results from the procedures under test, this is not in itself proof that they were measuring different things.

It is, however, of interest that sample C gave a value in the chick assays consistently about 13% below that of the standard E, and it also gave the lowest value in the rat NPU test, while in the chemical tests its value ranged from only 4% (total lysine) to 6.5% (direct FDNB test) below the value for sample E. It does appear therefore that the application of formalin as a firming agent immediately prior to processing, to ease the process of pressing liquor out of the fish after cooking, may have a greater biological effect than is detected by any of the chemical tests used here. The addition of somewhat higher levels of formalin has been found to reduce FDNB-reactive lysine in previous studies (Wessels et al., 1973; Carpenter, 1973), and it would be of interest to study the reactions occurring under different conditions and their effects on nutritional value. Sample B, in which formalin was added as a preserving agent prior to storage of the raw fish, had a higher value than sample C in the chick assays; this may be due to the formalin having been largely degraded during the storage period.

In general the results have been encouraging as to the usefulness of laboratory measures for predicting the value of fish meals as a source of lysine. They do, however, bring out the need for further standardization of the methods.

ACKNOWLEDGMENT

The authors thank P. Lerman for statistical advice and H. Mundheim for assistance in conducting biological assays.

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Received for review September 11, 1975. Accepted November 19, 1975. This work was supported in Norway by the Norwegian Fisheries Research Council and the International Association of Fish Meal Manufacturers defrayed traveling and shipping expenses incurred in the collaboration.

# Soybean Trypsin Inhibitor Activity of Soy Infant Formulas and Its Nutritional Significance for the Rat

Helen R. Churella,* Benita Co Yao, and William A. B. Thomson

The soybean trypsin inhibitor (SBTI) activity of five soy-based infant formulas was determined by a method specifically designed to test their relatively low SBTI levels. All formulas, except one, contained 15% or less of the SBTI activity of a soy-protein isolate source typically used in the manufacture of some formulas. Resterilizing two of these formulas did not significantly reduce residual SBTI activity. Weanling rats were fed adjusted diets containing an unprocessed soy formula, the formula after various stages of processing, the fully processed formula, and another commercially available soy formula. The rat diet groups did not differ from each other nor from the casein control in their caloric and protein utilization or pancreas weights. No pancreatic hypertrophy or hyperplasia was observed in any of the rats. Our results show that the level of SBTI in the soy infant formulas tested is low and of no nutritional significance for the rat.

Many years of clinical experience have demonstrated that soy-based and soy-protein-based infant formulas satisfactorily support the growth and development of infants (Cowan et al., 1969; Omans et al., 1963; Graham et al., 1970; Dean, 1973). Despite their apparent nutritional adequacy, it was reported that rats, when fed one such formula, showed poorer weight gain and caloric efficiency than rats fed other soy-based formulas (Theuer and Sarett, 1970). The relatively poor weight gain and caloric efficiency observed in the rats were attributed to the greater soybean trypsin inhibitor (SBTI) activity the investigators found in the formula in question than in the other formulas studied. SBTI activity is responsible, at least in part, for the poor growth, reduced protein and food efficiency, and pancreatic hypertrophy seen in rats which are fed raw soybean meal (Rackis et al., 1963; Booth et al., 1964; Rackis, 1965).

Appropriate heat treatment of soybean meal, however, readily inactivates SBTI, eliminating the antinutritional effects associated with the feeding of non-heat-treated soybean meal (Rackis, 1965; Leiner, 1962; Longenecker et al., 1964; Yen et al., 1969). The formula in question is heat treated during its manufacture. This formula also is manufactured from a soy-protein isolate which, due to the nature of its preparation, contains lower levels of SBTI

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Table I. Composition of Rat Diets

| Diet | Proximate anal., g per 100-g diet | | | | DL-Methionine added, g | SBTI act., TUI[e] |
|---|---|---|---|---|---|---|
| | Protein | Fat | Carbohydrate[d] | Ash | | |
| 1, base—formula A prior to heat processing[a] | 15.5[b] | 25.3[c] | 52.1 | 5.23 | 0.118 | 235 |
| 2, base—formula A after heat processing, but before sterilization | 14.7[b] | 26.1[c] | 52.2 | 5.35 | 0.114 | 40 |
| 3, base—formula A after sterilization | 14.8[b] | 25.5[c] | 52.0 | 5.37 | 0.116 | 10.4 |
| 4, 1 part of formula A base used in diet 1 and 1 part of formula A base used in diet 3 | 14.8[b] | 27.0[c] | 51.2 | 5.39 | 0.121 | 14.7 |
| 5, base—formula B concentrated form | 14.5[b] | 24.6 | 53.0 | 6.04 | 0.116 | 11.5 |
| 6, base—casein formula (control) | 15.4 | 22.4[c] | 55.1 | 4.03 | 0 | 1.5 |

