Int. J. Cancer: **23**, 542-546 (1979)

PRESERVED FOODS AS POSSIBLE CANCER HAZARDS: WA RATS FED SALTED FISH HAVE MUTAGENIC URINE

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Six batches of food traditionally and commonly consumed by southern Chinese, including two samples of dried shrimps and four samples of different species of salted fish, were tested for mutagenic properties using *Salmonella typhimurium* TA98 and TA1000. Mutagenic activities toward both tester strains were found in all preparations. In most cases, these activities were enhanced by liver microsomal activation. Urine collected from experimental rats regularly fed salted fish also showed mutagenic activity. The level of this activity decreased markedly when the experimental rats were transferred from a salted fish diet to Purina rat chow. Our data suggested the presence of mutagenic/carcinogenic substances in some local preserved foods and at least one of them, salted fish, has been suspected on epidemiological and experimental evidence to be a possible co-carcinogenic factor in the development of nasopharyngeal carcinoma in southern Chinese. Our finding is compatible with this hypothesis .

Nasopharyngeal carcinoma (NPC) has been known for well over 50 years to have a predilection for southern Chinese (Todd, 1921; Shanmugaratnam, 1971; Ho, 1972). From various epidemiological and experimental data, Ho (1975; 1976; 1978) postulates an aetiological hypothesis involving the interaction between three factors; a genetically determined susceptibility, early infection by the ubiquitous Epstein-Barr virus (EBV) and consumption of traditional preserved foods, particularly salted fish (Ho, 1971), by southern Chinese from early childhood.

As many human foods have been reported to harbour carcinogenic or procarcinogenic substances, notably nitrosamines and mycotoxins, a search for volatile nitrosamines in salted fish and other preserved food was initiated by Fong and Walsh (1971). Fong and Chan (1973*a*, *b*, 1976, 1977) later reported the finding of *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine by the gas chromatography mass spectrometry technique in several items of traditional southern Chinese food such as salted fish and dried shrimps. Huang *et al.* (1978*a*) confirmed the presence of NDMA in some samples of salted fish. Using combined gas chromatography and high resolution mass spectrometry, they further detected low levels (less than 1 μg/kg) of *N*-nitrosodiethylamine (NDEA); *N*-nitrosodibutylamine; *N*-nitrosodipropylamine and *N*-nitrosomorpholine in some salted fish samples (1978, unpublished data). The latter volatile nitrosamines are known to be potent in inducing tumours in the nasal and paranasal cavities or nasopharyngeal tube in certain animal species (Althoff *et al.*, 1974; Haas *et al.*, 1973; Pour *et al.*, 1973; Cardesa *et al.*, 1976). Four of 22 inbred Wistar Albino (WA) rats, when fed salted fish for a period of 12-24 months, developed carcinoma of the nasal cavity or maxillary sinus (Huang *et al.*, 1977 and unpublished data).

A number of rapid *in vitro* systems for detecting chemical carcinogens have been developed (Poirier, 1976). A recent evaluation of six short-term tests for predicting carcinogenicity of 120 organic chemical carcinogens by Purchase *et al.* (1978) concluded that the mutagenicity test reported by Ames *et al.* (1973, 1975) using the *Salmonella*/microsome system had the most extensive application. It is about 90% accurate in detecting a variety of chemical carcinogens as mutagens (McCann *et al.*, 1975; McCann and Ames, 1976; Purchase *et al.*, 1978). We therefore employed the Ames *Salmonella*/microsome mutagenicity test in the present study to detect mutagenic substances in some commonly consumed, locally preserved Chinese foods.

MATERIAL AND METHODS

Chemicals

Glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Sigma; Aroclor 1254 was a gift from Monsanto Chemical Co., St. Louis, Mo., USA; dimethylsulphoxide (DMSO), spectrophotometric grade, was purchased from Wako Pure Chemical Industries, Japan; aflatoxin B_1 from Calbiochem, San Diego, Calif.; NDMA from Eastman Kodak, Co., Rochester, N.Y., and XAD-2 resins from Applied Science Laboratories, Pa.

