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Mutagenic Activity of Nitrite-Treated Foods: Human Stomach Cancer May Be Related to Dietary Factors

Abstract. *By the* Salmonella typhimurium *test, extracts of Japanese raw fish treated in the laboratory with nitrite showed mutagenic activity which is prevented by addition of ascorbate. Extracts from similarly treated beef and hot dogs were nonmutagenic. The data conform to a working concept that the high stomach cancer incidence in Japanese and certain other populations may be due to specific dietary factors of an alkylnitrosamide type.*

We have described a working concept on the mechanism whereby gastric cancer in man may arise (*1*). It involves the endogenous formation of an alkylnitrosamide type of gastric carcinogen (*2*). Such chemicals can be formed in the stomach from nitrite and a suitable substrate (*3*). The sources of nitrite in the human environment have been described (*1*, *4*). In a search for the as yet unknown substrate, we have subjected to nitrosation under simulated gastric conditions several foods typically eaten by populations in countries with a high risk for gastric cancer, such as Japan, and with a low risk, such as the United States (*5*).

To provide preliminary information on the potential carcinogenicity, mutagenicity in indicator strains of *Salmonella typhimurium* was utilized (*6*). In this system, nitrosation of a type of fish commonly eaten in Japan, followed by extraction under mild conditions, yielded evidence of high mutagenic potential of this extract (Table 1). There was no increase in mutagenicity when the extract was exposed to the indicator organism in the presence of a 9000*g* supernatant of rat liver homogenate, indicating that a direct-acting mutagen, rather than one which requires metabolic activation, is formed in fish treated with nitrite. Furthermore, ascorbic acid in amounts twice equimolar to the nitrite used completely prevented the formation of the mutagenic principle. Similar treatment of homogenates of beef or hot dogs failed to give rise to measurable mutagenic activities.

Of all the *Salmonella* mutants tested, those sensitive to agents which yield base-pair substitutions were most responsive to the mutagenic principle in fish treated with nitrite (Table 2). The activity in base-pair sensitive strains and the lack of activity in the other strains is a useful guide for the future identification of the mutagenic principles present. It suggests that materials such as alkylnitrosamides and related structures can be candidates, and rules out compounds such as polycyclic or heterocyclic chemicals.

These results have dual significance. First, the absence of mutagenic activity in

Table 1. Mutagenic effects of various food extracts on *Salmonella typhimurium* strain TA 1535. Five grams of a food homogenate, including 5000 parts of sodium chloride per million (*10*), were incubated at *p*H 3 for 1 hour at 25°C with and without 5000 parts of sodium nitrite per million. Nitrosation was stopped by the addition of 5000 parts of ammonium sulfamate per million. Thereafter, the incubation mixture was extracted with hexane (twice), mainly to remove lipids, and then with ether (four times). Mutagenic activity of 10 μl of the reduced ether extract (total volume from 5 g of food homogenate, about 100 μl) was determined by spot-testing following the procedure described by Ames *et al.* (*6*). The results represent means ± standard deviations from two experiments with duplicate plates per point for hot dog and beef, from four experiments with duplicate plates per point for fish. The fish samples tested were purchased over an interval of 6 months and were from three distinct shipments. The S9 fraction is the 9000*g* supernatant of a liver homogenate from Aroclor-induced male CDF rats (*6*), with 400 μg of protein per plate. The hot dogs tested contained beef as well as pork. Beef and hot dogs were obtained from local stores. The Japanese fish (sanma hiraki) was imported deep-frozen from Japan and purchased from Main Street Foodstore, Flushing, New York. Ascorbic acid from Sigma Chemical Company was added to the fish homogenate to give a level of 28,000 parts per million, equivalent to twice the molarity of 5000 parts of nitrite per million.

Food extracts from	His^+ revertant colonies per plate	
	Without S-9 fraction	With S-9 fraction
Control	8 ± 3.5	8 ± 3.5
Methylnitrosourea (10 μg per plate)	280 ± 2.4	249 ± 8.5
Japanese raw fish	3 ± 2.1	7 ± 2.8
Japanese raw fish + nitrite	297 ± 8.5	290 ± 25.0
Japanese raw fish + nitrite + ascorbic acid	7 ± 0.7	
Hot dog	4 ± 2.4	6 ± 1.4
Hot dog + nitrite	6 ± 0	3 ± 2.1
Beef	9 ± 0.7	9 ± 2.1
Beef + nitrite	15 ± 5.7	9 ± 2.1

Table 2. Mutagenic effects of fish treated with nitrite on various strains of *Salmonella typhimurium*. Samples (10 μl) of extracts of Japanese fish treated with 5000 parts of sodium nitrite per million under the conditions given in Table 1 were applied to plates bearing various strains of *Salmonella* without metabolic activation system, and the revertants were determined as described (*6*). The results represent the mean number of his^+ revertant colonies ± standard deviation from four experiments with duplicate plates per point. No mutagenic activity in any strain was seen with extracts of hot dogs treated with nitrite.

