

Blood Fraction Effects on the Antibotulinal Efficacy of Nitrite in Model Beef Sausages

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ABSTRACT

Toxin production by *Clostridium botulinum* was studied in a model cured beef sausage containing 0-5% added dried bovine blood fractions. Controls which contained nitrite but without blood fractions, were toxic by bioassay in 3 wk at 27°C. Controls without nitrite or blood fractions were toxic at 1 wk, while model sausages supplemented with hemoglobin, red cells, or whole blood were toxic in 1-3 wk. Plasma yielded no detectable toxin for 4 to > 10 wk depending upon addition level. Sausages showing delayed toxigenesis had pH values lower than those which developed toxin earlier. These results demonstrated that use of blood fractions that increased iron levels in beef above 30 µg/g interfered with the antibotulinal efficacy of sodium nitrite.

INTRODUCTION

BLOOD is an underutilized animal by-product containing high levels of protein and iron, making it a potentially important nutritional supplement. Moreover, functional properties, such as emulsifying capacity, fat and water binding, can be enhanced by addition of blood products (Knipe, 1988). There is concern, however, that when used as an additive, the high iron content of certain animal blood fractions may interact with and compromise the antibotulinal action of sodium nitrite in cured meats (van Roon and Olsman, 1977; Tompkin et al., 1978a,b, 1979).

This view has evolved from four lines of evidence. First, iron is a required nutrient for clostridial spore germination and outgrowth, and botulinal toxin development (Siegel, 1981; Arnon et al., 1977; Francis and Dodge, 1987). Secondly, nitrite inactivates the *C. botulinum* phosphoroclastic system (Woods and Wood, 1982), which suggests that an interaction occurs between nitrite and intracellular iron-bound protein. A third line of evidence by Tompkin et al. (1978a,b; 1979) showed that added iron depleted residual nitrite levels in cured meats. They hypothesized that the extracellular iron bound nitrous oxide, depleted residual nitrite levels, and prevented the inhibitor from entering the cell. Finally, epidemiological data yielded an association between consumption of blood sausages, rich in iron-containing compounds, and foodborne botulism (Simunovic et al., 1985; Lücke, 1985). Since there is interest in using blood fractions in cured meat products, and because there are unresolved questions regarding safety of that practice, we used a model cured beef sausage to determine the effect of adding blood fractions on the antibotulinal efficacy of sodium nitrite.

EXPERIMENTAL

Cultures

A six strain spore mixture of *Clostridium botulinum* type A (33, 62A, 69) and proteolytic type B (999, 169, ATCC 7949) strains was used throughout the study. Each strain was prepared from cultures of heat shocked (80°C for 10 min) stock spores in botulinal assay medium

(Huhtanen, 1975) without thioglycolate (BAM) and was incubated at 35°C up to 2 wk in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing 10% CO₂, 5% hydrogen, and 85% nitrogen with a palladium catalyst. Periodically cultures were examined with phase contrast microscopy to determine the relative ratio of vegetative cells to spores. When greater than 50% of the cells were phase bright the spores were harvested by centrifugation and then washed 2X in sterile distilled water. Concentrated spore preparations were heat shocked (80°C for 10 min) prior to storage at 4°C. Spores were enumerated on BAM agar and also streaked onto egg yolk agar plates. Both sets of plates were incubated anaerobically at 35°C for 48 hr. Afterwards, the BAM plates were counted and the egg yolk agar plates observed for evidence of lipase activity. Supernatant fluid from the initial centrifugation was assayed for neurotoxin by the mouse bioassay (Hatheway, 1988). Spore mixes were prepared for two replicate trials from equal numbers of the 6 strains (9.7 X 10³ and 2.1 X 10³ spores/mL, respectively).

