

DETECTION OF GENOTOXICITY IN FRIED BACON BY THE SALMONELLA/MAMMALIAN MICROSOME MUTAGENICITY ASSAY

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Abstract—The potential for mutagen formation in fried bacon and the possible reduction or elimination of this hazard was examined in the Salmonella/mammalian microsome mutagenicity assay using *Salmonella typhimurium* strain TA98. Alkaline dichloromethane extracts were prepared from green pork bellies, commercial bacon (nitrite-treated and nitrite-free), and pilot-plant bacon (nitrite-free). When fried, all forms of bacon and the green belly samples gave positive mutagenic responses with the plate-incorporation technique. Unfried samples were not mutagenic. Aroclor-activated rat-liver S-9 fractions plus NADPH were essential to demonstrate a mutagenic response. When the frying temperature was held constant (171°C) maximum mutagen formation was observed in samples fried for 6 min; when samples were fried for 6 min a mutagenic response which increased with temperature, in a linear manner, was observed at temperatures above 125°C. Volatile nitrosamines were not detected in the bacon samples. The data indicate the generation of one or more mutagens in fried bacon and green pork belly, the levels of which can be reduced by decreasing heating temperature and/or time.

INTRODUCTION

Two lines of presumptive evidence suggest that certain dietary components may contribute to the aetiology of human cancer. The epidemiological data considered by Doll & Peto (1981), who reported on the avoidable causes of human cancer, suggested that, as a risk factor, diet contributed 35% toward all cancers in the United States. Second, mutagenic and carcinogenic compounds have been identified in some foods and it has been suggested that they may be oncogenic initiators, particularly in the aetiology of colon cancer (Weisburger, Cohen & Wynder, 1977).

Polycyclic aromatic hydrocarbons (Lijinsky & Shubik, 1964), mycotoxins (Wilson & Hayes, 1973), nitrosamines (Magee & Barnes, 1967), and amino acid and protein pyrolysis products (Nagao, Honda, Seino *et al.* 1977; Sugimura & Nagao, 1979) are the major classes of suspect human carcinogens that have been well-characterized in foods. In addition, mutagenic compounds have been detected, using the Ames Salmonella test, in various food products cooked at moderate temperatures. Spingarn, Kashi, Vuolo *et al.* (1980) isolated and identified a nitrogenous heterocyclic compound, which was generated during the

cooking of beef and caused frameshift mutations. This compound, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), and its methyl derivative (MeIQ) were also detected in broiled fish (Kasai, Yamaizumi, Wakabayashi *et al.* 1980). Unidentified mutagens have been observed in a variety of foods including: chicken broth, beef broth, evaporated milk, bakery and pasta products, breakfast cereal and Worcestershire sauce (Levin, Blunt & Levin, 1981; Pariza, Ashoor & Chu, 1979a).

Various investigators have proposed that Maillard reactions may be responsible for the formation of mutagens observed in cooked beef. Spingarn & Garvie (1979) observed mutagenic end-products when sugars and ammonia were heated together in a model system. Ashoor, Dietrich, Chu & Pariza (1980), demonstrated that more mutagen was formed in proline-fortified fried ground beef than in unfortified control samples.

Temperature is an important factor for the initiation of mutagen formation during cooking. Dolora, Commoner, Vithayathil *et al.* (1979) showed that mutagen formation occurred at the surface, but not in the centre portions of hamburgers fried at 190°C to 200°C. Mutagenic activity was not detected at either location when samples were heated in a microwave oven.

Since bacon is thinly sliced, its internal temperature during cooking readily approaches that of the surface, a fact which would be expected to increase the potential for mutagen formation. Moreover, bacon is cured with a brine solution containing significant amount of sugar which contributes to the character

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Abbreviations: B[a]P = Benzo[a]pyrene; DCM = dichloromethane; DMSO = dimethylsulphoxide; NDMA = nitrosodimethylamine; NPYR = nitrosopyrrolidine.

browning and flavour development in the product. Because bacon incorporates those factors thought to be important in the generation of mutagens during cooking, we undertook the present investigation to assess the product's mutagen-forming potential, and establish how the level of genotoxic activity could be reduced or eliminated.

