J. Agric. Food Chem. 1983, 31, 428–431

Differences in Observed Mutagenicity Associated with the Extraction of Mutagens from Cooked Fish

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The effect of using different types of extraction procedures on the apparent mutagenicity of fried sole was examined by using the Ames *Salmonella* mutagenic assay. By employing different types of solvents, by varying the method of protein removal, and by changing the pH of the organic extraction, we observed a 7–8-fold change in apparent mutagenicity. The various types of extraction procedures did not appear to produce mutagenic compounds, but under certain conditions an inhibitor-type compound seemed to be extracted which interfered with the mutagenicity assay. The mutagen was also found to bind to precipitated proteins and the strength of binding appeared to be pH dependent. The use of organic solvents such as ethanol, methanol, and acetone for protein precipitation and initial separation extracted almost 2 times the amount of mutagens as the most effective aqueous extraction procedure. Our studies show that the type of extraction procedure used for isolating mutagens from fish and probably food in general plays an important role in correctly assessing the mutagen content of a particular food.

Since the development of the Ames *Salmonella* mutagenicity assay, many reports have appeared in the literature on the production of mutagenic (sometimes carcinogenic) substances during the heating of foods. Although the Ames test has the ability to detect many carcinogens as mutagens (McCann et al., 1975; McCann and Ames, 1976; Sugimura et al., 1976), a positive result in the test does not necessarily indicate that the assayed substance is a carcinogen. In the case of mutagenic food extracts (like those from the fried fish described here), the compounds responsible for the mutagenicity must first be isolated, identified, and tested in mammalian systems before their carcinogenicity can be evaluated. An important step in this evaluation process is the extraction procedure employed to concentrate the mutagens from foods. A number of different procedures ranging from simple solvent extraction and centrifugation processes to complex, multistep procedures have been used for this purpose.

The simpler extraction systems have been used to study mutagen formation in charred fish and meat. Nagao et al. (1977) and Sugimura and Nagao (1979) suspended the charred surfaces of beefsteak and four species of fish in dimethyl sulfoxide (Me_2SO) and, after centrifugation, incorporated the Me_2SO supernate directly into a mutagenicity test. A simple water extraction and centrifugation procedure was used by Stich et al. (1981) in their study of compounds extracted from dried fruits which possess clastogenic activity in Chinese hamster ovary cells.

Commoner and his colleagues (Commoner et al., 1978; Dolara et al., 1979; Vithayathil et al., 1978) used a more complex extraction method which involved the use of acid, base, salts, and organic solvents and reported on the presence of mutagenic substances in fried ground beef and in beef extract.

Briefly, their procedure involves precipitation of proteins with ammonium sulfate, acidification with HCl, extraction of the aqueous portion with CH_2Cl_2, basification with ammonium hydroxide, and then reextraction with CH_2Cl_2. Several other investigators using this procedure or modifications of it have reported on the presence of mutagens in cooked ground beef (Spingarn and Weisburger, 1979; Pariza et al., 1979b; Iwaoka et al. 1981a) and in volatiles from the frying of ground beef (Rappaport et al., 1979). Felton et al. (1981) have also confirmed the presence of mutagens in hamburgers by using an acetone (or 2-propanol) protein precipitation and extraction procedure.

Processed and cooked foods other than fried ground beef were extracted and tested by the method of Pariza et al. (1979b) and were found to contain varying amounts of mutagens (Pariza et al., 1979a). Spingarn et al. (1980) have used the Commoner et al. (1978) extraction procedure described above and reported mutagens to be present in foods with high starch content such as breads, biscuits, pancakes, and potatoes.

Thus several different extraction procedures have been used to study mutagen formation in one type of food (e.g., ground beef), and one extraction procedure (Commoner's) has been applied to a variety of raw and cooked foods. However, foods are complex mixtures of chemical compounds, and problems can arise during the isolation of mutagens due to harsh pH conditions used for extraction or due to reactions between food components and reagents used in the extraction. Artifactual mutagenicity and over- or underestimation of mutagen content can occur.

In our studies dealing with mutagen formation in a high starch food (biscuits), it was reported that artifactual mutagens could be produced when ammonium salts but not sodium salts were used to precipitate proteins (Iwaoka et al., 1981b). It was also reported that a slight variation in an extraction procedure can lead to a greater than 2-fold variation in the apparent mutagenicity of fried hamburger (Iwaoka et al., 1981a).

