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Colonic Protein Fermentation and Promotion of Colon Carcinogenesis by Thermolyzed Casein

Denis E. Corpet, Ya Yin, Xue-Ming Zhang, Christian Rémésy, Denis Stamp, Alan Medline, Lilian Thompson, W. Robert Bruce, and Michael C. Archer

Abstract: *Thermolyzed casein is known to promote the growth of aberrant crypt foci (ACF) and colon cancer when it is fed to rats that have been initiated with azoxymethane. We speculated that the promotion was a consequence of increased colonic protein fermentation (i.e., that the thermolysis of the casein decreases its digestibility, increases the amount of protein reaching the colon, and increases colonic protein fermentation and that the potentially toxic products of this fermentation promote colon carcinogenesis). We found that the thermolysis of casein reduces its digestibility and increases colonic protein fermentation, as assessed by fecal ammonium and urinary phenol, cresol, and indol-3-ol. Thermolysis of two other proteins, soy and egg white protein, also increases colonic protein fermentation with increased fecal ammonia and urinary phenols, and thermolysis of all three proteins increases the levels of ammonia and butyric, valeric, and i-valeric acids in the cecal contents. We found, however, that the increased protein fermentation observed with thermolysis is not associated with promotion of colon carcinogenesis. With casein, the kinetics of protein fermentation with increasing thermolysis time are clearly different from the kinetics of promotion of ACF growth. The formation of the fermentation products was highest when the protein was thermolyzed for one hour, whereas promotion was highest for protein that had been thermolyzed for two or more hours. With soy and egg white, thermolysis increased colonic protein fermentation but did not promote colon carcinogenesis. Thus, although thermolysis of dietary casein increases colonic protein fermentation, products of this fermentation do not appear to be responsible for the promotion of colon carcinogenesis. Indeed, the results suggest that protein fermentation products do not play an important role in colon cancer promotion. (Nutr Cancer 23, 271-281, 1995)*

Introduction

Considerable attention has been given to the possible importance of macro- and micronutrients in the origin of colon cancer and less to the manner in which food is prepared (1-3). Experimental studies show, however, that diets that contain thermolyzed proteins, carbohydrates, or fats can markedly enhance colon carcinogenesis (4-8). In particular, casein that has been thermolyzed in a manner to simulate household cooking is known to promote the growth of aberrant crypt foci (ACF) and colon cancer (4,5). The mechanism responsible for this promotional effect is not known.

We hypothesized that thermolysis of the dietary protein could lead to increased colonic protein fermentation (9) and that products of this fermentation promote colon carcinogenesis. Thermolysis makes proteins less digestible as a consequence of cross-linking and the formation of unnatural amino acids (Reference 10, pp 332-338). As a result, more dietary protein could reach the large bowel, where it could be fermented by bacteria to yield fermentation products such as ammonia and a variety of phenols (11). Ammonia and phenol are toxic to mammalian cells and are known tumor promoters (12-14). Indeed, one phenol produced by the fermentation of tryptophan, indole-3-ol (indican), has been reported to initiate myelogenous leukemia in mice (15).

This colonic protein fermentation hypothesis could explain several known associations between diet and colon cancer. On the one hand, proteins in foods such as meats that are usually cooked might be expected to increase colonic protein fermentation. On the other hand, starches resistant to digestion will reach the colon and might be expected to increase colonic carbohydrate fermentation, increase the level of energy reaching the colonic bacteria, and reduce the level of protein fermentation (Ref. 11, p 80). Thus meat would increase the concentration of fermentation products, whereas fiber and starch would reduce their concentrations, and the hypothesis would explain a risk associated with meat consumption and a protection associated with foods containing starch.

In the present studies, colonic fermentation was assessed on the basis of fecal ammonia and urinary phenols and on the basis of the concentration of ammonia and branched short-chain fatty acids formed on in vitro incubation of the cecal contents. Colon cancer promotion was assessed with the use of ACF (8). ACF are putative precursors of colon cancer (16) that are induced by colon carcinogens (17). The growth of ACF as measured at 100 days correlates with the appearance of tumors at one year (5). The diets included casein that was thermolyzed at 180°C for one, two, and four hours and, in addition, soy and egg white protein thermolyzed for two hours.