Product formulation and inoculation

Model sausages were formulated by blending 1 cm³ beef cubes (under blade chuck) with dried bovine blood fractions: hemoglobin from Sigma Chemical Corp. (St. Louis, MO) and plasma, red blood cells, and whole blood, all obtained from the American Protein Corporation (Ames, IA). Each blood fraction was substituted for beef at levels from 0-5% (w/w) in the formulations. The meat and blood fraction total weights were 500g/batch. Fortification levels were based on findings of Guzman et al. (1988), as well as the decision to exceed normally incurred levels, in an attempt to establish a risk potential. Additional additives to each batch formulation included 10% ice (50g), 2.5% NaCl (12.5g), 156 µg/g NaNO₂ (78mg), and 550 µg/g (275mg) NaAscorbate. The mixture was homogenized for 2 min in a food processor. Formulations were inoculated with a target level of 300 spores/g and thoroughly mixed. Five gram (±0.05g) aliquots were removed and vacuum sealed in low oxygen-permeable film (IKD All-Vak #13, O₂ permeation = 1.0 cc/100 in²/24 hr @ 25°C, International Kenfield Distributing Co, Rosemont, IL); these were heated at 80°C for 20 min. Samples were then double bagged in the same film under vacuum and stored for 1-10 wk at 27°C.

Product analysis

Viable spore counts in samples were estimated by a 5-tube MPN (USFDA, 1984). Sample weights for MPN analysis were 0.1, 0.01, 0.001g. Samples for aerobic and anaerobic bacterial counts were prepared using pasteurized samples and blending with a Stomacher 400 Lab Blender (A.J. Seward, London) located in a Coy anaerobic chamber. Samples were anaerobically diluted and surface plated (Spiral Systems, Inc., Cincinnati, OH) onto BAM agar plates. Petri dishes were incubated at 35°C inside a Coy anaerobic chamber or transferred to a 35°C aerobic incubator. Bacteriological determinations were performed at day 0 and at least two other sampling intervals.

Samples for chemical and neurotoxin determinations were removed and analyzed weekly. Nitrite levels were estimated by the AOAC (1984) procedure. Determination of pH was performed using a combination electrode attached to a Radiometer PHM82 meter (Radiometer, Copenhagen, Denmark) on liquid homogenates of samples after heating (to inactivate toxin) and cooling to room temperature. Botulinal neurotoxin was determined using the mouse bioassay. Iron levels of meat samples (dry ash) and blood fractions (wet ash) were determined by atomic absorption spectroscopy using AOAC (1984) procedures; statistical analyses and curve fittings were performed using RS/1 (BBN Software Products, Cambridge, MA).

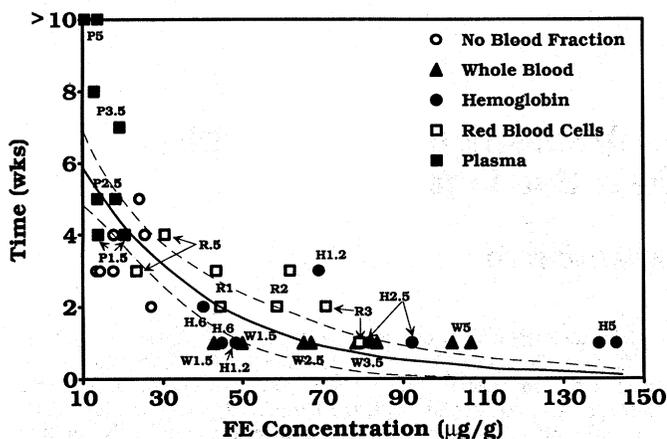


Fig. 1—Relationship between iron content and time to toxin detection. Numbers following letters indicate percent added blood fraction. (—) Best fit curve: $Y = 8.7 - 0.03x$; (---) 95% confidence interval.

RESULTS & DISCUSSION

Iron analysis

Iron concentrations of dried bovine hemoglobin, red blood cells, whole blood, and plasma were 0.27% ($2687 \pm 222 \mu\text{g/g}$), 0.24% ($2442 \pm 231 \mu\text{g/g}$), 0.16% ($1644 \pm 222 \mu\text{g/g}$), and 0.01% ($63 \pm 1 \mu\text{g/g}$) (w/w basis), respectively. Values (expressed as means \pm standard deviations of triplicates) reflected the varying amounts of heme iron in the fractions. Iron levels in eight beef chuck samples averaged $24 \pm 5 \mu\text{g/g}$ (range $13\text{--}28 \mu\text{g/g}$).