[a] Formula A represents a blend of equal quantities of two separate soy formula batches. [b] The amino acid profile of the protein in formula A was similar to that of formula B. [c] Fat was a blend of corn and coconut oils except for diet 5, which contained soybean oil. [d] Carbohydrate was 50% sucrose and 50% corn syrup solids. [e] TUI trypsin units inhibited.

than do soybean meal and other soy products (Rackis, 1966). Nonetheless, the authors of the above reference implied that the heat treatment of the formula in question may not be adequate to reduce its SBTI activity to a nutritionally insignificant level.

The amount of heat processing a soy formula receives is critical for reaching its optimal nutritional quality (Hackler and Stillings, 1967). Recently, the accuracy of the conventional assay for measuring low levels of SBTI as found in heat-treated soy products has been questioned (Smith and Circle, 1972). Considering these two factors, we deemed it necessary to re-examine the formula in question for SBTI activity and the nutritional significance of this activity to determine whether recommendation of an increase in the heat treatment of the formula was appropriate.

Rackis (1965) showed that less SBTI activity is needed to cause pancreatic hypertrophy than to retard growth in a rat being fed raw soybean meal. Consequently, we undertook to determine SBTI activity of the formula and the effect of this activity not only on weight gain and caloric efficiency, but also on the pancreatic weight and development of rats receiving the formula.

## METHODS AND MATERIALS

SBTI activity was determined by modified methods of Erlanger et al. (1961) and Kakade et al. (1969) for (1) five infant soy-based formulas, (2) the soy protein used in the manufacture of the formula in question (formula A), (3) this formula after various stages of heat processing, (4) twice-sterilized formula, and (5) the diets fed in the rat studies. The modifications of the methods included suggestions by Rackis (1972) and the following: concentration of the extracts of the soy formulas or soy isolates by lyophilization; subsequent serial dilution of the lyophilized extract and determination of SBTI activity of each dilution; calculation of SBTI activity of the sample by using only those extract dilutions which inhibited between 20 and 80% of a standard trypsin activity.

The trypsin (from Nutritional Biochemicals Corporation, Cleveland, Ohio) used in the assay was found to be 52% pure when evaluated by active-site titration using *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride (Cyclo Chemical Division of Travenol Lab., Inc., Los Angeles, Calif.) according to the method of Chase et al. (1967).

By the modified assay we employed, 1 g of purified SBTI (purified Kunitz soybean trypsin inhibitor from Nutritional Biochemical Corporation) inhibited 1.64 g of trypsin.

The SBTI activity is expressed as trypsin units inhibited (TUI). One trypsin unit is defined as an increase of 0.01 absorbance units at 410 nm per 10 ml of the reaction mixture as described in the method of Kakade et al. (1969). The rat diets were prepared from lyophilized (1) non-heat-treated soy formula A, (2) soy formula A after various stages of heat processing, (3) formula A after sterilization, (4) equal parts of diets 1 and 3, (5) commercially available formula B, and (6) a casein control diet. Lyophilization of the formulas was accomplished within 48 hr in a Vacudyne freeze drier at 200-$\mu$m vacuum and less than 40 °C.