In the course of our study on mutagen formation during the cooking of fish (Krone and Iwaoka, 1981), we observed that the use of milder pH conditions for protein precipitation and extraction of mutagens (i.e., pH 6.8 vs. pH 2.5) yielded significantly lower levels of mutagenicity than expected. This paper reports on a systematic study of the effects of pH and different solvents on the extractability of mutagens from fried fish.

EXPERIMENTAL SECTION

Materials. Sole fillets obtained from a local market were fried for 6 min/side at 190 °C without the use of cooking oil in an electric skillet having a nonstick coating. After grinding in a food grinder, portions were taken for each of the various procedures described below. All chemicals used were reagent grade or better and the dichloromethane (CH_2Cl_2) was Mallinckrodt "Nanograde".

Aqueous Extraction Procedure. Ground fried sole was homogenized with 4 volumes of distilled water in a Waring blender and the homogenate was divided into three portions. One portion was adjusted to pH 2.5 with HCl, another portion was adjusted to pH 4.5, and the pH of the

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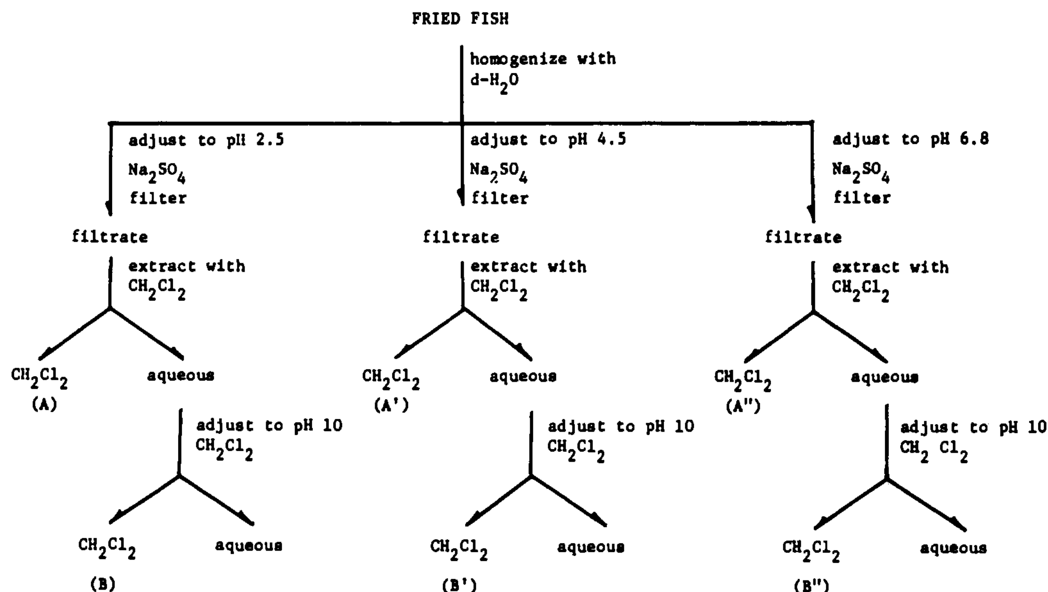

Figure 1. Procedures used to investigate the effect of the pH of protein precipitation on the extraction of mutagens from fried fish.