EXPERIMENTAL

Bacon samples. Initial studies used commercially processed nitrite-treated and nitrite-free bacon. Subsequent experiments used nitrite-free bacon produced in our pilot plant to control composition and processing variables. The pilot plant bacon was prepared by pumping 4–5 kg pork bellies to a weight 10% above green (unprocessed) weight using a Koch stitch pump injector. The curing solution consisted of 77% distilled/deionized water, 3% sodium tripolyphosphate, 5% sucrose, and 15% food-grade sodium chloride by weight. The bellies were cured overnight at 0.5°C, and then processed by a 5-hr cooking/smoking schedule to an internal temperature of 53°C. The finished product was stored overnight at 0.5°C, tempered the following day to approximately –4°C, and then sliced into 3-mm strips and stored at 4°C.

Final preparation of the bacon samples involved equilibration of the bacon slices to room temperature and then frying in a preheated Teflon-coated electric skillet calibrated to 171°C. Strips were cooked for a total of 6 min, and were turned at 2-min intervals. Modifications to this basic regime in some experiments are noted in the experimental design section. Between batches the skillet was cleaned of bacon drippings and char. Fried bacon samples were stored at –18°C until extracted.

Mutagen extraction. The mutagen extraction scheme was modified from that of Felton, Healy, Stuermer *et al.* (1981). Frozen bacon slices were reduced to a powder by blending with solid CO₂ in a food processor. The powder sample was transferred to a beaker and homogenized in acetone (5 ml/g) with a Brinkmann polytron homogenizer (Brinkman/Sybron, Westbury, NY). The homogenate was filtered, and the solids were re-extracted and refiltered. The acetone extracts were pooled and stored overnight at –18°C, and the cold extracts were filtered to remove precipitated proteins. Solvent was removed by evaporating *in vacuo*.

The residue was dissolved in 0.01 N-HCl (pH 2), and extracted three times with 2 ml DCM/g. This fraction was discarded since preliminary studies had indicated that this fraction exhibited no mutagenic activity. The aqueous portion was adjusted to pH 10 with NaOH and re-extracted with DCM. This alkaline/organic extract was pooled and concentrated *in vacuo*. Extracted samples were stored at –18°C in 1 ml of DCM. Before the mutagenicity assay, a small portion of the sample was removed, evaporated under nitrogen, and redissolved in dimethylsulphoxide (DMSO).

Mutagenicity assay. The histidine-requiring strain TA98 of *Salmonella typhimurium* (the gift of Professor B. N. Ames) was used as the tester organism. Strain TA98 has a *his* D3052 frameshift (–2) mutation incorporated into its *his* operon, rendering the organism incapable of synthesizing histidine. Addition or

deletion mutations that correct the lesion permit endogenous histidine anabolism. The plate incorporation method of Ames, McCann & Yamasaki (1975) with minor modifications was used to detect mutagen-induced His⁺ revertants. Minimal medium consisted of 400 mM-K₂HPO₄, 150 mM-MgSO₄, 15 mM-sodium citrate, 75 mM-(NH₄)₂SO₄, 1.5% agar, and 220 mM-glucose. Top agar (0.5%) consisted of 0.6% NaCl, and trace amounts of histidine and biotin. Molten (50°C) top agar was distributed in 2-ml portions into tubes, and then supplemented with 10⁸ cells, 0.1 ml DMSO containing control or test fractions, and 0.5 ml S-9 mix (2.8 mg protein) or phosphate buffer. The S-9 mix was prepared from Aroclor 1254-induced rat livers. The top agar mixtures were immediately overlaid on preprepared minimal medium plates. Plates were scored for histidine prototrophy after incubation at 37°C for 48 hr. B[a]P (10 µg) and 2-acetylaminofluorene (10 µg) were used as positive controls yielding 288 ± 102 (\bar{x} ± SD) and 458 ± 75 revertants/plate, respectively. Solvent controls yielded 25 ± 5 revertants/plate. A sample was considered positive if it produced a two-fold increase above the spontaneous reversion rate, and exhibited a dose-response relationship. Prototrophy on experimental plates was confirmed by randomly picking colonies, replating on biotin-supplemented, histidine-free plates, and observing growth after 48 hr at 37°C. Results are expressed as numbers of TA98 revertants per gram equivalent (gE) of cooked bacon.