Food samples

The samples tested were traditional Chinese foods purchased from a local market. They included two samples of dried shrimps and samples of four different species of salted fish. In each experiment, food extracts were prepared by mincing the samples into fine particles and suspending them in DMSO at a concentration of 2 ml DMSO/g sample. The suspension was then shaken vigorously for 24 h and centrifuged at 2,000 *g* for 10 min at room temperature. The supernatants were then subjected to the *Salmonella*/mammalian microsome mutagenicity test (Ames *et al.*, 1975).

Received: November 22, 1978 and in revised form January 22, 1979.

TABLE I

IN VITRO MUTAGENIC ACTIVITIES OF DMSO FOOD EXTRACTS

Food	Amount (mg/plate)	Induced revertants [1]/plate					
		TA98			TA100		
		$-S_9$	$+S_9$	$+S_9+P$ [2]	$-S_9$	$+S_9$	$+S_9+P$
Dried shrimps Sample A	1.25	NS	NS	0	NS	84	105
	5	NS	NS	0	NS	105	190
	10	NS	235	0	88	68	90
	15	83	171	NS	180	68	NS
	25	73	NS	NS	115	0	NS
Dried shrimps Sample B	1.25	54	NS	NS	Sp	NS	193
	5	54	154	NS	NS	156	255
	10	NS	82	144	NS	169	167
	15	NS	Sp [3]	Sp	NS	104	121
	25	NS	129	81	NS	NS	145
Salted fish Sample C (yellow croaker)	1.25	46	127	84	NS	94	120
	5	36	82	176	NS	180	210
	10	82	99	86	NS	104	92
	15	NS	Sp	Sp	NS	117	90
	25	NS	84	NS	70	68	NS
Salted fish Sample D (white herring)	1.25	0	85	NS	0	NS	60
	5	NS	138	69	110	90	90
	10	64	128	0	80	104	120
	15	95	NS	0	80	184	140
	25	48	NS	0	82	65	66
Salted fish Sample E (croaker)	1.25	206	113	NS	NS	84	59
	5	154	143	120	NS	154	132
	10	154	NS	144	NS	182	196
	15	Sp	0	Sp	69	144	117
	25	NS	0	0	NS	129	78
Salted fish Sample F (white croaker)	1.25	NS	77	NS	130	116	110
	5	NS	125	80	192	188	126
	10	81	151	NS	126	109	183
	15	51	0	NS	110	114	60
	25	0	0	0	120	NS	61

[1] The number of spontaneous revertants (varying from 40 for TA98 to 70 for TA100) have been subtracted from the revertant values. DMSO food extracts, bacteria and, where indicated, S-9 mix, were incorporated into top agar as described in " Methods ". — [2] P, Pre-incubation procedure of Yahagi *et al.* (1977). — [3] Sp: Spoiled. — [4] Values greater than spontaneous colonies × 2 are interpreted as significant and recorded. NS: not significant.

Urine samples

Urine samples from experimental and control male and female inbred WA rats (matched for sex, age and body weight) were collected as follows:

(1) Experimental and control male and female rats were housed individually in metabolic cages especially designed to minimize contact between urine and faeces. Seventy-two-hour urine samples, from four rats of the same sex and receiving the same treatment, were collected. Experimental animals were fed daily with 25 g steam-cooked salted fish and tap water, whereas the control rats were given tap water and Purina rat chow salted to approximately the concentration found in the salted fish.

(2) Seventy-two-hour pooled urine samples were first collected from experimental rats which had been fed regularly steamed salted fish and tap water during weekdays and Purina rat chow during weekends for a period of 5 months. These same experimental rats were then transferred to a salted Purina rat chow for 10 days, after which period another 72-h pooled urine sample was collected.

The urine samples (300-400 ml) were then concentrated about 100-fold by passing through a XAD-2 column in which mutagenic substances were adsorbed and later eluted according to the method described by Yamasaki and Ames (1977). Urine concentrates were then dissolved in 3-4 ml DMSO and subjected to the mutagenicity test.

Bacterial strains

Tester strains TA98 and TA100 of *S. typhimurium*, for the detection of frameshift and base-pair substitution mutations respectively, were generously supplied by Prof. Bruce N. Ames, University of California, Berkeley, Calif. Overnight culture of bacteria in nutrient broth were used for mutagenesis assay.