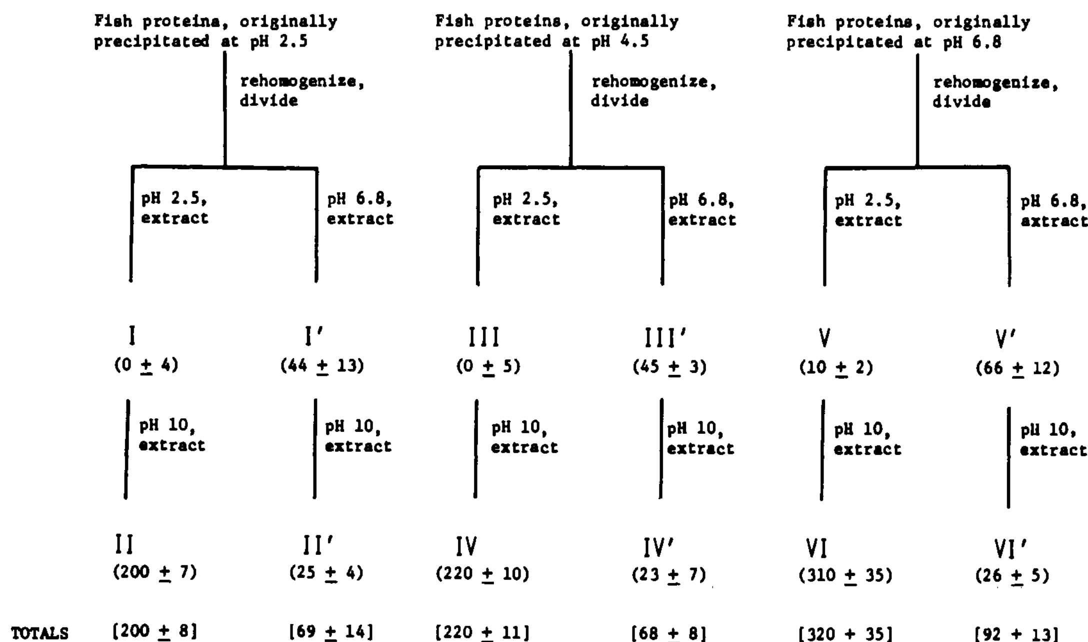

Figure 2. Schematic diagram of the procedure used to reextract fish proteins originally precipitated at pH 2.5, 4.5, and 6.8. The numbers of revertant colonies produced by the various organic fractions (shown in parentheses below each Roman numeral) are for the extract from 10 g of fried sole and have been calculated by using the regression coefficients of linear dose–response curves for each extract. Total revertants obtained by each procedure are shown in brackets at the bottom. *S. typhimurium* strain 1538 with 80 μL of S9 was used for testing of the extracts. The spontaneous revertants (24) are not included.

remaining portion was left unchanged (pH 6.8). Each portion was saturated with Na_2SO_4 (30 g of Na_2SO_4/100 mL of homogenate) and when the proteins had precipitated they were removed by filtration through glass wool in a Büchner funnel with slight suction. These proteins were retained for further study. The aqueous filtrates were each partitioned 3 times with CH_2Cl_2 (20 mL of CH_2Cl_2/100 mL of filtrate) to produce the acidic organic extracts A, A′ and A″ in Figure 1. These correspond to the CH_2Cl_2 extracts of the pH 2.5, pH 4.5, and pH 6.8 filtrates, respectively. The aqueous phases were each adjusted to pH 10 with 50% NaOH and again partitioned 3 times with CH_2Cl_2 (basic extracts B, B′, and B″). Extract B was obtained from the basic aqueous phase which was initially at pH 2.5. In the same way, extracts B′ and B″ were obtained from basic aqueous phases which were initially at pHs 4.5 and 6.8, respectively.

Reextraction of Precipitated Proteins. The proteins which were precipitated and filtered in the above procedures were themselves reextracted by using an aqueous extraction very similar to that described for fried fish. Figure 2 shows an abbreviated diagram of the extraction procedure. The proteins that were precipitated at pH 2.5 were homogenized with 4 volumes of distilled water and divided into two equal portions. One portion was adjusted to pH 2.5 and the other to pH 6.8. These homogenates were saturated with Na_2SO_4 and filtered as above. The filtrates were partitioned with CH_2Cl_2 to produce acidic organic extracts I and I′. The aqueous phases were adjusted to pH 10 and again partitioned with CH_2Cl_2 to produce basic organic extracts II and II′. The proteins initially precipitated at pH 4.5 were treated in the same manner as above to give acidic extracts III and III′ and basic extracts IV and IV′. Last, the proteins initially

Table I. Comparison of Mutagenic Activities in Organic Extracts of Fried Sole Using Three Different pHs for Protein Precipitation

symbol[a]	pH of partition	no. of revertant colonies[b]	symbol	pH of partition	no. of revertant colonies	symbol	pH of partition	no. of revertant colonies
A	2.5	7 ± 8	A′	4.5	63 ± 6	A″	6.8	62 ± 7
B	10	421 ± 33	B′	10	143 ± 19	B″	10	28 ± 5
A + B		428 ± 34	A′ + B′[c]		206 ± 20	A″ + B″[c]		90 ± 9

[a] Symbols refer to different organic extracts in Figure 1. Each extract is equivalent to 10 g of fried fish. [b] *S. typhimurium* strain 1538 with 80 μL of S9/plate. Spontaneous revertants (24) have been subtracted from these numbers. [c] The totals A + B, A′ + B′, and A″ + B″ are significantly different at the $p = 0.01$ level.

precipitated at pH 6.8 were also reextracted to give acidic extracts V and V′ and basic extracts VI and VI′. In total, 12 separate extracts were obtained. All pairs of extracts labeled with Roman numerals (I and II, III and IV, V and VI) are pH 2.5 acid and pH 10 basic extracts. All paired extracts with primed Roman numerals (I′ and II′, III′ and IV′, V′ and VI′) are pH 6.8 and pH 10 basic extracts, respectively.

