Unexpected mutagen in fish

SIR — We have previously observed powerful mutagenic activity towards *Salmonella typhimurium* TA 1535 in extracts of the Japanese fish, Sanma hiraki, treated in the laboratory with salt and nitrite at pH 3, a technique similar to the salting–pickling method for preserving fish in northern Japan, a high-risk area for stomach cancer[1,2]. Gavage of such extracts to rats induced cancer of the glandular stomach[3]. It has been generally accepted that *N*-nitroso compounds are involved in the aetiology of gastric cancer[4–6]. We have now made the unexpected observation that the mutagen isolated from salt–nitrite-treated Sanma hiraki fish is not an *N*-nitroso compound, but 2-chloro-4-methylthiobutanoic acid, as verified by techniques including NMR, infrared spectrometry and low- and high-resolution mass spectrometry.

Frozen Sanma hiraki, purchased from a local Japanese food store, was homogenized (1:2, w/v) in water. After centrifugation, NaCl was added to the supernatant to give a concentration of 2%. The solution was adjusted to pH 3 with 12 M HCl, centrifuged again, and sodium nitrite was added to a concentration of 73 mM, while the pH was maintained at 3 with 12 M HCl. After 1 h at 23 °C, excess nitrite was eliminated with an equimolar amount of ammonium sulphamate. Aliquots were taken during this and subsequent procedures to monitor mutagenicity towards *S. typhimurium* TA 1535 without rat liver S9 fraction. The mutagen was extracted from the mixture with ethyl acetate. The organic phase was partitioned with 0.1 M NaOH to move the mutagen into the aqueous layer, in order to eliminate lipids. The clear, aqueous phase was adjusted to pH 3 and extracted with methylene chloride. After removing methylene chloride *in vacuo*, the residue was dissolved in methylene chloride/hexane (1:1) and passed through a silica Maxi-Clean solid-phase extraction cartridge (Alltech, 20988). The mutagen was eluted from this cartridge with a mixture of hexane: methylene chloride: ethyl acetate (60:30:10) and further purified by three steps of high-performance liquid chromatography (HPLC), giving a single homogeneous peak. As determined by gas chromatography/mass spectrometry, the mutagen had apparent molecular ion peaks at *m/z* 168 and 170, the latter with 35% intensity of the former, indicating the presence of Cl. A possible formula, $C_5H_9ClO_2S$, was indicated by high-resolution mass spectrometry. The NMR spectrum showed signals consistent with the presence of one CH_3, two CH_2 and one CH group. The assignments were confirmed by decoupling experiments. There were no aromatic hydrogens. The infrared spectrum displayed a carbonyl group (1,726 cm^{-1}). Evaluation of the combined results revealed the fish mutagen to be 2- chloro-4-methylthiobutanoic acid (CMBA), which has not been previously described in *Chemical Abstracts*. The chemical is a slightly viscous, colourless liquid.

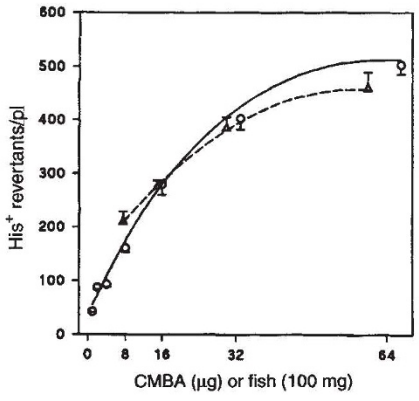

FIG. 1 Direct-acting mutagenicity in *S. typhimurium* TA 1535 produced by 2-chloro-4-methylthiobutanoic acid (μg CMBA, three plates at each dose level; circles) or extracts of Sanma hiraki fish treated with salt and nitrite at pH 3 (see text; data shown from four plates at each dose level in units of 100 mg wet weight fish; triangles). Error bars are ± s.d.

The structure suggested that the mutagen might be derived from methionine. Therefore, L-methionine was taken through the same reactions. The HPLC procedures, as applied to the fish extract, gave a peak with a retention time identical to that of the mutagen from the fish. Mass spectral and NMR data confirmed the identity of the product as CMBA, providing unambiguous, independent support for the structure of the mutagen isolated from fish (see Fig. 1). La Vecchia *et al.*[7] found a relative risk of gastric cancer of about 2.40 for the highest quintile of dietary methionine intake in a northern Italian population.