Table II. Trypsin Inhibitor Activity in Various Commercial Infant Soybean Formulas

| Soy formula[a] | Trypsin units inhibited (TUI)/g of protein, ± SD | $N$[b] |
|---|---|---|
| Concentrated forms | | |
| A | 0.9 ± 0.1 | 5 |
| B | 0.7 ± 0.1 | 5 |
| C | 1.1 ± 0 | 2 |
| D | 2.1 ± 0.2 | 2 |
| E | 2.1 ± 0.5 | |
| Ready-to-feed forms | | |
| A | 1.6 ± 0.4 | 5 |
| B | 1.7 ± 0.2 | 5 |
| C | 8.1 ± 0.7 | 2 |
| Soy protein isolate used in the manufacture of formula A | 14.4 ± 2.8 | 7 |

[a] (A) Isomil, Ross Laboratories, Columbus, Ohio; (B) ProSobee, Mead Johnson Lab., Evansville, Ill.; (C) Neo-Mull-Soy, Syntex Lab., Inc., Palo Alto, Calif.; (D) Mullsoy, Syntex Lab., Inc., Palo Alto, Calif.; (E) Soyalac, Loma Linda Foods, Riverside, Calif. [b] $N$ = number of samples or batches analyzed.

The heat processing of formula A followed the standard manufacturing procedure set for this product. The lyophilized formulas were adjusted with carbohydrate, fat, minerals, vitamins, and DL-methionine to give diets of the following composition: protein, 15%; fat, 25%; carbohydrate, 52%; minerals, 4%; vitamin mixture, 2.2%; moisture, 2%; DL-methionine, 0.12% (American Roland, Inc.).

Approximately 2 g of minerals accompanied the lyophilized formulas used in preparation of the diets. This amount of mineral supplied 100 g of diet with approximately 0.6 g of calcium, 0.4 g of phosphorus, 0.2 g of sodium, 0.6 g of potassium, 0.4 g of magnesium, 9 mg of iron, 2 mg of zinc, and 0.7 mg of copper. Salt mix USP XIV was added to bring the total mineral content of the diet

Table III. SBTI Activity of Soy Formula A at Various Stages of Manufacture

| | TUI/g of protein ± SD | | Cumulative destruction of trypsin inhibition, % | |
|---|---|---|---|---|
| | Concentrated form | Ready-to-feed form | Concentrated form | Ready-to-feed form |
| Formula A | | | | |
| (1) Prior to processing (no heat treatment) | 13.6 ± 3.1 | 16.5 ± 0.7 | 0 | 0 |
| (2) After processing prior to final sterilization | 3.0 ± 0.7 | 2.3 ± 1.2 | 77.6 | 85.4 |
| (3) Final commercial product | 1.1 ± 0.2 | 1.3 ± 0.4 | 91.8 | 92.1 |

Table IV. Decrease in Trypsin Inhibitor Activity of Soy Formulas by Resterilization

| Soy formula | Before resterilization, TUI/g of protein | TUI reduction, % | After resterilization,[a] TUI/g of protein | Total TUI reduction, % |
|---|---|---|---|---|
| Concentrated form | | | | |
| A | 0.9 | 94 | 0.7 | 95 |
| | 0.9 | 94 | 0.8 | 94 |
| Ready-to-feed form | | | | |
| A | 1.6 | 89 | 1.1 | 92 |
| | 1.0 | 93 | 0.7 | 95 |
| B | 1.8 | 88 | 1.5 | 90 |

[a] Heat treatment in resterilization equivalent to $F_{250}^{18} = 8.5$.

to an estimated 4 g/100 g. In addition to vitamins accompanying the formulas, all diets were supplemented with 2.2 g/100 g of Vitamin Fortification Mixture (Nutritional Biochemical Corporation, Cleveland, Ohio).

Approximate analysis, SBTI activity, and actual DL-methionine levels of the diets are shown in Table I.

Weanling (21-day-old) male rats of the Sprague-Dawley strain were used in the animal study. The rats were sorted into six groups of 12 rats with similar body weight distribution in each group. The average initial body weight for the groups ranged between 49.8 and 50.8 g. The rats were housed in individual galvanized cages and fed ad libitum (animal study was conducted by WARF Laboratories, Inc., Madison, Wis.). The food consumption and weights of the rats were measured weekly over a 3-week period. At the end of this period, the animals were sacrificed. Pancreas and liver were excised and weighed while still fresh and preserved in buffered formalin for later histopathological examination by a pathologist (associated with WARF Laboratories).

## RESULTS

SBTI activities for the five commercially available soy infant formulas examined are given in Table II. Except for one of the formulas (formula C in the ready-to-feed form), SBTI activity was less than 15% of the SBTI activity of a soy-protein isolate that is typical of isolates used in the manufacture of some of these formulas.