Experimental design. Preliminary experiments with commercial bacon samples were used to establish the conditions required to demonstrate mutagenic activity and to determine the fraction in which it was present. Two commercial nitrite-free bacon samples and five nitrite-treated samples from separate processing plants were obtained from a local store and fried at 200°C for 12 min to a well-browned state. Extracts of the cooked bacon were tested for mutagenicity using *S. typhimurium* strain TA98 with and without S-9 mix. In subsequent experiments two matched pairs of pork bellies from the pilot plant were tested, one side of each pair being tested green (unprocessed) while the other was manufactured into nitrite-free bacon. All of the samples were tested both fried and unfried.

The activation requirements for the detection of mutagenic activity in fried bacon were studied in a series of tests in which the constituents of the S-9 mix were varied (see Results section).

Further experiments were performed to determine the optimum temperature and cooking time for mutagen production. In the first experiment nitrite-free bacon strips were fried at 171°C for 0, 2, 4, 6, 8 or 10 min. In the second experiment the bacon strips were fried for 6 min at 25, 125, 150, 175, 200 or 225°C.

Nitrite and nitrosamine assay. Residual sodium nitrite content was determined using 10 g samples assayed by the Fiddler modification of the Griess–Saltzman procedure (1977). The minimum detectable level was 1 µg/g. Volatile nitrosamines were analysed in selected samples by the mineral oil distillation procedure described by Fiddler, Gates, Pensabene *et al.* (1981), and were measured using a gas chromatograph (Varian Series 2700, Palo Alto, CA) equipped with a thermal energy analyser (Thermo Electron 502,

Waltham MA). The chromatograph conditions were as follows: column 9ft \times 1/8 in. 15% Carbowax 20-M TPA 60/80 mesh gas chron P; program; from 120°C to 220°C at 4°/min; flow-rate, He:35 ml/min; injection port temperature, 200°C; detector, TEA—cold temperature -115°C (LN₂/EtOH). The minimum detectable level was 1 ng/g.

Thin-layer chromatography. Thin-layer chromatography of B[a]P (Aldrich Chemical Co., Milwaukee, WI) and the alkaline/organic fraction of bacon samples was carried out to ascertain where B[a]P was a constituent of the mutagenic fraction. Silica-gel plates (Analtech, Newark, DE; 250 μ m) were used in conjunction with an ether-hexane-acetic acid (20:80:1) solvent system, 50% sulphuric acid followed by heating was used to detect the organic compounds present on the TLC plate.

RESULTS

Preliminary experiments using fried commercial bacon extracts showed that one or more mutagenic components were present in the basic/organic (DCM) fraction, while no mutagenic activity was apparent in the acidic and aqueous fractions. No mutagenic activity was detected in unfried bacon extracts, nor was activity present in the frying drippings. Genotoxicity was evident with frameshift-sensitive tester strains, and biotransformation by S-9 preparations was essential for detecting the activity.

When extracts from cooked commercial samples of bacon (up to 55 gE) were tested for mutagenicity using TA98 with and without S-9, plates treated with S-9 and bacon extracts showed a roughly 35-fold increase in revertants at 55 gE as depicted in the dose-response curve in Fig. 1. Without S-9 all concentrations of bacon extracts showed only background revertant levels. No significant differences in mutagenic activity were noted with five nitrite-treated and two nitrite-free commercial bacon samples that were fried under similar conditions.