D. E. Corpet is affiliated with the Université de Toulouse, ENVT Ecole Nationale Vétérinaire, UMR INRA Xenobiotiques, 31076 Toulouse, France. C. Rémésy is affiliated with the INRA Institute National de la Recherche Agronomique, Laboratoire Maladies Métaboliques, Theix, France. Y. Yin, X.-M. Zhang, D. Stamp, W. R. Bruce, and M. C. Archer were affiliated with the Ontario Cancer Institute, Toronto, Ontario M4X 1K9, Canada, when the work was carried out. A. Medline is affiliated with the Department of Pathology, University of Toronto, Toronto, Ontario M5S 1A8, Canada. L. Thompson, W. R. Bruce, and M. C. Archer are presently affiliated with the Department of Nutritional Sciences, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

Table 1. Experimental Groups and the Protein Component of Their Diets		
Group No.	No. of Animals	Protein Content of Diet, % by weight^a
<i>Digestibility of thermolyzed casein</i>		
1	4	27.5% Untreated casein
2	4	12.5% Untreated casein + 15% casein thermolyzed for 1 hr
3	4	12.5% Untreated casein + 15% casein thermolyzed for 2 hrs
4	4	12.5% Untreated casein + 15% casein thermolyzed for 4 hrs
5	4	27.5% Untreated casein replaced with 27.5% starch
<i>Colonic fermentation with thermolyzed casein</i>		
1	6	27.5% Untreated casein
2	7	12.5% Untreated casein + 15% casein thermolyzed for 1 hr
3	7	12.5% Untreated casein + 15% casein thermolyzed for 2 hrs
4	7	12.5% Untreated casein + 15% casein thermolyzed for 4 hrs
<i>Colonic promotion with thermolyzed casein</i>		
1	28	27.5% Untreated casein
2	16	12.5% Untreated casein + 15% casein thermolyzed for 1 hr
3	17	12.5% Untreated casein + 15% casein thermolyzed for 2 hrs
4	17	12.5% Untreated casein + 15% casein thermolyzed for 4 hrs
<i>Colonic fermentation with other proteins</i>		
1	11	12.5% Untreated casein + 15% untreated soy
2	11	12.5% Untreated casein + 15% soy thermolyzed for 2 hrs
3	10	12.5% Untreated casein + 15% untreated egg white
4	10	12.5% Untreated casein + 15% egg white thermolyzed for 2 hrs
<i>Colonic promotion with other thermolyzed proteins</i>		
1	9	23.5% Untreated casein
2	9	3.5% Untreated casein + 20% casein thermolyzed for 2 hrs
3	9	3.5% Untreated casein + 20% untreated soy
4	9	3.5% Untreated casein + 20% soy thermolyzed for 2 hrs
5	9	3.5% Untreated- casein + 20% untreated egg white
6	9	3.5% Untreated casein + 20% egg white thermolyzed for 2hrs

Note a: Remainder of diet is as defined in Reference 5.

Materials and Methods

Animals

Five-week-old female F344 rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN) or Iffa-Credo (Lyon, France). They were acclimatized in the colony for one to two weeks, where they were housed in plastic cages on wood chips at 23°C with controlled 12:12-hour light-dark cycles and provided laboratory chow (6% fat; Teklad Premier Laboratory Diets, Madison WI or UAR, Villemoisson, France) and water ad libitum for one week before the studies with the experimental diets. Animals were weighed at the beginning and termination of the experiments. Animal care was in accordance with the guidelines of the Canadian Council on Animal Care.

Diets

The control and experimental diets were based on the standard AIN-76 diet (18), which was modified to contain 20% beef tallow and 3.5% corn oil. The fat was added to the diet at the expense of carbohydrate on a caloric basis, and the diets differed only in their protein component, as previously described (4,5) and detailed in Table 1. Components of the diets were obtained from ICN (St. Laurent, Canada, or Buckinghamshire, UK). Casein was

thermolyzed for one, two, or four hours as a thin layer on a Pyrex pan in a domestic oven at 180°C (5). Soy and egg white protein were obtained from ICN and were similarly thermolyzed for two hours.

Digestibility of Thermolyzed Casein

The rats were randomized into groups that were fed diets containing the untreated or thermolyzed casein for 14 days. The 24-hour food intake during the last day was recorded for each animal, and feces and urine were collected for the saine period of time. The nitrogen values of food and feces and urine were determined by micro-Kjeldahl techniques (19). The endogenous nitrogen was determined from a group of rats fed a diet containing no protein. The calculation of the digestibility (net protein utilization) was calculated from Equation 36 in Reference 10.