Organic Extraction Procedures. The fried sole was homogenized with 4 volumes of any one of three water miscible organic solvents: acetone, ethanol, or methanol. These homogenates were filtered through glass wool by using a Büchner funnel and slight suction. The solvents were removed from each filtrate by rotary evaporation, the residues were taken up in distilled water, and the pH was adjusted to pH 6.8 (from an initial pH of 5.5) with 50% NaOH. These aqueous solutions were partitioned 3 times with CH_2Cl_2 (20 mL of CH_2Cl_2/100 mL of aqueous) to produce three pH 6.8 organic extracts. The aqueous phases were then adjusted to pH 10 with 50% NaOH and partitioned 3 times with CH_2Cl_2 to give basic organic extracts.

Mutagenicity Testing. All CH_2Cl_2 extracts from the above experiments were dried over Na_2SO_4 and reduced to about 5 mL by rotary evaporation. The extracts were quantitatively transferred to sterile test tubes (having Teflon-lined screw caps) and made up to a known volume. Aliquots of these solutions were placed in sterile disposable test tubes (13 × 100 mm), the solvent was removed under a stream of dry nitrogen, and 200 μL of Me_2SO was added. These solutions were then tested for mutagenicity according to the procedure described by Ames et al. (1975) using *Salmonella typhimurium* strain 1538 (provided by B. Ames), with the addition of 80 μL of Arochlor-induced rat liver microsome preparations. The standard mutagen used in this study was 0.5 μg of 2-aminoanthracene with 20 μL of S9/plate which produced a mean and standard deviation of 699 ± 102 revertants ($n = 10$).

Dose–response experiments using five dose levels (i.e., the extract from 0, 5, 10, 15, and 20 g of fish/plate) were performed on each extract of fried sole. Duplicate plates for all dose levels were included in each run of the Ames assay.

Statistical Analysis. The product–moment correlation coefficient was calculated for each dose–response experiment. The regression coefficients were also calculated, and the standard deviation of the number of revertants produced by a given dose (10 g in all cases) was determined by using these parameters (Dixon and Massey, 1969). A *t* test was used to compare the regression lines from duplicate runs of each procedure and also to compare the total number of revertants produced by each procedure.

RESULTS AND DISCUSSION

All of the mutagenic extracts obtained in these studies exhibited linear dose–response relationships and had product–moment correlation coefficients of ≥0.97. Each extraction method was performed in duplicate, and no significant differences were found between replicate runs even at the $p = 0.3$ level. The results presented in all tables and figures are from one representative experiment and are expressed as the revertants (±standard deviation) produced by an amount of extract derived from 10 g of cooked fish.

The procedure used to examine the effect of pH on the extraction of mutagens is shown in Figure 1, and the results of the study are shown in Table I. Essentially no mutagenic activity was seen in the pH 2.5 extract (A) while the pH 4.5 (A′) and pH 6.8 (A″) extracts contained significantly higher amounts. The quantity of mutagens found in the pH 10 extracts was greatest for extract B and decreased in extracts B′ and B″. The most interesting result was an almost 5-fold decrease in total mutagenic activity observed when the pH of protein precipitation was increased from pH 2.5 to pH 6.8. It was expected that total mutagenicity of each procedure (A + B, A′ + B′, and A″ + B″) would be the same or differ slightly, but this was not the case. There seemed to be several possible explanations for this variation in mutagenicity: (1) the pH 4.5 (A′) and pH 6.8 (A″) extracts could have contained, in addition to mutagens, substances which inhibited mutagenesis in the Ames assay; (2) the low pHs may have caused mutagenic substances to form during the extraction which could later be extracted at higher pH; (3) the mutagens were bound to the precipitated proteins to a greater extent as the pH of precipitation increased.

To test the first possibility, mixing experiments were performed. The pH 2.5, 4.5, and 6.8 extracts were combined with other extracts known to contain mutagens and these mixtures were tested in the Ames assay. The pH 6.8 extract (but not the 2.5 or 4.5) was able to inhibit mutagenicity by about 40% and thus appeared to contain inhibitory substances. However, this reduction in mutagenicity was not enough to account for the 5-fold difference seen above.