The rate of SBTI destruction occurring during manufacture of formula A is shown in Table III. SBTI activity of the non-heat-treated formula was similar to that of its parent soy-protein isolate. After heat processing, but prior to final sterilization, as much as 77.6 and 85.4% of the original SBTI activity of the concentrated and ready-to-feed forms, respectively, were destroyed. Final sterilization reduced the SBTI activity of both formula forms to less than 10%.

Resterilizing formulas A and B with a thermal process similar to that used in the commercial sterilization of evaporated cow milk ($F_{250}^{18} = 8.5$) reduced SBTI activity in each by only an additional 3% (Table IV).

Results of the rat feeding study are given in Table V. Weight gains and pancreas and liver weights were statistically evaluated by analysis of covariance. After the 3-week feeding period, weight gains of the six experimental diet groups were not statistically different from each other or from the control group when adjusted to calories or protein consumed, nor were the mean pancreas weights of any of the experimental diet groups different from each other or from the control group when these weights were adjusted to 100 g body weight (BW). None of the diets produced pancreatic hypertrophy and/or hyperplasia in any of the rats. Histologic examination of the pancreas confirmed the absence of either pancreatic hypertrophy or hyperplasia.

Mean liver weights per 100 g BW of the experimental diet groups were not statistically different from one another. Mean liver weights per 100 g BW of diet groups 2 and 5, however, were statistically smaller than the mean liver weights per 100 g BW of the control group diet 6. Livers of all the rats were normal in gross appearance and histologically.

## DISCUSSION

In preliminary studies, we found by using the method conventionally employed for determining SBTI activity (Erlanger et al., 1961) that SBTI values per unit of an extract of soy formula were low. After converting these values to units of SBTI per gram of formula protein, however, they seemed unusually high and variable. Other laboratories also have found it difficult to accurately determine SBTI activity of heated soy products (Smith

Table V. Effect of Diets Varying in SBTI on the Weight Gain, Liver and Pancreas Weights, and Caloric and Protein Efficiencies of Rats

| Diet group | Mean wt gain, g, ± SD | Mean g liver wt/100 g BW[a] ± SD | Mean g pancreas wt/100 g BW[a] ± SD | Caloric efficiency g wt gain/100 cal consumed ± SD | Protein efficiency g wt gain/g protein consumed ± SD |
|---|---|---|---|---|---|
| 1 | 70.83 ± 8.81 | 4.47 ± 0.42 | 0.681 ± 0.111 | 7.81 ± 0.60 | 2.59 ± 0.20 |
| 2 | 77.42 ± 12.29 | 4.37 ± 0.26 | 0.669 ± 0.076 | 8.13 ± 0.43 | 2.78 ± 0.17 |
| 3 | 77.08 ± 8.75 | 4.57 ± 0.11 | 0.648 ± 0.124 | 8.17 ± 0.47 | 2.75 ± 0.14 |
| 4 | 73.42 ± 8.60 | 4.70 ± 0.39 | 0.668 ± 0.116 | 7.85 ± 0.42 | 2.69 ± 0.14 |
| 5 | 70.00 ± 10.25 | 4.34 ± 0.31 | 0.647 ± 0.114 | 7.98 ± 0.87 | 2.70 ± 0.30 |
| 6 | 80.75 ± 5.24 | 4.85 ± 0.36 | 0.713 ± 0.117 | 8.93 ± 0.51 | 2.82 ± 0.17 |

[a] Grams body weight.

and Circle, 1972). By concentrating the extract by lyophilization and thus increasing the amount of SBTI available for assay, the percentage of trypsin inhibition obtained per unit of extract was increased manyfold. In addition, analyzing serial dilutions of the concentrated extract also reduced the variability of results we had encountered previously. In this latter modification, as given by Kakade et al. (1969), the trypsin units inhibited (TUI) by 1 ml of the extract (SBTI solution) are plotted as a function of the actual volume of extract assayed, and the resultant curve is extrapolated to zero level of extract. The value (TUI) obtained at zero is considered to most accurately represent the SBTI activity of 1 ml of extract. In deriving the curve, only those concentrations of extracts (SBTI) which inhibited between 20 and 80% of trypsin were considered. Extract concentrations which were outside this range of inhibition deviated from the linear plot.

