CLOSTRIDIAL GROWTH INHIBITORS, DERIVED FROM NITRITE.

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INTRODUCTION.

Since years the use of nitrite, a curing agent for meat products, is subject of discussion. Our research was conducted to find compounds, derived from nitrite, which may partially or fully replace this agent, without increasing the risk of botulism. The first investigations concerned Perigo-type inhibitors. A second group consisted of some C- and S-nitrosation products. Thirdly, ethylnitrolic acid, formed by reaction with sorbic acid, was investigated.
At first the selected compounds were tested for anticlostridial action in a culture medium. Those substances which passed this screaning test were further investigated in a pasteurized pork product. The tests were conducted with Clostridium sporogenes spores.
This paper is an abstract from the author's thesis (van Roon, 1979).

MATERIALS AND METHODS.

Chemical procedures.

The Perigo-type reaction products black Roussin salt and dicysteyl-di-nitric oxide-ferrate were prepared according to the procedures of Pawel (1882) and van Roon and Olsman (1977), respectively. The methods of Mirna and Hofmann (1969) and Meyer (1875) were used for the preparation of S-nitric oxide-cysteine and ethylnitrolic acid respectively. The two Perigo-type compounds were analyzed quantitatively by spectrophotometry (van Roon, 1979). Free nitrite was estimated by a Griess reagent method (ISO 2918, 1975), non-heme protein bound nitrite by a procedure of Olsman and van Leeuwen (1977).

Bacteriological procedures.

The Clostridium sporogenes strain 945 spore suspensions were prepared and stored according to Grever (1975). Before inoculation the spores were heat shocked (10 min at 80°C). The culture medium experiments were all conducted in Miller-Prickett tubes containing (in g/l) tryptone (15), yeast extract (10) and agar (15). This medium will be abreviated TYA. The tubes were inoculated with spore numbers ranging from 10^7 to 10 per tube (with 15 ml TYA) and incubated at 30°C.
The inhibitory level was defined by the concentration that allowed development of only a few colonies in the 10^7 - and 10^6-tubes after 2 weeks of incubation. Clostridial counts in the pork product were made by a pour plate technique, applying TYA at pH 7.0 (Grever, 1975) and incubated anaerobically.

Experiments with the pasteurized pork product.

The test compounds and spores were added together with the brine to minced lean ham meat (8 mm plate). After mixing during 5 min of 150 or 400 g of brine with 1.0 or 2.0 kg of meat respectively in a bowl chopper, the dough was canned (100 or 200 g cans respectively) and stored overnight at 8°C.
First of all the uninoculated batches were prepared, followed by those with an inoculum of 10^3 heat shocked spores per g of product. Before storage at least three of the cans were equipped with thermocouples. The next day the cans were pasteurized to a centre temperature of 68 to 69°C. Cooling took place with running tap water until centre temperatures below 30°C were measured. The cans were placed in an incubater at 30°C. The moment of swelling of the incubated cans was the criterion of the inhibitory effect of the tested compounds. Enumeration of samples of the swollen inoculated cans always showed 10^4 to 10^8 clostridia per g of product. The final product had the following composition: protein 175 g/kg, moisture 750 g/kg, polyphosphates 3 g P_2O_5/kg and NaCl 22 - 26 g/kg. The input concentration of test compounds is given per experiment.

RESULTS.

Perigo-type inhibitors.

Earlier research with culture media proved the involvement of sulfur-iron-nitric oxide-complexes in this type of inhibition (van Roon, 1979). Two types of complexes were studied. The inhibitory level of black Roussin salt in TYA at pH 7.0 was 42 µmol/l (25 mg/l). Dicysteyl-di-nitric oxide-ferrate inhibited clostridial growth in a concentration of 180 µmol/l (64 mg/l) at pH 7.0 and 63 µmol/l (22 mg/l) at pH 6.2.
The research was continued with the emphasis on the dicysteyl-ferrate. This complex was formed in a solution containing cysteine, Fe(II)-ions, nitrite and ascorbic acid at pH 6.0 at all test temperatures: 1, 18, 30, 75, 110 and 115°C. However, at the latter two temperatures also black Roussin salt was produced (van Roon, 1979). These experiments indicated that in cured meat products, which contain non-heme bound iron in the muscle tissue (Hamm and Bünnig, 1974) formation of the dicysteyl-ferrate may be expected and in sterilized meat products black Roussin salt too.
Experiments in which cysteine was replaced by myofibrillar protein, proved that the dicysteyl-ferrate was also formed, but tightly bound to the cysteyl residues of the protein (van Roon, 1979). Addition of this complex to the pork product (0,58 mmol/kg, 208 mg/kg) showed no clostridial growth inhibition at all (van Roon and Olsman,