Aqueous reextraction of the precipitated proteins was performed to test the other possible explanations mentioned above. The results of these reextraction experiments are shown in Figure 2. Regardless of the pH of the initial precipitation of proteins (either 2.5, 4.5, or 6.8), low levels of mutagenicity were recovered when the proteins were reextracted at pH 6.8 and 10 (samples I′ and II′, III′ and IV′, and V′ and VI′). This was true even for the proteins which had originally been salted out at very low pHs (pH 2.5). Therefore, very acidic conditions did not appear to be causing the formation of mutagens during the extraction.

However, significant quantities of mutagenic substances were recovered when these same proteins were reextracted at pH 2.5 and 10. The total mutagenicity (I plus II, III plus IV, and V plus VI in Figure 2) appears to be inversely proportional to the amount of mutagenicity that was originally extracted from the fish (Table I). For example,

Table II. *S. typhimurium* Revertant Colonies Produced by pH 6.8 and pH 10 CH_2Cl_2 Extracts Obtained by the Precipitation of Proteins by Various Organic Solvents

solvent used for protein precipitation	revertants[a] produced by $MeCl_2$ extracts at		
	pH 6.8	pH 10	total[b]
acetone	613 ± 25	135 ± 10	748 ± 27
ethanol	493 ± 30	253 ± 24	746 ± 38
methanol	474 ± 45	195 ± 15	669 ± 47
water	62 ± 7	28 ± 5	90 ± 9

[a] *S. typhimurium* strain 1538 was used with metabolic activation on the extract from 10 g of fish. Spontaneous revertants (24) have been subtracted from totals. [b] Totals for acetone, ethanol, and methanol procedures are not significantly different.

when fried *sole* was extracted at pH 6.8 and 10 (A″ and B″), the lowest levels of mutagenicity were observed (90 revertants from the extract of 10 g of fish), while reextraction of the pH 6.8 *proteins* at pH 2.5 and 10 (V and VI) produced the highest number of revertants (320). This phenomenon suggested that the mutagen was an organic basic compound and may have been binding to the precipitated proteins. The extent of binding appeared to increase as the pH of precipitation was increased. The association could be due to ionic interactions between the basic, positively charged mutagen and negatively charged groups on the proteins.

At pH 2.5 nearly all the carboxyl groups on the acidic amino acid side chains (glutamic and aspartic acids) would be unionized while the side chains of the basic amino acids would be positively charged. The basic mutagen would also be positively charged and electrostatic interactions between the protein and mutagen would be minimal. As the pH was increased from 2.5 to 4.5 or 6.8, the carboxyls would become negatively charged, allowing ionic interactions to occur. Because significant mutagenicity could be extracted from all the proteins, even those from sole originally precipitated at pH 2.5, other forces such as hydrophobic interactions could also be involved in the association of the mutagens with proteins.

Because of the difficulty encountered in extracting the mutagens using an aqueous system, the need to develop other extraction procedures became apparent. Organic solvents such as acetone, ethanol, and methanol are commonly used to precipitate proteins, and the extraction behavior of the mutagens indicated that they should be more soluble in these organic solvents than in water. Felton et al. (1981) showed that the mutagens in fried hamburger were more efficiently extracted when acetone or 2-propanol were used as the extracting solvent.

The numbers of revertant colonies produced by extracts from fried sole obtained by using three different organic solvents are shown in Table II. All three solvents were able to extract mutagenic substances more effectively than any of the aqueous procedures. In fact, between 7 and 8 times more mutagenicity was obtained when using these organic solvents than when using a water extraction procedure (pH 6.8 and pH 10).

In summary, this work has demonstrated that the type of extraction procedure used to concentrate mutagens is an important step in assessing a food's mutagenic potential. Since each type of food is different and contains a unique number and amount of chemical substances, a mutagen extraction procedure which is appropriate for one class of foods should not be indiscriminately applied to other foods without first investigating the possible effects of pH on apparent mutagenicity, the interaction of solvents, the reactions of salts, etc.

Because of the possible carcinogenicity of mutagens in foods, it is important that the procedures used for studying mutagen formation in a food be thoroughly tested for that food product. This will help decrease the dissemination of misleading information.

Registry No. Ethanol, 64-17-5; methanol, 67-56-1; acetone, 67-64-1.

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Received for review April 13, 1982. Revised manuscript received November 15, 1982. Accepted December 13, 1982. This research was supported in part by the Egtvedt Food Research Fund of the University of Washington Institute for Food Science and Technology.