitor of carbonic anhydrase. However, both by two-dimensional paper chromatography and automatic amino acid analysis of authentic *S*-methylcysteine with a hydrolysate of the active through fraction, we were unable to demonstrate the presence of this amino acid in the carbon dioxide active fraction. This would be expected since Rinderknecht used retention of a Dowex 50 column in a similar manner as our fractionation technique in his original isolation of the bean peptide.

Another possible candidate was eliminated when the active through fraction gave a negative Lieberman-Buschard test to positive tests with authentic alfalfa saponins. The saponin compounds are widely distributed in the legume family and have been implicated in bloat problems in sheep and cattle, producing foaming, hyperemia, and increased motility of the intestine. Alcoholic extracts of California Small White beans containing bean saponins were reported by DeEds (1963) to produce similar conditions when injected into sections of rat intestine.

The flatulence response to a given weight of beans differs in volume and composition between subjects and is relatively constant within the same test subject. For example, a bean control meal was fed with each pair of bean fractions tested to evaluate this subject's flatulence response and the standard deviation was 270 ± 18, $n = 5$, over a 2-yr period. Since the gases responsible for bean flatulence are of microbiological origin, this is a reflection of the well established fact (Haenel, 1961; Speck, 1970) that the intestinal flora based on stool counts varies widely between people, while remaining remarkably constant for the same person. This permits the use of one test subject, who is representative and constant in flatulence response, to be used in a survey study such as this, while requiring a larger number of subjects in any flatulence evaluation for the general population. These data are presented with this qualification.

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