Omission of sodium chloride during the reaction failed to produce the mutagen. The reaction of the amino group of methionine with nitrite generates a reactive carbonium ion that in water yields the expected 2-hydroxy-4-methylthiobutanoic acid (Fig. 2). However, when chloride is present, it reacts with the carbonium ion to yield the 2-chloroderivative.

Protein hydrolysates in some commercial products contain mutagenic and genotoxic glycerol chlorohydrins[8,9]. Salt enhances the mutagenicity of nitrite-treated extracts of black beans, perhaps through a similar mechanism, in model studies bearing on aetiological factors for gastric cancer in Latin America[9]. Monochloracetic acid, the simplest compound in this series, is neither mutagenic[10] nor carcinogenic in mice and rats[11]. Thus, the appreciable mutagenicity and activity in the DNA repair test in hepatocytes of Williams of 2-chloro-4-methylthiobutanoic acid indicate that this molecule has genotoxic attributes based on the possible formation of reactive carbonium or sulphonium ions.

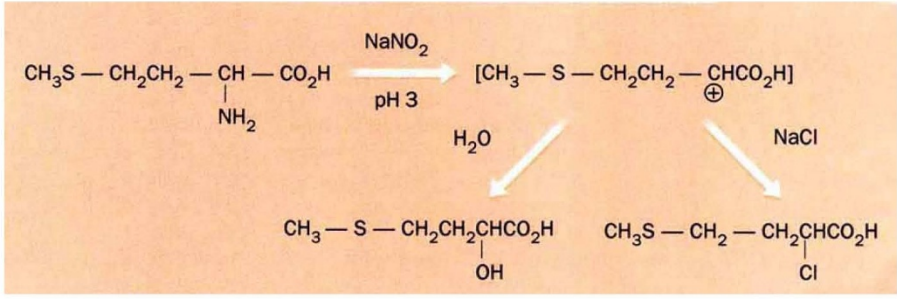

FIG. 2 Suggested scheme for the conversion of methionine to mutagen.

Thus, treatment of fish with both salt and nitrite generates a novel mutagen, an α-chlorobutanoic acid. We are now studying the role of this compound in the induction of gastric cancer.

Wei Chen
John H. Weisburger*
Emerich S. Fiala
Steven G. Carmella
Di Chen
Thomas E. Spratt
Stephen S. Hecht
American Health Foundation, Valhalla, New York 10595, USA

*To whom correspondence should be addressed.

1. Marquardt, H.F., Rufino, F. & Weisburger, J.H. *Science* **196**, 1000–1001 (1977).
2. Binkerd, E.F. & Kolari, O.E. *Fd Cosmet. Tox.* **13**, 655–661 (1975).
3. Weisburger, J.H., Marquardt, H., Hirota, N., Mori, H. & Williams, G.M. *J. natn. Cancer Inst.* **64**, 163–167 (1980).
4. Wakabayashi, K., Nagao, M. & Sugimura, T. *Cancer Surv.* **8**, 385–399 (1989).
5. O'Neill, I.K., Chen, J. & Bartsch, H. (eds) *IARC Sci. Publ. 105* (Int. Ag. Res. Cancer, Lyon, 1991).
6. Forman, D. & Shuker, D.E.G. (eds) *Cancer Surv.* **8**, 201–492 (1989).
7. La Vecchia, C., Ferraroni, M., D'Avanzo, B., Decarli, A. & Franceschi, S. *Cancer Epid. Biomarkers Prev.* **3**, 393–398 (1994).
8. Piasecki, A. Ruge, A. & Marquardt, H. *Arzneimittel-Forsch.* **40** (II), 9, 1054–1055 (1990).
9. Rojas-Campos, N., Sigaran, M.F., Bravo, A.V., Jimenez-Ulate, F. & Correa, P. *Nutr. Cancer* **14**, 1–3 (1990).
10. Ashby, J. & Tennant, R.W. *Mutagenesis* **9**, 7–15 (1994).
11. National Toxicology Program *Tech. Rep. 896* (NIH Publ. 92–2851, 1992).