Colonic Protein Fermentation Measurement

Colonic protein fermentation was assessed in two ways. First, rats were randomized to appropriate control and experimental diets for 14 days. During the last week of these diets, they were placed in metabolic cages, and feces and urine were collected through Day 14, frozen rapidly, and stored at -25°C before assay. Colonic protein fermentation was assessed by measuring fecal ammonium and urinary phenols. Fecal ammonia was measured by

Berthelot's indophenol reaction, as described by Gips and co-workers (20). Urinary phenol and cresol were analyzed with a method based on that of Bucket (21). Briefly, 10 ffl of glucuronidase solution (10 times dilution) was added to 2 ml of urine that was incubated at 37°C for 18 hours; then 10 μ l of nitrobenzene, an internal standard (500 mg/l), and 1 ml of concentrated HCl were added, and the mixture was heated at 100°C for 30 minutes. It was then cooled, neutralized with 1 ml of 10 M NaOH, and extracted with 2 ml of diethyl ether. The organic phase was collected and dried. The residue was dissolved in 50 ffl of methanol and analyzed by gas chromatography on a stainless steel column (1.5 m x 2 mm ID), packed with 20% ethylene glycol succinate on Chromosorb W-AW-DMCS 80-100 mesh, and monitored with a flame ionization detector. The concentrations of phenol and cresol were calculated on the basis of the ratio of their peak areas to that of nitrobenzene. The identities of the peaks corresponding to phenol and cresol on the gas chromatograph were confirmed by gas chromatography-mass spectrometry. Urinary indol-3-ol was measured using the method described by Curzon and Walsh (22). p-Dimethylaminobenzaldehyde (0.6 ml of a 2% solution) was added to 0.2 ml of urine and kept at room temperature for 10 minutes. The mixture was then added to 0.4 ml of 40% NaOH on ice. After the mixture was cooled, 2 ml of petroleum spirit were added and it was shaken for 30 minutes; 1 ml of supernatant was used to measure absorbance spectrophotometrically at 465 nm.

The second method for assessing fermentation was based on cecal contents, which were fast frozen with liquid nitrogen and kept at -25°C before assay. Ammonia and volatile fatty acids were determined as previously described (9). Briefly, ammonia was determined in the supernatant of centrifuged diluted samples by a spectrophotometric method (kit 640, Sigma Chemi-cal, St. Quentin, France). Volatile fatty acids were determined by gas chromatography by the method of Jouany (23) by combining the peaks of i-valeric acid with those of methyl butyric acid as branched-chain fatty acids that are markers of protein fermentation (11).

Measurement of Promotion

In the studies of ACF promotion, the animals were initiated with one injection of azoxymethane (AOM; Sigma Chemical, Mississauga, Canada, or St. Quentin, France; 20 mg/kg ip). They were maintained on laboratory chow for a further seven days and were then randomly allocated to the appropriate control or experimental diets. After 100 days, they were sacrificed, and their colons were prepared flat in formalin, coded, and scored blindly for ACF number and multiplicity (aberrant crypts per ACF) with the use of methylene blue (16).

Statistical Methods

Group means for fermentation measures were compared by Student's t test. The statistical analysis of aberrant crypt assays for colon cancer promotion was based on ACF size, as defined by mean number of aberrant crypts per focus. Comparisons between groups were made making use of the quadratic nature of the variance of these values (24), although the calculated variances differed little from values calculated by conventional methods.

Results

Digestibility of Thermolyzed Casein

As noted above, it was our expectation that thermolysis of casein would decrease its digestibility, that the decrease in digestibility would lead to increased protein residue reaching the colon and increased colonic protein fermentation, and that the products of this fermentation would lead to colon cancer promotion. To test the first step of this hypothesis, we measured the digestibility of casein that had been thermolyzed. For a two-week period, groups of animals were given diets containing 27.5% untreated casein or 12.5% untreated casein plus 15% casein thermolyzed for one, two, or four hours, as shown in Table 1. Nitrogen balances were then carried out to assess the digestibility of the thermolyzed casein. The results (Figure 1) show that, as expected, the digestibility of casein drops significantly when it is thermolyzed. It decreases substantially after one hour of thermolysis and then decreases to near zero when the casein is thermolyzed for two or four hours.