Fried samples from the pork bellies prepared in our pilot plant showed a four-fold increase in revertants, at 4 gE, fried for 6 min at 171°C to 106 ± 15 rever-

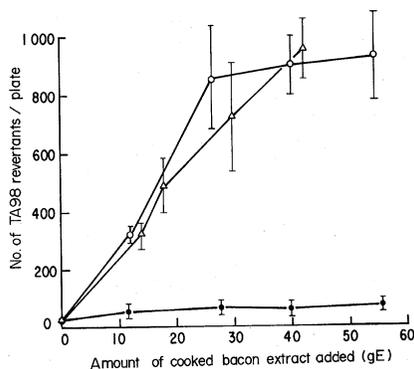


Fig. 1. The dose-response relationship between the concentrations of commercial bacon extracts and mutagenic activity as detected using *Salmonella typhimurium* strain TA98: nitrite-free bacon with S-9 mix (○); nitrite-free bacon without S-9 mix (●); nitrite-treated bacon with S-9 mix (△). The bacon was cooked for 12 min at 200°C.

tants/plate (mean \pm SD) while unfried samples exhibited background mutation levels (27 ± 5 revertants/plate). Matched pairs of green bellies and treated bacon yielded similar mutagenicity profiles when fried.

The results of the studies of the cofactors required for activation of the mutagenic component(s) showed a strict requirement for metabolic activation. Metabolizing mixtures without NADP⁺ did not activate the promutagenic fraction. S-9 from uninduced liver produced background mutation rates, while heat-treated (boiled) Aroclor-activated liver S-9 produced fewer revertants than did the controls. Elimination of either KCl, MgCl₂, or glucose-6-phosphate (G-6-P) did not reduce biotransformation significantly, indicating either that these cofactors were not required for activation or they were available from the bacterial medium or liver cytosol. Maximum mutagenic response required an active induced mixed-function oxygenase system plus a source of NADPH. It was also determined that 5.7 mg protein/plate yielded maximum mutagenic response (Fig. 2). Deviations from this concentration resulted in decreased activity.

When nitrite-free bacon strips were fried at 171°C for 0 to 10 min, turning the strips at 2-min intervals, mutagenic activity was maximal after 6 min (Fig. 3),

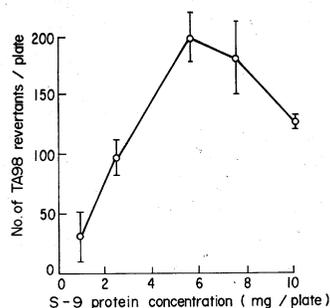


Fig. 2. The effect of the amount of S-9 protein on the level of mutagenic activity detected in fried bacon. The nitrite-free bacon (4 gE/plate) was fried for 6 min at 17°C and tested using *Salmonella typhimurium* strain TA98.

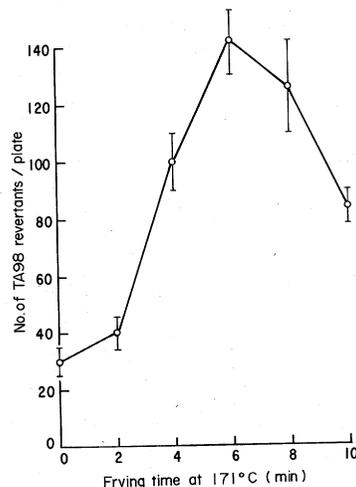


Fig. 3. The effect of frying time at 171°C on mutagenic activity in nitrite-free bacon (4 gE/plate) tested using *Salmonella typhimurium* strain TA98 with S-9 mix.

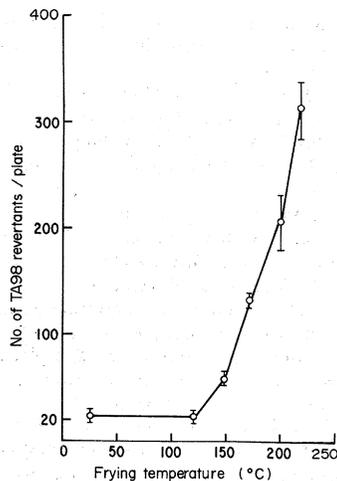


Fig. 4. The effect of frying temperature on the formation of mutagenic activity in nitrite-free bacon (4 gE/plate) fried for 6 min tested using *Salmonella typhimurium* strain TA98 with S-9 mix.

and further frying decreased the number of revertants/plate. Reduced mutagenicity associated with extended cooking times may have been due to thermal decomposition or volatilization of the mutagen(s). When cooking time was held constant at 6 min while cooking temperature was varied between 125° and 225°C unfried samples and samples fried at 125° C yielded background reversion rates, while mutagen formation increased linearly with temperature at cooking temperatures between 150° and 225°C (Fig. 4).