Preparation of S-9 Mix

Liver homogenates (S-9) (9,000 *g* supernatant) from rats induced with a polychlorinated biphenyl

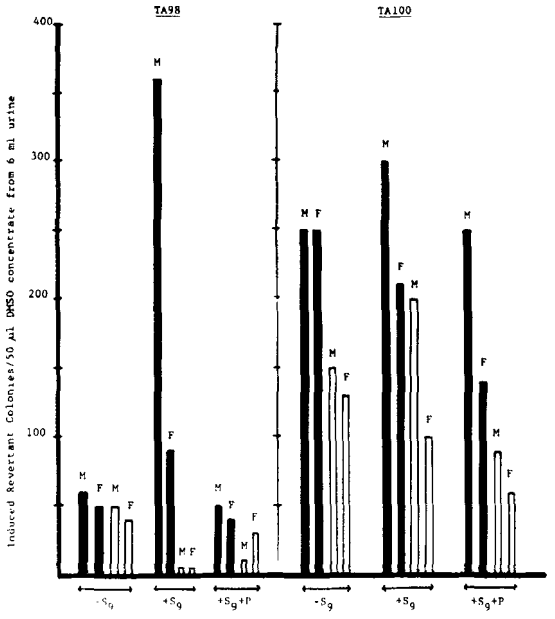

FIGURE 1 — Mutagenic activities of urine from male and female experimental and control rats. Induced revertant colonies: colonies counted minus spontaneous colonies. Solid bars represent the number of induced revertant colonies when the pooled 72-h urine concentrates from four experimental rats were added to the culture plates. Open bars represent the number of induced revertant colonies when the pooled 72-h urine concentrates from four control rats were added. P: Pre-incubation procedure of Yahagi *et al.* (1977).

mixture (Aroclor 1254) were prepared according to a method described by Ames *et al.* (1973, 1975). Briefly, a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg was given to a 200 g male Sprague-Dawley rat 5 days before killing. S-9 mix contained per ml: S-9 (0.3 ml), $MgCl_2$ (8 µmoles), KCl (33 µmoles), G-6-P (5 µmoles), NADP (4 µmoles) and sodium phosphate buffer pH (100 µmoles), 7.4. S-9 mix was freshly prepared each time and bacterial contaminants removed by passage through a sterile Swinex Filter unit (Millipore, Corp., Bedford, Mass.) equipped with a Millipore filter (0.45 µm pore size).

Mutagenesis assay

The assays were carried out as described by Ames *et al.* (1973, 1975). One-tenth ml of the bacterial tester strain and the sample to be tested and, where appropriate, 0.5 ml S-9 mix were added to 2 ml molten top agar. The contents were mixed and poured onto minimal-glucose agar plates containing a limited amount of L-histidine. After 2 days' incubation at 37° C, the revertant colonies to histidine prototrophy were counted. The sterility of the microsomal preparations and samples tested was routinely checked to ensure that there was no bacterial contamination. The validity of the bacterial strains was also checked by using known mutagens such as aflatoxin B_1 and NDMA. For the detection of mutagenicity due to *N*-nitrosamines, a procedure described by Yahagi *et al.* (1977) was employed. The test substance, S-9 mix and bacteria, were pre-incubated at 25° C for 20 min, then mixed with 2 ml