It may not be possible to assay protein-containing products without encountering slight trypsin inhibition. This is evident in our analysis of the casein diet, which is known not to contain SBTI (Table I). The protein present in the extracts of formulas may compete in the assay with the artificial substrate (benzoyl-DL-arginine *p*-nitroanilide) for trypsin, resulting in an apparent trypsin inhibition; thus, it is probably not possible to determine when SBTI activity of a soy product has been completely destroyed.

Development of pancreatic hypertrophy in a rat has been shown to be a more sensitive indicator of the presence of SBTI activity in the diet than reduced protein efficiency or depression of growth in the rat (Rackis, 1965). Through a preliminary study, we found pancreatic hypertrophy was most evident in rats which had been fed unheated soy protein at 14% of their diet for a 3-week period. For this reason, the rat study presented here was confined to a 3-week period.

That none of the rats in any of the diet groups, even those fed diet 1, which contained 235 TUI per 100-g diet, developed pancreatic hypertrophy or hyperplasia is consistent with the findings of Rackis et al. (1963). These investigators found that soy-protein isolates which contained 260 mg of SBTI per 100-g diet and which were fed at 14% protein in the diet produced small but insignificant increases in pancreatic weight in rats. That none of the experimental diets, even diet 1, contained nutritionally significant levels of SBTI activity may be substantiated further by the lack of significant differences in weight gains, and in calorie and protein efficiencies between any of the experimental groups and the control group.

It is difficult to explain the lower liver weights of diet groups 2 and 5 than of the control group. The lower protein level of diets 2 and 5 may have been responsible.

We found the soy isolate which is used in the manufacture of formula A to contain 14.4 ± 2.8 TUI per g of isolate. This level of SBTI activity represents 30% or less of the SBTI activity of raw soybean meal (Rackis, 1966; Cogan et al., 1968). Consequently, what appears to be a 10% residual level of SBTI in a soy infant formula actually may represent less than 3% of the SBTI found in the original soybean meal. Heat treatment given soy formulas as they are prepared for marketing apparently lowers the concentration of SBTI to an almost irreducible level. The heat treatment formula A receives during processing is comparable to or greater than the heat treatment which has been shown to destroy about 95% of SBTI activity in soybean meal or soy flour (Baker et al., 1973; Rackis, 1966; Albreckt et al., 1966), and which provides maximum nutritive value and 90% reduction of SBTI activity of soy milks (Hackler and Stillings, 1967).

To completely destroy the apparent residual SBTI activity in a soy formula would be difficult because of our inability to accurately detect very low levels of SBTI. Also, the additional heat treatment needed to destroy the residual SBTI may well cause a reduction in the product's protein quality. Such a reduction in protein quality would be of far greater nutritional significance than would the supposed presence of an insignificant amount of SBTI activity.

Theuer and Sarett (1970) attributed the lower weight gain and caloric efficiency of rats receiving formula A than for those receiving formula B to the higher SBTI activity they found in formula A compared to formula B. In our study, when rats were fed formula A (diet 3), which was adjusted to contain the same level of protein and methionine as formula B (diet 5), no statistical differences in weight gain, caloric and protein efficiency, and pancreas weight per 100 g BW were observed between rats fed either formula. None of the rats, when fed either formula, developed pancreatic hypertrophy or hyperplasia, nor did formula A, even prior to any processing (diet 1), contain sufficient SBTI activity to produce the pancreatic hypertrophy or hyperplasia and growth depression seen in rats fed raw soybean meal.

It is concluded that the residual SBTI activity in all soy infant formulas analyzed is low and that, at this low level, the inhibitor has no nutritional significance for the rat.

ACKNOWLEDGMENT

The authors thank Joseph J. Rackis, Northern Regional Research Laboratories, U.S. Department of Agriculture, Peoria, Ill., for his invaluable suggestions concerning the assays, both in vitro and in vivo, used in the study. We are grateful to Duane Benton and Bruce Johnson for their guidance and advice and to James E. Jeffries for editorial assistance.

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Received for review November 12, 1974. Accepted September 26, 1975.