Assays for volatile nitrosamines indicated that nitrosodimethylamine (NDMA) levels were < 1 ng/g, and nitrosopyrrolidine (NPYR) was not detected in pilot-plant bacon. Nitrite levels were < 1 µg/g in nitrite-free bacon. This suggests that the detected mutagenic response cannot be attributed to a volatile nitrosamine.

Thin-layer chromatographic assays of B[a]P (Aldrich, Milwaukee, WI) and the alkaline/organic fraction of a bacon sample demonstrated that B[a]P was more nonpolar than any of the constituents of the bacon fraction, and had a significantly different R_f value.

DISCUSSION

Recent research has established that mutagens can be formed during the cooking of a variety of foods (Felton *et al.* 1981; Nagao *et al.* 1977; Pariza *et al.* 1979a; Sugimura & Nagao, 1979). It seems probable that most fried meat, fish, and poultry products tested in the future will show various levels of mutagenic activity. Bacon was selected for our study because it conformed to the predicted requirements for food mutagen formation (i.e. it contains browning precursors and is heated thoroughly).

Various investigators have suggested that products resulting from Maillard reactions may be the precursors responsible for mutagen formation in cooked meats (Ashoor *et al.* 1980; Shibamoto, 1980; Spingarn & Garvie, 1979). The present results however did not discern any difference between the mutagenicity in

matched pairs of cured bacon and green bellies. It is possible that sucrose, a nonreducing sugar present in the curing solution, was not involved in the browning reaction and that only endogenous reactive compounds reacted during browning. This would explain the similarity of the mutagen levels found in processed and unprocessed bellies. An alternative hypothesis is that the mutagen did not result from Maillard reactions, but from a different mechanism, e.g. generation of heterocyclic compounds from amino acids and/or proteins. We are currently investigating the precursors and mechanism of formation of the mutagenic fraction in fried bacon.

Results from this study indicate that the mutagenic activity detected is not attributable to the presence of a volatile nitrosamine. In the nitrite-free bacon only trace levels were observed of residual nitrite or of the volatile nitrosamines commonly associated with fried bacon (NDMA and NPYR). Moreover, both volatile and nonvolatile nitrosamines are generally base-pair substitution mutagens, not detected using strain TA98 (Rao, Young, Lijinsky & Epler, 1979). Finally, the detection of the mutagenic activity from nitrosamines generally requires a liquid preincubation modification of the assay (Rao *et al.* 1979), which was not included in the present study. Similarly, the organic/alkaline extract does not contain B[a]P. The fraction exhibited activation and detection characteristics similar to those noted for mutagens formed in fried beef (Spingarn *et al.* 1980). However we have not determined whether the mutagenic activity detected in the present study is attributable to a previously identified compound.

This study demonstrates that there is a critical temperature range required for mutagen formation. This is consistent with studies on cooked ground beef (Dolara *et al.* 1979; Pariza, Ashoor, Chu & Lund, 1979b). Thus, low-temperature cooking could reduce or eliminate mutagen formation. Dolara *et al.* (1979) reported that microwave-irradiated beef did not produce mutagenic activity in the Ames test. Preliminary evidence from our laboratory suggests that microwave-irradiated or steamed bacon does not contain detectable mutagenic activity. Selection of appropriate cooking methods and conditions may be a practical method of reducing genotoxins in fried bacon. We are currently investigating other cooking regimes for the potential to form and eliminate mutagens.

The health significance of dietary genotoxins has not been adequately assessed. Their presence might explain the epidemiological observations that the consumption of specific dietary components (such as fat and protein) is linked to the occurrence of some cancers (Kolonel, Hankin, Lee *et al.* 1981). Genotoxic activity has been detected in foods containing high levels of these components and investigators have attempted to link the two lines of evidence.

An assessment of the toxicological significance of the genotoxic activity observed in fried bacon first requires the isolation and identification of the active component(s). This work is currently in progress.

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