1977; van Roon, 1979). The product displayed a greish colour. Huhtanen et al. (1977).proved the inability of black Roussin salt to inhibit clostridial growth in a heated, cured meat product.

The determination method for non-heme, protein bound nitrite not only estimates the content of S-nitrosation products (Olsman and van Leeuwen, 1977) but also the concentration of the dicysteyl-ferrate (van Roon, 1979). As the S-nitrosation products in the heated cured meat are labile (Olsman, 1977), the protein bound nitrite content will decrease during the incubation period of the cans, until a constant level is obtained. This level originates from the dicysteyl-ferrate formed in the product from the non-heme bound iron fraction in the muscle tissue (about 0.1 mmol/kg). The constant level of non-heme, protein bound nitrite was found to be between 0.1 and 0.2 mmol/kg, which corresponds with 0.05 to 0.1 mmol dicysteyl-ferrate/kg.
Apparently a major part of the non-heme protein bound iron-fraction is converted into this Perigo-type compound, which is not inhibitory in the meat product.

C-and S-nitrosation products.

Four C-nitrosation products were tested for their anticlostridial abilities in TYA at pH 6.2. All compounds, 3-nitrotyrosine, 3,4-dihydroxyphenylalanine (both derived from tyrosine) and creatinine-5-oxime and 1-methyl-hydantoin-5-oxime (both derivatives from creatinine) showed no inhibition (van Roon, 1979).
In earlier experiments by Incze et al. (1974) and Moran et al. (1975) S-nitric oxide-cysteine proved to inhibit clostridial growth in culture media. Experiments with the pasteurized pork product showed this compound to be active. In one experiment 1,43 mmol/kg (216 mg/kg) had the same efficacy as 1,45 mmol $NaNO_2$/kg (100 mg/kg). Both are input concentrations. In another one 3.00 mmol S-NO-cysteine/kg (450 mg/kg) was not as good as 2.90 mmol $NaNO_2$/kg (200 mg/kg). However, two combinations of both inhibitors (the sum of the input concentrations was 2.96 mmol inhibitor/kg) were as effective as nitrite (2.90 mmol/kg) alone (van Roon, 1979). All products displayed a pink colour, except for the batches without nitrite. The pH of the products ranged from 5.9 to 6.2. S-NO-cysteine might offer a possibility to replace nitrite. However, this compound is labile and it appears to have transnitrosating abilities (Massey et al., 1979).

Reaction products with sorbic acid.

Namiki and Kada (1975) discovered that one of the reaction products (ethylnitrolic acid) was a good inhibitor for E. coli in a culture medium. It proved to inhibit the clostridial spores in TYA at pH 6.2. A concentration of 0.35 mmol ethylnitrolic acid/l (37 mg/l) had an equal inhibitory action as 2.7 mmol $NaNO_2$/l (187 mg/l). Addition of 0.96 mmol ethylnitrolic acid/kg test product (100 mg/kg) however did not inhibit clostridial growth (van Roon, 1979).
In the same experiment 1 g sorbic acid/kg product was tested for inhibition in combination with increasing input concentrations of $NaNO_2$ (0, 50, 100 and 150 mg/kg) and $NaNO_2$ alone (150 mg/kg). Compared with the inhibitor control batch in all other batches inhibition was observed. The more nitrite was added the better the anticlostridial action, but addition of nitrite alone resulted in the strongest inhibition. All batches containing nitrite or ethylnitrolic acid displayed a pink colour. The pH of the product was 6.3.

Residual free nitrite level and inhibition.