Salmonella typhimurium strain	His^+ revertant colonies (No.)	
	Extract	Spontaneous
TA 1535	252 ± 29.0	14 ± 2.8
TA 100	320 ± 42.0	80 ± 2.8
TA 1537	7 ± 5.7	4 ± 2.8
TA 1538	14 ± 4.2	8 ± 5.0
TA 98	30 ± 5.7	17 ± 3.5

meats commonly eaten in the United States after treatment with sizable amounts of nitrite (*7*), that is, amounts considerably larger than commonly used in the processing of such foods (*4*), is noteworthy. This may be due to the fact that nitrite is preferentially bound to myoglobin (*8*). Second, the mutagenic activity found in nitrosated fish might be relevant to the question of the etiology of human gastric cancer.

Our observation that ascorbic acid prevents the formation of mutagenic activity in nitrite-treated fish is in agreement with earlier findings of the protective effect of vitamin C in other systems (*9*). It also supports the reported inverse association of the consumption of foods rich in ascorbate and gastric cancer (*5*). Thus, vitamin C and foods rich in this essential micronutrient may be useful as an easily available means of prevention of this cancer.

Further work is needed to isolate the mutagenic principles.

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Lithium Ion Entry Through the Sodium Channel of Cultured Mouse Neuroblastoma Cells: A Biochemical Study

Abstract. *Lithium ion entry at low concentrations (1 to 5 m*M*) into an electrically active adrenergic clone of mouse neuroblastoma cells was stimulated by veratridine; and this stimulation was blocked by tetrodotoxin. These data provide biochemical evidence that lithium ions enter by way of the sodium channel which may be a major pathway for entry of this ion into electrically active cells.*

The efficacy of lithium salts for treating affective illness (mania and depression) has been well established since Cade (*1*) first used them for this purpose in 1949. Although many investigators have sought to determine the biochemical effects of Li^+ which might lead to a better understanding of these psychiatric disorders, the mechanism of action of Li^+ remains uncertain (*2*).

Interest has focused recently on the entry of Li^+ into cells. In studies with pharmacologic concentrations of Li^+, many investigators have used red blood cells (*3*, *4*) rather than nervous tissue, the probable site of lithium's therapeutic effects. The red blood cell is easily accessible, and electrolyte transport by its membrane is similar to that of a neuron (*3*). However, electrolyte transport by a membrane of a neuron is different from that of a red cell in that the neuronal membrane has properties that allow the initiation and propagation of an action potential. The basis of one of these properties is thought to be sodium channels or ionophores which gate the flow of sodium ions to make the membrane electrically excitable (*5*).

Electrophysiological studies with very high concentrations of Li^+ (usually total Na^+ replacement by Li^+) have shown that Li^+ will support an action potential for a brief time (*6*). Such results suggest that Li^+ enters through the sodium channel. Whether Li^+ enters through the sodium channel at therapeutic concentrations and in the presence of normal concentrations of Na^+ is unknown.

The sodium channel may be studied biochemically in cultured cells with the use of the alkaloid veratridine (*7*) which selectively increases the permeability of electrically excitable membranes to Na^+ (*8*). With these biochemical techniques, Catterall and Nirenberg (*9*) showed that veratridine-stimulated Na^+ uptake occurred only in cells that were electrically excitable. These results were confirmed by others (*10*). Using similar pharmacologic tools, we have obtained biochemical evidence for the entry of Li^+ into mouse neuroblastoma cells through the sodium channel at low concentrations of Li^+ and high concentrations of Na^+. The entry of Li^+ into these cells by way of the sodium channel occurs more rapidly and to a greater extent than the entry of Li^+ into the resting cell.

We used a clone of mouse neuroblastoma cells, N1E-115, as a model for the adrenergic neuron (*11*). These cells have electrically excitable membranes (*12*), enzymes that participate in the synthesis and catabolism of catecholamines (*11*), and muscarinic acetylcholine receptors that are blocked by certain psychotropic drugs (*13*).

The time course for the entry of Li^+ into cultured neuroblastoma cells was linear for approximately 30 minutes in the absence or presence of veratridine (Fig. 1). This alkaloid, however, caused a stimulation of Li^+ entry at all time points. The intracellular volume of the cells was determined by using [^{14}C]inulin and [^{3}H]OH as external and internal volume markers, respectively; this volume was 8.1 μl per milligram of cell protein or 5.6 μl per 10^6 cells. Thus, the cell to medium ratio of Li^+ at 30 minutes was ten- and fourfold with and without veratridine, respectively.

The magnitude of this stimulation of Li^+ entry by veratridine was dependent on the concentration of veratridine bathing the cells (Fig. 2A). At the highest concentration tested (0.5 m*M*), veratridine stimulated Li^+ entry more than six times above control levels. In electrophysiological studies with this clone (*14*), 50 μM veratridine, a concentration that caused a threefold stimulation of lithium ion entry (Fig. 2A), caused a reversal of polarization of the transmembrane potential in the absence of any electrical stimulus.

Tetrodotoxin (*8*) inhibited the veratridine-stimulated Li^+ entry (Fig. 2B). Tetrodotoxin, which blocks the action potential by blocking the "fast" sodium channel, inhibited by about 50 percent the veratridine-stimulated Li^+ entry at a concentration of about $10^{-7}M$. Tetrodotoxin completely blocked the sodium-dependent phase of the action potential in this clone at 3 μM, a concentration that would nearly completely block the veratridine-stimulated Li^+ entry (Fig. 2B) (*14*). Ouabain (5 m*M*), an inhibitor of Na^+- and K^+-dependent adenosinetriphosphatase, had no effect on Li^+ entry