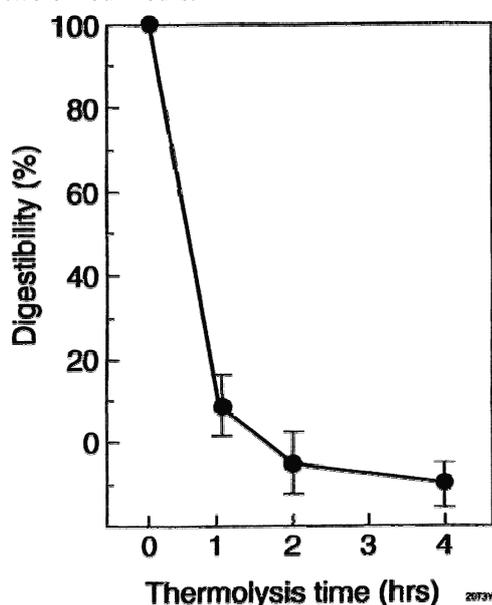


Figure 1. True digestibility (net protein utilization) of casein plotted as a function of duration of thermolysis (180°C) of a fraction of casein in diet. Results were calculated from data obtained from feces collected after 14 days on diets (Table 1). Error bars, SEM.

Colonic Fermentation With Thermolyzed Casein

We next determined whether the poorly digested protein that reached the colon is fermented by colonic bacteria to increase the exposure of the colon to potentially toxic fermentation products. To assess the formation of the products, rats were fed diets containing casein thermolyzed for zero, one, two, or four hours (Table 1), and fecal and urine samples were collected and assayed for ammonia, phenol, cresol, and indol-3-ol, respectively. These estimates of daily exposure to the putative promoting compounds after two weeks on the diets are shown in Figure 2. A marked increase in the exposure of colonic cells to these fermentation products is evident when casein is thermolyzed. However, in each case, the level of the fermentation product increases markedly when casein is thermolyzed for one hour but then decreases for the longer thermolysis times.

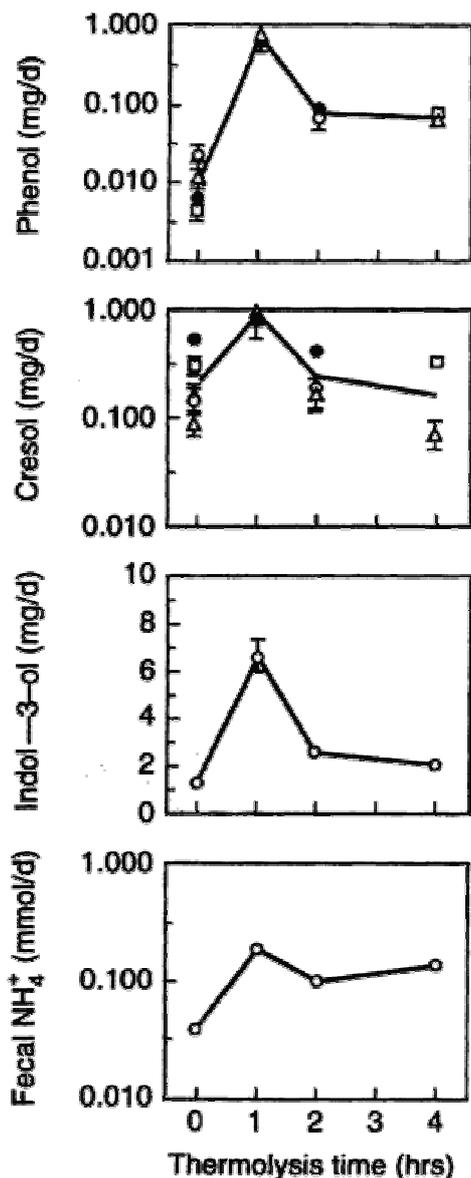


Figure 2. Quantity of phenol, cresol, and indol-3-ol excreted in urine and of ammonia excreted in feces of rats over 24 hrs plotted as a function of duration of thermolysis (180°C) of casein in diet. Results were calculated from data obtained from feces and urine collected after 14 days on diets (Table 1), as described in **Materials and Methods**. Separate experiments are shown with different symbols. Error bars, SEM for animals in groups calculated on logarithms of values for phenol, cresol, and ammonia. Error bars are smaller than symbols for ammonia.