In several batches of products with added nitrite and S-NO-cysteine a significant correlation ($r = 0.936$, $n = 8$; $r = 0.995$, $n = 4$) was observed between this level at the moment the incubation was started and the average swelling time of cans of these batches in days. The higher this level, the better was the inhibition (van Roon, 1979). The other batches could not be used for the calculations as the incubation period was terminated before all cans were swollen. The results agree with the hypothesis of Christiansen et al. (1978) that clostridia and product compounds compete for residual free nitrite. A similar indication was observed by Tompkin et al. (1978[a]) in their study on the effect of refrigeration of the cans during several days prior to incubation.

DISCUSSION.

In heated cured meats iron-nitric oxide complexes are formed. A part of the added nitrite is bound to heme-iron. According to Bousset and Fournaud (1976) for full saturation 0.5 mmol $NaNO_2$/kg lean pork is required. Another part reacts with non-heme iron. This reaction produces a dicysteyl-ferrate in the heated products and black Roussin salt in sterilized products only. Both complexes are tightly bound to meat protein. The latter compound was detected in a luncheon meat in the small concentration of 14 µmol/kg (van Roon, 1979). The applied heat treatment ($F_9 = 0.5$) releases only a small amount of H_2S, necessary for the production of this complex. Non-heme, protein bound nitrite determinations showed that 0.05 to 0.1 mmol dicysteyl-ferrate/kg pasteurized product was present. The iron-nitric oxide-complexes consume input nitrite, resulting in a reduction of the residual free nitrite concentration in the product after heat treatment. This concentration seems to be important for the clostridial inhibition in the product. The effect of Fe(II)-addition (50 mg/kg) on clostridial inhibition in a pasteurized beef product was reported by van Roon and Olsman (1977). This addition resulted in a higher protein bound nitrite level, a lower residual free nitrite level and a poorer anticlostridial action. A similar result was observed by Tompkin et al. (1978[b]). Indeed, both heme and non-heme iron are involved in the formation of complexes at the cost of nitrite necessary for growth inhibition. In heated pork products about 0.7 mmol input nitrite/kg lean meat (50 mg/kg) is involved in the production of these complexes.
Besides iron-nitric oxide-complexes also S-nitrosation products are formed in heated cured meats. Our experiments proved that added S-nitric oxide-cysteine had about the same efficacy in controling clostridial growth as nitrite itself. Experiments by Lee et al. (1978) showed that S-nitrosation products are not essential for growth inhibition . Olsman (1977) postulated that S-nitrosation products merely act as a temporary reservoir of nitric oxide, to be followed by other reactions (e.g. transnitrosation), which mainly are still unknown. It is interesting to observe that addition of S-NO-cysteine to the pork product resulted in the formation of residual free nitrite. The anticlostridial action in the incubated cans could be related to the free nitrite level after heat treatment. Probably not only nitrite itself, but also other reaction products (some with inhibitory abilities?) from S-nitrosation compounds may react with the Griess reagent.
The inhibitory behaviour of ethylnitrolic acid in the test product was very disappointing. This compound is known

to become unstable at 80°C (Meyer, 1875). Decomposition products are acetic acid, hydroxylamine, nitrous acid and nitrous oxide. (Maschka and Mirna, 1951).
Our experiments with sorbic acid produced disappointing results. Only one g sorbic acid/kg product was used, as to comply with Dutch regulations,for other foods (sorbic acid addition to meat products is not allowed). The main effect of sorbic acid seems to be the inhibition of spore germination (Sofos et al., 1979), but apparently this effect needs a lower pH than 6.3 and a higher sorbic acid concentration. Our experiments showed that nitrite was the more important factor to control clostridial growth.
Our research has not given a solution for an adequate replacement of nitrite by other compounds. It may give a contribution to the knowledge and the mysteries of the effect of nitrite on the control of clostridial growth in heated cured meat products.

REFERENCES.

Bousset, J., Fournaud, J., 1976. L'emploi des nitrates et des nitrites pour le traitement des produits carnés: aspects technologiques et microbiologiques. - Ann. Nutr. Aliment. 30: 707-714.

Christiansen, L.N., Tompkin, R.B., Shapiris, A.B., 1978.Fate of Clostridium botulinum in perishable canned cured meat at abuse temperature. - J. Food Prot. 41: 354-355.