# Inhibition of Nitrosamine Formation in Fried Bacon by Propyl Gallate and L-Ascorbyl Palmitate

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It was shown that treatment, just prior to frying, of normal nitrite-cured bacon with 1000 ppm of propyl gallate, piperazine, sodium ascorbate, or ascorbyl palmitate markedly reduces the formation of nitrosopyrrolidine during cooking. Propyl gallate, piperazine, and ascorbyl palmitate were more effective than sodium ascorbate in this respect. When nitrosoproline was added to lard or nonnitrite bacon (bacon prepared without nitrite) and the mixture cooked, the formation of nitrosopyrrolidine was demonstrated but the yield was extremely low (0.06–0.21%). Addition of the above-mentioned additives did not inhibit the formation of nitrosopyrrolidine from nitrosoproline. It was, therefore, concluded that these additives inhibit nitrosopyrrolidine formation in normal bacon by interfering with reactions other than the decarboxylation step of nitrosoproline. The possibility of using ascorbyl palmitate in cured bacon is discussed.

Studies with laboratory animals have indicated that many *N*-nitrosamines are strong carcinogens (Magee and Barnes, 1956; Druckrey et al., 1967; Lijinsky et al., 1969). The reported occurrence (Sen, 1972; Crosby et al., 1972; Fiddler et al., 1974) of nitrosamines in food products, especially nitrite-treated meats, is, therefore, a matter of concern. Previous studies (Sen et al., 1973; Fazio et al., 1973; Crosby et al., 1972) have shown that traces of nitrosopyrrolidine (NPy) are formed during frying of bacon although none can be detected in the uncooked product. It is believed that NPy is formed by decarboxylation of nitrosoproline (NPro) which could arise from the interaction of added nitrite and the naturally occurring amino acid, L-proline (Lijinsky and Epstein, 1970; Sen et al., 1973). An alternative pathway would be via direct interaction of pyrrolidine and nitrite. The results of model system experiments by Pensabene et al. (1974) and Bills et al. (1973) have provided support to the theory that NPy can be formed from NPro under simulated conditions of bacon frying. It has also been established that putrescine, spermine, spermidine, and collagen, all of which are known to occur in pork bellies, can react with nitrite to form NPy (Bills et al., 1973; Huxel et al., 1974). Among all the precursors tested NPro seemed to produce NPy in highest yields.

Nitrite is used as a curing agent for bacon as it imparts an attractive red color to the meat. In combination with sodium chloride, it also induces a particular type of flavor (Brooks et al., 1940; Cho and Bratzler, 1970; Parr and Henrickson, 1970). In addition, the combination of salt and nitrite plays a significant role in controlling the outgrowth of *Clostridium botulinum* spores (Pivnick et al., 1967; Greenberg, 1972). Although the exact mechanism of action of nitrite against the growth of *botulinum* spores is not known, recent studies (Greenberg, 1972; Perigo and Roberts, 1968) have indicated that the initial level of nitrite used, and not the residual level, is the important factor. Nitrite-treated canned pork luncheon meat with only 2 ppm of residual nitrite has been shown to possess considerable antibotulinum effect (Pivnick and Chang, 1973).

Studies by Fiddler et al. (1973) have shown that the addition of 550–5500 ppm of sodium ascorbate (NaAsc) in wieners can markedly reduce the formation of dimethylnitrosamine (DMN). Similar studies (Herring, 1973) with bacon have indicated that NaAsc at 500–2000-ppm levels is effective in reducing the formation of NPy in cooked bacon but the results have been reported to be erratic. Moreover, the possibility exists that the addition of excess ascorbate at the initial stage of the curing process may destroy the added nitrite and reduce its inhibitory effect against *C. botulinum*. It was, therefore, thought that it would be more desirable to add the ascorbates or other nitrite-scavenging food additives at the end of the curing process, because by that time the bacon would already contain the *botulinum*-inhibitory factor which is believed to be formed (from nitrite) during the curing process. In this paper we wish to report the results of a study in which samples of commercial bacon prepared with normal levels of nitrite were treated with various food additives, and their effect on the formation of NPy during cooking was investigated.

## MATERIALS AND METHODS

**Bacon Samples.** Vacuum-packed side bacons were purchased from the local supermarkets. The nonnitrite bacons were obtained through the courtesy of a commercial firm, and these were prepared by the company's standard method except that no nitrite was used during curing.

**Chemicals.** Propyl gallate (PG) (Nutritional Biochemicals Corporation), L-ascorbyl palmitate (AP) (ICN-K&K Laboratories, Inc.), and NaAsc (Hoffmann-La Roche Ltd.) were used without further purification. NPro

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