Colonic Promotion With Thermolyzed Casein

The kinetics of colonic promotion as a function of casein thermolysis time were determined next (Table 1). Animals were given a dose of the colon carcinogen AOM and then fed diets containing casein that had been thermolyzed for zero, one, two, or four hours. After 100 days, the colons were scored for ACF. There was no effect of thermolysis on the number of ACF (data not shown), but there was a significant increase in the size of the ACF with increasing thermolysis time after one hour (Figure 3). This result was consistent with earlier results, which also showed no increase in promotion with one hour of

thermolysis (5). There thus appears to be a clear discrepancy between the kinetics of colonic protein fermentation and promotion with increasing thermolysis time. The former peaks at one hour, whereas the latter plateaus after two hours of thermolysis. The results of the promotional studies are thus not consistent with the notion that colonic promotion is a consequence of exposure to the putative colonic promoters.

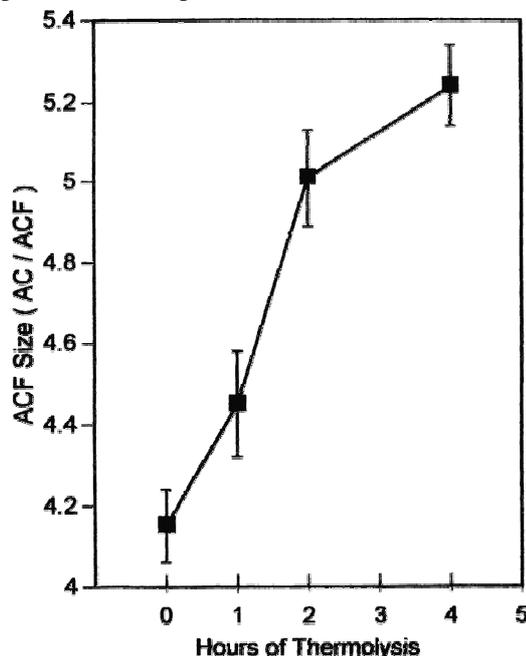


Figure 3. Average crypt size or crypt multiplicity measured as number of aberrant crypts (AC) per focus (ACF) at 100 days plotted as a function of duration of thermolysis (180°C) of casein in diet (Table 1). Error bars, SEM.

Colonic Fermentation With Other Thermolyzed Proteins

We next explored the effects of thermolyzing two other proteins, soy and egg white protein, on colonic fermentation and promotion. We began by comparing the effect of diets containing untreated proteins and proteins thermolyzed for two hours on the formation of fermentation products (Table 1). Groups of animals were given diets containing the untreated protein or the thermolyzed protein for two weeks, and the feces and urine were collected for assessment of fecal ammonia and urinary phenols. The results (Figure 4) show that thermolysis of the soy and egg protein increases these measures of colonic fermentation. Presumably, two hours of thermolysis decreased the digestibility of the proteins, and more protein residue reached the colon to be fermented.

Colonic Promotion With Other Thermolyzed Proteins

The promoting effects of untreated and thermolyzed casein and soy and egg white protein were then compared (Table 1). Groups of rats given AOM were then placed on diets containing the untreated proteins or the proteins thermolyzed for two hours. After 100 days, the cecal contents were collected to assess colonic fermentation and the colons were examined for ACF. The results (Figure 5, top 4 graphs) show that, for each of the proteins, thermolysis led to a marked increase in protein fermentation, as assessed by cecal formation of ammonia

and branched-chain fatty acids as well as by cecal butyric and valeric acids. This result is consistent with our previous results where protein fermentation was assessed by urinary phenols after two weeks (Figures 2 and 4). The results (Figure 5, bottom graph) also show again that thermolyzed casein increases colonic promotion, a result consistent with the previous studies (5) and the results shown in Figure 3. However, in contrast to the results with casein, thermolyzed soy and egg white protein do not increase colonic promotion of ACF. Indeed, thermolysis decreased the size of ACF in animals consuming egg white protein (Figure 5, bottom graph).