Iron levels in the model beef sausages are shown in Fig. 1. Iron levels of the model sausages generally increased with iron content of the blood fraction, as expected. For example, at the highest fortification levels, iron concentrations averaged 143 ± 9 , 104 ± 3 , 75 ± 6 , 12 ± 5 , and $\mu\text{g/g}$ for hemoglobin (5%), whole blood (5%), red blood cell (3%), and plasma (5%), respectively. Control samples containing 0% blood fractions averaged $20 \pm 5 \mu\text{g/g}$ iron. Control samples had less iron than raw beef because of the dilution with curing agents and water. For the same reason, plasma samples were generally lower in iron than controls.

Residual nitrite

Although $156 \mu\text{g/g}$ NaNO_2 was added to the formulations, residual nitrite levels after cooking (day 0) were $<10\text{--}40 \mu\text{g/g}$. Despite the various treatments, nitrite levels in all samples declined to $<10 \mu\text{g/g}$ within 2 wk (data not shown). A low correlation ($R^2 = 0.06$, $P > 0.05$) existed between residual nitrite level and total iron, indicating that residual nitrite was unaffected by iron addition alone. A similar finding was reported by Kim et al. (1987). Using a similar system at 27°C , nitrite levels fell below $10 \mu\text{g/g}$ with 8 d. They indicated that the rapid depletion of nitrite resulted from the combined effect of added iron, ascorbate, naturally occurring meat reductants, and clostridial spores. Those investigators concluded that total reducing capacity of the system was the major factor affecting nitrite depletion in meat systems. The observations from our study supported that conclusion since nitrite levels fell very rapidly in all samples irrespective of iron load.

pH

Changes in pH values of sausages containing the highest levels of blood fractions (3 or 5%) are shown in Fig. 2. (Values are means of duplicates from two trials.) In general, throughout

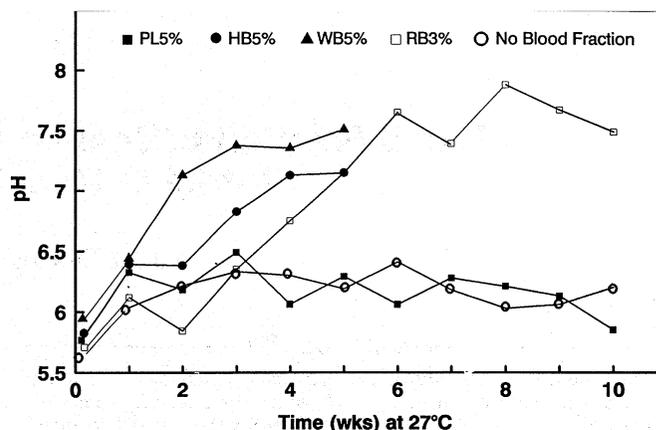


Fig. 2—Relationship between incubation time at 27°C and sausage pH. PL=plasma; HB=hemoglobin; WB=whole blood; RB=red blood cells. Numbers following letters indicate percent added blood fraction.

the testing period plasma and control pH values remained lower than either whole blood or hemoglobin. Red blood cell pH values were initially 5.7, similar to plasma and control samples (5.6 and 5.75, respectively). By wk 4, however, pH of the 5% red blood cell samples rose to a level similar to the whole blood and hemoglobin samples ($\text{pH} > 6.6$). pH values for the lower levels of blood fraction addition showed similar trends, although, as expected, less markedly.