Grever, A.B.G., 1975. Onderzoek naar de vorming van remstoffen voor Clostridium sporogenes in natriumnitriet bevattende voedingsbodems en in vleeswaren. - CIVO-rapport R4692.

Hamm, R., Bünnig, K., 1974. Die Verteilung des Eisens im Muskelgewebe von Schwein und Rind. - Z. Lebensm. Unters. Forsch. 156: 9-15.

Huhtanen, C.N., Dymicky, M., Wasserman, A.E., 1977. Inhibition of Clostridia by iron nitrosylsulfides and citric acid in canned ham, p. 47-52. In: Tinbergen, B.J. and Krol, B.(ed). Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist 1976. Pudoc, Wageningen, The Netherlands.

Incze, K., Farkas, J., Mihályi, V., Zukal, E., 1974. Antibacterial effect of cysteine-nitrosothiol and possible precursors thereof. - Appl. Microbiol. 27: 202-205.

ISO 2918, 1975 - Meat and Meat Products. The determination of nitrite content.

Lee, S.H., Cassens, R.G., Sugiyama, H., 1978. Factors affecting inhibition of Clostridium botulinum in cured meats - J. Food Sci. 43: 1371-1374.

Maschka, A., Mirna, A., 1951. Kinetische Untersuchungen über den Zerfall der Äthylnitrolsäure in säurer Lösung. - Monatsh. Chem. 82: 84-94.

Massey, R.C., Dennis, M.J., Crews, C., Davies, R., Mc Weeny, D.J., 1979. Model system studies on N-nitrosamine formation in relation to cured meat: the non-polar phase and S-nitroso peptides - Sixth Meeting Anal. Formation N-nitroso compounds, Budapest, 1979.

Meyer, K., 1875. Ueber die Nitroverbindungen der Fettreihe - Justus Liebigs Ann. Chem. 175: 88-140.

Mirna, A., Hoffmann, K., 1969. Über den Verbleib von Nitrit in Fleischwaren. I. Mitteilung. Umsatz von Nitrit mit Sulfhydryl-Verbindungen. - Fleischwirtschaft 49: 1361-1366.

Moran, D.M., Tannenbaum, S.R., Archer, M.C., 1975. Inhibitor of Clostridium perfringens formed by heating sodium nitrite in a chemically defined medium. - Appl. Microbiol. 30: 834-843.

Namiki, M., Kada, T., 1975. Formation of ethylnitrolic acid by the reaction of sorbic acid with sodium nitrite. - Agric. Biol. Chem. 39: 1335-1336.

Olsman, W.J., 1977. Chemical behaviour of nitrite in meat products. I. The stability of protein-bound nitrite during storage, p. 101-109. In:Tinbergen, B.J. and Krol, B.(ed). Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist 1976. Pudoc, Wageningen, The Netherlands.

Olsman, W.J., Leeuwen, C.M. van, 1977. The assay of protein-bound nitrite in meat products. - Z. Lebensm. Unters. Forsch. 164:234-242.

Roon, P.S., van, Olsman, W.J., 1977. Inhibitory effect of some Perigo-type compounds on Clostridium spores in pasteurized meat products, p.53-61. In:Tinbergen, B.J. and Krol, B. Proc. 2nd int. Symp. Nitrite Meat Prod., Zeist 1976. Pudoc, Wageningen, The Netherlands.

Roon, P.S. van, 1979. Effect of reaction products from nitrite on Clostridium sporogenes in heated cured meats.- Dissertation, University of Utrecht, Utrecht. The Netherlands.

Sofos, J.N., Busta, F.F. Allen, C.E., 1979. Botulism control by nitrite and sorbate in cured meats: a review.- J. Food Prot. 42: 739-770.

Tompkin, R.B., Christiansen, R.N., Shapiris, A.B., 1978[a]. Effect of prior refrigeration on botulinal outgrowth of perishable canned cured meat when temperature abused. - Appl. Environ. Microbiol. 35: 863-866.

Tompkin, R.B.,Christiansen, R.N., Shapiris, A.B., 1978[b]. The effect of iron on botulinal inhibition in perishable canned cured meat.-J. Food Technol. 13: 521-527.