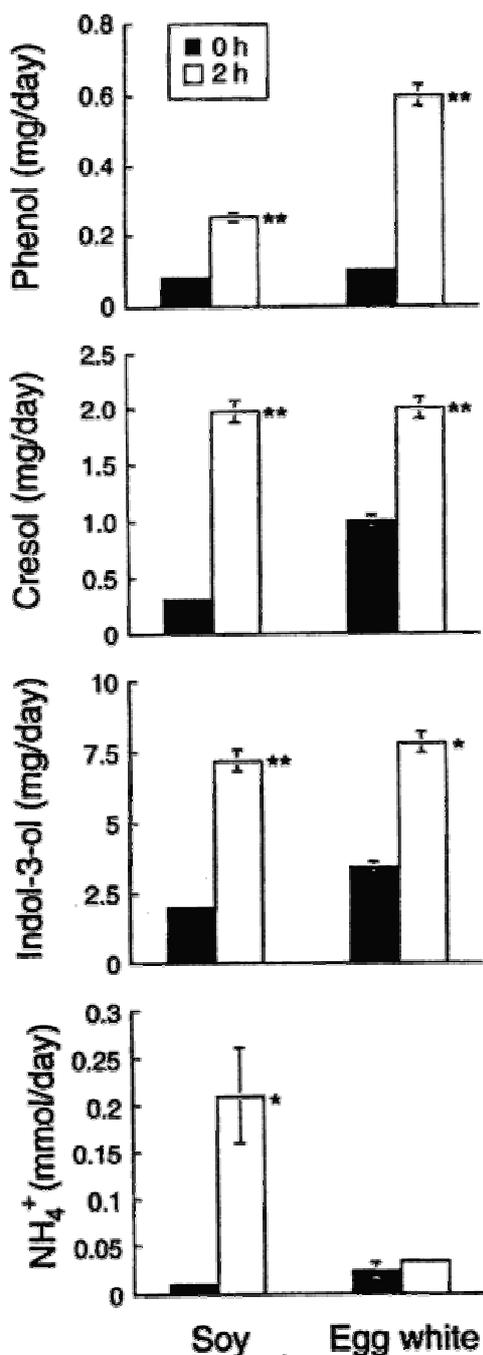


Figure 4. Quantity of phenol, cresol, and indol-3-ol excreted in urine and of ammonia excreted in feces of rats over 24h when these animals were consuming soy or egg white protein untreated or thermolyzed (2 hrs at 180°C) (Table 1). Error bars, SEM. Symbols: *, $p < 0.05$; **, $p < 0.01$.

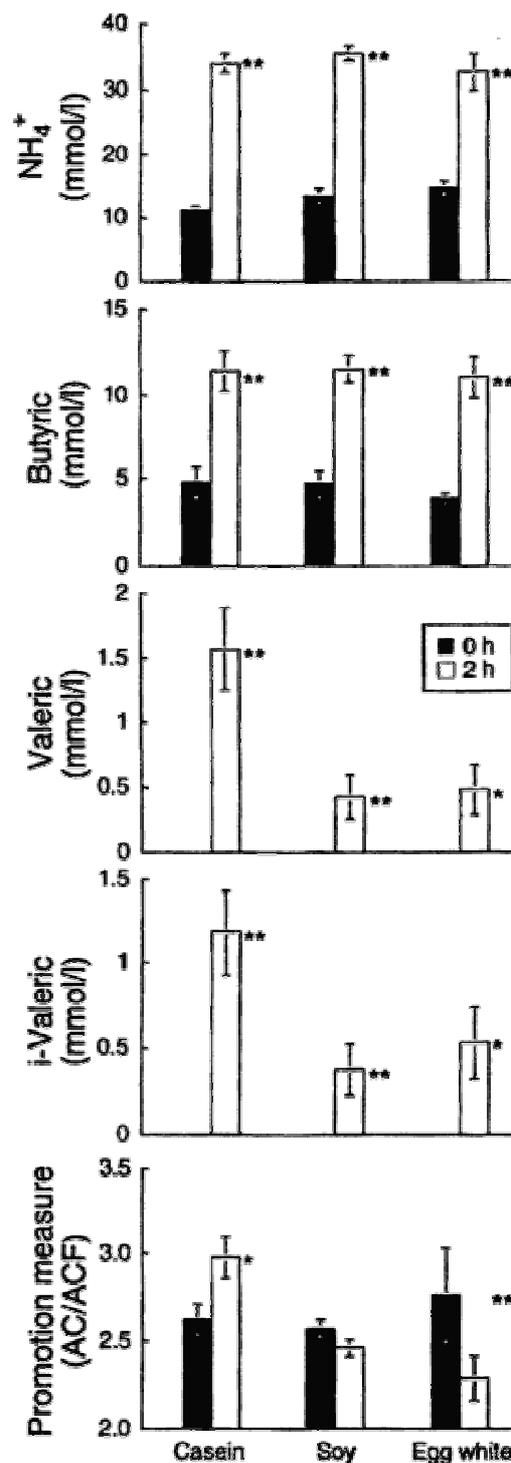


Figure 5. Top four graphs: ammonia and butyric, valeric, and i-valeric acids in cecal samples from animals consuming casein, soy protein, and egg white protein, untreated or thermolyzed (2 hrs at 180°C) (Table 1). Bottom graph: average crypt size, measured as no. of aberrant crypts (AC) per focus (ACF) at 100 days, for same proteins. Error bars, SEM. *, $p < 0.05$; **, $p < 0.01$.