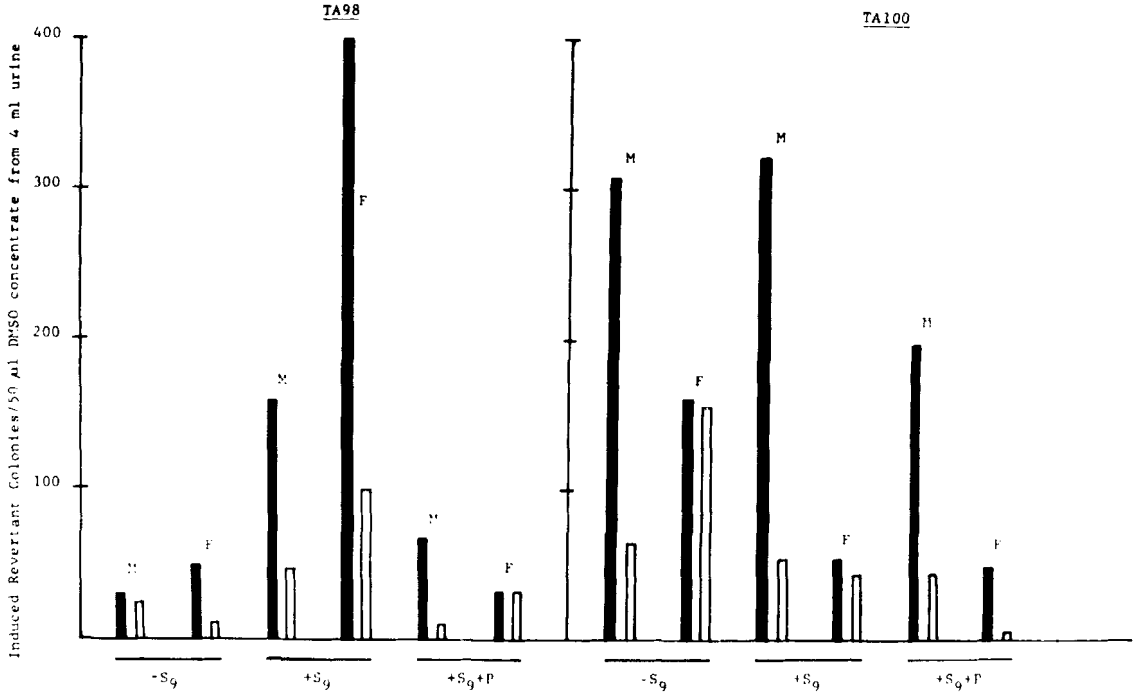

FIGURE 2 — Mutagenic activities of urine from male and female rats fed regularly with salted fish and after such feeding had been suspended. Solid bars represent induced revertant colonies while rats were being fed with salted fish. Open bars represent induced revertant colonies after the rats were transferred from a salted fish diet to salted Purina rat chow.

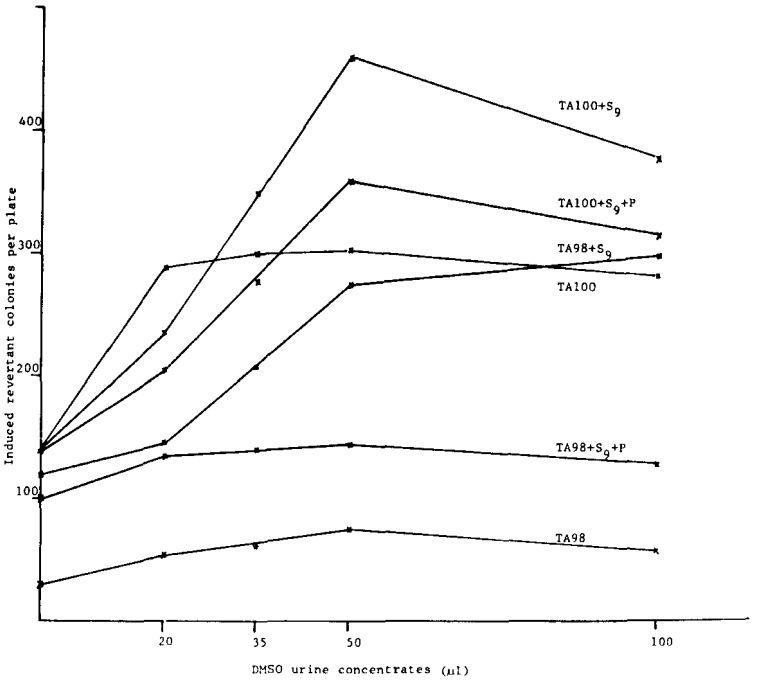

FIGURE 3 — Mutagenic dose-response curves of pooled urine from four male rats fed regularly with salted fish.

molten top agar and finally poured onto a minimal glucose agar plate.

RESULTS

The results of our Ames/microsome test on the DMSO extracts from two samples of dried shrimps and four samples of salted fish on *S. typhimurium* TA98 and TA100 are shown in Table I. All six samples showed mutagenic activities towards both tester strains.