The pH of the model sausages was affected by the native buffering capacity of the meat, additives, microbial acid production, and proteolytic products, such as ammonia. The higher pH values observed in hemoglobin, whole blood, and red blood cell samples, with respect to the control, may have been influenced by the predominating effect of proteolytic activity. Dried plasma contains about 6% carbohydrate (Knipe, 1988), which should provide a sufficient level of fermentable sugars to stimulate acid production by native flora. The acid production may have overcome any tendency for these samples to become more alkaline with time.

Bacteriology

Raw beef samples had aerobic loads ranging between $10^3\text{--}10^4$ CFU/g. The cooking step (80°C for 20 min) reduced levels to $10^0\text{--}10^1$ CFU/g. Five-tube MPN analysis indicated that spore loads in the model sausage were 388 ± 111 spores/g (range 230–525 spores/g) for the 8 tests. Plate count enumerations demonstrated that facultative/anaerobic growth generally increased over time to a maximum density of 10^8 CFU/g (data not presented). The increase in numbers did not correlate significantly ($P > 0.05$) with either iron level, residual nitrite, or time to toxin detection.

Neurotoxin detection

Pooled data from trials 1 and 2 are shown in Table 1. (Values are the number of toxic samples, 2 mice injected/sample, over the total number of samples.) Selected samples were neutralized with types A, B, and polyvalent CDC antitoxin; only type A was observed when toxin was present. Control (no blood fractions) samples, without NO_2 became toxic after 1 wk of incubation at 27°C . When nitrite, but no blood, was added, the time to neurotoxin detection was extended to 3 wk. Hemoglobin and whole blood samples, regardless of addition level, became toxic at 2 wk. Red blood cell samples exhibited neurotoxin at 3, 2, 2, and 1 wk, for 0.5, 1, 2, 3% additions,

Table 1—Effect of added blood fractions on time to botulin neurotoxin detection

Time (Wk)	Hemoglobin				Red Blood Cells				Whole Blood				Plasma			Controls (No Blood Fractions)		
	0.6%	1.25%	2.5%	5%	0.5%	1%	2%	3%	1.5%	2.5%	3.5%	5%	1.5%	2.5%	3.5%	5%	+NO ₂	-NO ₂
0	0/4*	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/16	0/16
1	2/4	2/4	4/4	4/4	0/4	0/4	0/4	2/4	4/4	4/4	4/4	2/4	0/4	0/4	0/4	0/4	0/16	15/16
2	3/4	4/4	4/4	4/4	0/4	0/4	2/4	4/4	4/4	4/4	4/4	3/4	0/4	0/4	0/4	0/4	0/16	15/16
3	3/4	3/4	3/4	4/4	1/4	4/4	3/4	4/4	4/4	4/4	4/4	4/4	0/4	0/4	0/4	0/4	7/16	16/16
4					1/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	2/4	2/4	0/4	0/4	9/16	
5					1/4		4/4						0/4	0/4	0/4	0/4	16/16	
6					1/4								0/4	2/4	0/4	0/4	16/16	
7					2/4								2/4	1/4	2/4	0/4		
8					4/4								1/4	3/4	4/4	0/4		
9					4/4								1/4	3/4	4/4	0/4		
10					4/4								0/4	4/4	4/4	0/4		

* Toxic samples/Total samples (duplicate samples, duplicate trials)

respectively. All plasma samples were free of detectable toxin longer than any of the controls. Time to neurotoxin detection were 4, 4, 7, and > 10 wk for added bovine plasma concentrations of 1.5, 2.5, 3.5, and 5%, respectively. The observation that some of the plasma samples remained toxin-free for 10 wk at 27°C suggested that there was some inhibitory factor in this blood fraction. Such a factor may create an unsuitable environment for growth, deprive the organism of required nutrients, or inhibit the organism directly. A lowered pH was not likely sufficient to produce the effect. One possible explanation is that this blood fraction contained lactoferrin or transferrin, iron-binding proteins which are found in serum and other physiological fluids, and would be concentrated in dried plasma. Both proteins have bacteriostatic activity toward Gram-positive organisms (Payne et al., 1990). The mechanism of action has been ascribed to iron chelation with resulting iron starvation of susceptible organisms (Rainard, 1986).