Discussion

We have examined the possibility that the mechanism responsible for the promotion of colon cancer by thermolyzed casein involves colonic protein fermentation, that thermolysis of the protein leads to increased colonic protein fermentation, and that the products of this

fermentation promote colon carcinogenesis. Certainly our first expectation was met. We found that thermolysis of casein decreased its digestibility (Figure 1) and led to significant increases in the formation of the fermentation products ammonium, phenol, cresol, and indole-3-ol (Figure 2). In these studies, digestibility and formation of fermentation products were assessed for dietary casein that had been thermolyzed for one, two, and four hours. The most significant changes were seen after the first hour of heating, when the color of the thermolyzed casein was pale yellow and the digestibility was reduced to <20%. Further heating for two and four hours decreased the digestibility further but resulted in a decrease in the formation of fermentation products. Presumably, with heating, protein digestibility decreases, more protein residue reaches the colon, and protein fermentation and the products of fermentation increase. With further heating, the protein residue still reaches the colon but is so denatured and cross-linked that it resists bacterial digestion, and the products of fermentation decrease. The two-hour thermolysis of egg white and soy protein led to an increase in protein fermentation similar to that seen with casein. The increase was seen with the fecal ammonia and the urinary phenol measures (Figure 4) and for ammonia and the branched-chain, as well as the unbranched, volatile fatty acids in the cecal contents (Figure 5).

There have been few systematic studies of the effect of thermolysis on colonic protein fermentation. Chacko and Cummings (25) observed that fecal ammonia was increased when subjects consumed baked beans. Urinary indican has been used as a measure of stasis in the gastrointestinal tract and of colonic fermentation (26). This evidence and our work described here thus appear to be consistent with the notion that protein thermolysis leads to increased levels of colonic protein fermentation.

Our second expectation, that the products of protein fermentation promote colon carcinogenesis, however, was not supported by our studies. If the products of protein fermentation had been the promoters, we would have expected that the levels of fermentation products would have been reflected in the degree of promotion. This was not the case. Fermentation products reached a maximum for protein thermolyzed for one hour and were lower for longer thermolysis times (Figure 2), whereas promotion continued to increase with thermolysis time through two hours (Figure 3). Furthermore the thermolysis of soy and egg white proteins led to increased levels of protein fermentation products (Figures 4 and 5) but did not promote ACF growth (Figure 5). Thermolysis of the proteins thus appears to lead to reduced digestibility and increased colonic protein fermentation in each case, but only in one case to promotion of colon carcinogenesis.

The lack of association between the toxic protein fermentation products ammonia and the phenols with colon cancer promotion was unexpected. Previous studies suggested that ammonia given intrarectally can act as a colon tumor promoter (12). Perhaps the sudden exposure to intrarectal ammonia leads to more toxicity than exposure to ammonia that is continuously formed from the deamination of amino acids. The slow formation of phenols could also be less toxic than acute exposures to

these agents. The phenols are usually conjugated and excreted by the colonic epithelial cells (27), and it is possible that the actual exposure of normal colonocytes to these compounds is minimal.

The lack of association between colonic protein fermentation and ACF promotion leaves us without an explanation for the promoting effect of thermolyzed casein. Our studies have shown that the promotion is not likely associated with the presence of mutagenic compounds such as the heterocyclic amines that can be formed in the pyrolysis of proteins or of the physical properties of the thermolyzed protein (unpublished observations). Our studies with soy and egg white proteins suggest that the mechanism may be rather unique for casein. A major difference between casein and the other proteins is the high level of phosphorylated serine in casein (28). Thermolysis of phosphoserine could lead to the formation of different kinds and amounts of products. For instance, phosphorylated serine is readily dehydrated to dehydroalanine, a derivative of acrylic acid that might act as an alkylating agent *in vivo* (29) or could interact with important components of the diet.

Acknowledgments and Notes

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Note added to Author's version.

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