The results of our analysis of the urine samples collected from the experimental rats fed salted fish daily for 72 h showed that their mutagenic activities were uniformly higher than those of control rats (Fig. 1). This is consistent with the results depicted in Figure 2, which showed a marked decrease in the levels of mutagenic activity of urine from experimental rats (regularly fed salted fish) after they were transferred to a salted rat chow for 10 days. Stronger mutagenic activities on TA98 were exhibited in urine concentrates with liver microsomal activation. Again, as in the case of the food preparations tested, TA100 appeared to be more sensitive than TA98 when the assay employed the procedure of Yahagi *et al.* (1977). This is also the experience of Yahagi *et al.* A linear dose-response relationship has been observed up to 50 µl DMSO urine concentrates (equivalent to about 5-6 ml urine) in the mutagenicity of the urine collected from experimental rats regularly fed a diet of salted fish (Fig. 3).

DISCUSSION

Using two tester strains of *S. typhimurium* TA98 and TA100 to detect frameshift and base-pair mutations, we have demonstrated the presence of mutagenic activity at low levels in all six items of commonly consumed local preserved food (Table I). In most cases, the activity was enhanced by liver microsomal activation and in some cases also by Yahagi's procedure of pre-incubating the bacteria with S-9 mix at 25° C for 20 min before proceeding to the test. The latter procedure has been reported to exhibit enhancement selectively in the case of *N*-nitroso compounds. It is, therefore, very likely that some of the mutagens in the samples tested contained such compounds.

A linear dose-response relationship was evident in the food extracts at low concentrations, but at high concentrations the number of induced revertant colonies decreased (Ho *et al.*, 1978). Since DMSO, in the amounts used in our experiments, had no toxic or mutagenic effects on both tester strains, this lack of dose-response relationship at high concentrations shown by the food extracts could be due to the toxic effect inherent in the extracts manifesting at such concentrations. Therefore, in on-going experiments, the dose-response relationship at higher as well as lower concentrations than those reported here is being investigated.

The mutagenic activity was even more evident in the urine from rats regularly fed the salted fish diet; since the activity decreased markedly when the same rats were transferred to a salted rat chow, it may be presumed that the mutagenic activity of their urine was derived from the consumption of salted fish.

Huang *et al.* (1978*b*) were successful in inducing carcinomas in the nasal cavity and maxillary sinus of 4/22 inbred WA rats by feeding them with salted fish for a period of 12-24 months. Subsequently Huang *et al.* (unpublished data) found squamous carcinoma developing in the maxillary sinus in one of another two WA rats similarly fed. Our present findings may explain the induction of carcinoma in

the nasal and paranasal regions of rats fed with salted fish. They also support Ho's hypothesis that salted fish is a possible co-carcinogenic factor in the development of NPC in southern Chinese, although one has to be cautious in extrapolating events in animals, especially under experimental conditions, to the human situation.

ACKNOWLEDGEMENTS

The authors are grateful for the financial assistance of the World Health Foundation (Hong Kong) and the Hong Kong Anti-Cancer Society. We thank Mrs. Seto for secretarial help and Mr. K. W. Leung for photographic assistance.

ALIMENTS CONSERVÉS ET RISQUE DE CANCER: DES RATS WA NOURRIS AVEC DU POISSON SALÉ ONT UNE URINE MUTAGÈNE

Six lots d'aliments consommés traditionnellement et communément par les Chinois du Sud, dont deux échantillons de crevettes séchées et quatre de diverses espèces de poissons salés, ont été testés du point de vue de leurs propriétés mutagènes, en utilisant les TA98 et TA100 de *Salmonella typhimurium*. Avec l'une ou l'autre souche, toutes les préparations avaient une activité mutagène. Dans la plupart des cas, cette activité était accélérée par activation microsomale au niveau du foie. L'urine des rats utilisés pour l'expérience, qui avaient régulièrement absorbé du poisson salé, avait aussi une activité mutagène, qui a sensiblement diminué lorsqu'on leur a donné une autre nourriture. Il semble donc que certaines préparations alimentaires locales contiennent des substances mutagènes/carcinogènes. D'après les données épidémiologiques et expérimentales que nous avons recueillies, nous soupçonnons que l'une d'elles au moins, le poisson salé, est un facteur cocarcinogène dans le développement du cancer du nasopharynx chez les Chinois du Sud. Nos observations sont compatibles avec cette hypothèse

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