Relationship among iron content, residual nitrite and time to neurotoxin detection

Time to initial detection of neurotoxin was plotted vs iron concentration of the corresponding beef sausage sample (Fig. 1). Nonlinear regression analysis indicated a high inverse correlation between these two factors, with an exponential decay curve yielding the best fit: $Y = 8.7^{-0.03x}$ ($P < 0.01$, $R^2 = 0.91$), where X is the iron concentration in $\mu\text{g/g}$, and Y the wks to neurotoxin detection by bioassay. Sausages with iron levels below 30 $\mu\text{g/g}$ were toxic after ≥ 3 wk at 27°C, while samples with iron levels above 30 $\mu\text{g/g}$ became toxic in 1–2 wk. Note that this iron content exceeded only marginally that of endogenous iron levels in the beef used in our study (24 $\mu\text{g/g}$). Thus, at customary usage levels, the antibotulinal effectiveness of nitrite may be compromised by minimal levels of added iron-containing compounds.

The correlation was poor between time to toxin detection and nitrite levels ($R^2 = 0.01$), indicating that residual nitrite per se was not the critical determinant offering protection against botulin toxin development. Kim et al. (1987) concluded that the chemical nature of protein-bound nitrite was altered by iron, which, in turn stimulated botulin growth. Our current research supported this assertion, since, despite persistently low residual nitrite levels in all samples, the time to toxin detection varied considerably. The multiplicity of factors contributing toward nitrite depletion suggested that iron exerted an indirect effect on residual nitrite and subsequent botulin growth and toxigenesis.

The results of this study were in general agreement with the observations of Tompkin et al. (1978a,b). In their work, canned ham, which contained added powdered hemoglobin, had lowered residual nitrite levels, and enhanced *C. botulinum* growth. In addition, Vahabzadeh et al. (1983) reported that cured ground pork, containing added myoglobin or ferric chloride, had reduced antibotulinal activity of nitrite. Data further demonstrating that iron and nitrite interactions are important for the

inhibition of *C. botulinum* were presented by other research groups. After showing that nitrite inactivated the phosphoroclastic system (Simmons and Costilow, 1962), Woods and Wood (1982) hypothesized that nitrite interacted with intracellular iron-bound protein in *C. botulinum*. The phosphoroclastic system converts pyruvate to acetate through a series of ATP-generating coupled oxidation-reduction reactions. The inactivation of this system by nitrite deprives the organism of energy and results in its death. Data supporting the interaction of nitrite and intracellular iron were provided by Reddy et al. (1983) who demonstrated the binding of sodium nitrite to clostridial iron-sulfur centers. Carpenter et al. (1987) extended the work by showing that nitrite inactivated clostridial ferredoxin and pyruvate-ferredoxin oxidoreductase, key proteins in the phosphoroclastic system.

Published reports on the mechanism of bacterial inhibition by nitrite, especially of *C. botulinum*, are numerous (see Benedict, 1980 for review). In particular, chemical interactions occurring between iron and nitrite have been studied both as a botulin inhibitor (Perigo and Roberts, 1968; Huhtanen and Wasserman, 1975; Woods and Wood, 1982) and stimulant (van Roon and Olsman, 1977; Tompkin et al., 1978a,b; 1979). Generalizations derived from these and other works become confounded when ionic or covalently and coordinately bound iron are substituted into otherwise identical matrices (Vahabzadeh et al., 1983). The data from our present experiment demonstrated that blood fractions interfered with antibotulinal activity of sodium nitrite. They were consistent with the hypothesis that iron in blood fractions extracellularly binds to nitrite and prevent its entry into the clostridial spore. Our data suggested, however, that the effect on nitrite depletion may be indirect. Our results may explain Lücke's (1985) observation that blood sausages were associated with food borne botulism in Germany. Thus, it is advisable to include additional microbial growth barriers when iron-containing compounds are added to cured meats.

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