inert. Similar protection of the carotenoids in Pro-Xan (Witt et al., 1971), other leaf protein concentrates (Arkcoll, 1973), and dehydrated alfalfa (Hoffman et al., 1945; Livingston et al., 1955) has been reported, and commercial Pro-Xan is now stored under inert gas (Edwards et al., 1979).

Cold Storage. Temperature effects were not studied, but Witt et al. (1971) reported that after 19 weeks of storage, Pro-Xan containing ethoxyquin retained 96% of its xanthophyll at 2 °C but only 50% at 38 °C.

Light Effects. Leaf protein concentrates should always be stored in the dark since oxidation of carotenoids in the light is strongly catalyzed by the chlorophyll present (Arkcoll, 1973).

ACKNOWLEDGMENT

We are indebted to Gene Whitaker and Donald Kuzmicky for assistance in preparing leaf protein concentrates, to Sandra Tillin for mass spectral analyses, to Bruce Mackey for statistical analysis, and particularly to Katie Summers for carotene and xanthophyll analyses.

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Received for review January 4, 1982. Accepted April 26, 1982.

Mutagens in Dried/Salted Hawaiian Fish

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Mutagenic substances were found in dried/salted skipjack, *Katsuwonus pelamis*, Indo-Pacific jackfish, *Caranx ignoblis*, and big-eyed scad, *Trachuroks crumenophthalamus*. These mutagens were detected by *Salmonella* tester strain TA100 in the absence of rat liver microsomes. The mutagens can be obtained as purified materials by extraction with selective solvents, absorption on silica gel, and high-pressure liquid chromatography. The mutagens are not detected in freshly caught fish or fish that is dried/salted soon after being caught. The dried/salted product, which contains mutagens, is prepared from fish that has been frozen at sea and stored for several months or chilled fresh fish that has remained unsold for 6–8 days.

Dietary components are believed to have an important influence in the causation of some cancers (Armstrong and Doll, 1975). Because of this, and because most carcinogens are also mutagens, a systematic search was undertaken for mutagens among unusual food items eaten in Hawaii. We report here that dried, salted fish frequently contain high levels of mutagens as detected by Ames tester strain TA100 in the absence of rat liver microsomes.

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MATERIALS AND METHODS

The dried fish were purchased in local food markets and are common food items sold in the local food section of many supermarkets in Hawaii. The items studied were dried/salted skipjack or striped tuna, *Katsuwonus pelamis* (local name Aku), small Indo-Pacific jackfish, *Caranx ignoblis* (local name Papio), and big-eyed scad, *Trachuroks crumenophthalamus* (local name Akule). All fish were

0021-8561/82/1430-0937$01.25/0

caught in Hawaiian waters and dried and salted by local fish processors.

Preparation of Extracts. Extracts were prepared by first cutting the dried fish into small pieces of 0.25–0.5 g and placing, typically, 15 g, in a Virtis homogenizer, Model 23, with a 3-fold weight excess of hexane. The mixture was homogenized at a setting of 50 for 3 min with cooling in an ice bath. The resulting mixture was centrifuged at 30000*g* for 10 min, and the supernatant hexane phase was decanted. The process was repeated by using ethyl acetate and finally with 0.1 M, pH 7.2, phosphate buffer. The extracts in hexane were passed through a silica Sep-PAK cartridge, and extracts in H_2O or ethyl acetate were passed through a C-18 Sep-PAK cartridge (Waters Associates, Milford, MA 01757, part no. 51910). The material passing through each column was evaporated to dryness and dissolved in dimethyl sulfoxide (Me_2SO) to give a final solution that contained the extract of 3 g of fish product in each milliliter of Me_2SO solution. The components of the fish extract retained by the C-18 Sep-PAK cartridge were eluted by 8 mL of hexane and those retained by silica Sep-PAK by 8 mL of acetone. These eluates were each evaporated under a stream of N_2 gas at room temperature and the residues dissolved in Me_2SO as described above.

Measurement of Mutagenicity. *Fish Extracts.* The mutagenicity of the extracts was measured by the pour plate assay of Ames et al. (1975). Extracts were added in variable amounts to 3.0 mL of soft agar containing 0.1 mL of a 14-h culture of the Ames tester strain. The solution was mixed well, poured over a hard agar plate, and incubated at 37 °C for 48 h as described in Ames et al. (1975). All assays were carried out in the absence of rat liver microsomes. All experiments were carried out in triplicate, and the results are reported as the mutation ratio, i.e., the number of revertant colonies on the experimental plate divided by the number on the control plate. A mutation ratio of 2.0 or greater must be observed for an extract to contain mutagens. Each experiment included a solvent control in which a volume of solvent equal to that used in the extract was evaporated under the same conditions used to produce the extracts. The residue was dissolved in Me_2SO and tested with each of the Ames tester strains. In addition controls were carried out in which solvent was added directly to the soft agar tubes. These solvent control experiments were carried out over a dose range covering concentrations low enough to produce no effect to those high enough to exert a profound toxic effect. Each extract was also tested in multiple doses ranging from the no effect level to severe toxicity. Tests to determine the true revertant character were carried out by transfer of colonies from experimental plates to plates containing no histidine. At least 80% of these colonies were histidine auxotrophs. Examination of the background lawn with a dissecting microscope at the concentrations of extracts that produced the maximum number of revertants did not show any indication that the extracts were toxic to the test bacteria at these doses.

Each tester strain was continually monitored to be certain it did not deviate from prescribed characteristics as described by Ames et al. (1975), and numbers of spontaneous colonies and responses to known mutagens were within the range of values described by de Serres and Shelby (1979). Rat liver ribosomes (S-9) were prepared as described by Ames et al. (1975) from aroclor-induced Fisher rats.

In this study Ames strains TA100, TA98, and TA1537, as well as *Escherichia coli* WP-2 (Venitt et al., 1977) and *E. coli* rec⁻ (Ichinotsubo et al., 1977), were used to measure the mutagenicity of the fish extracts.

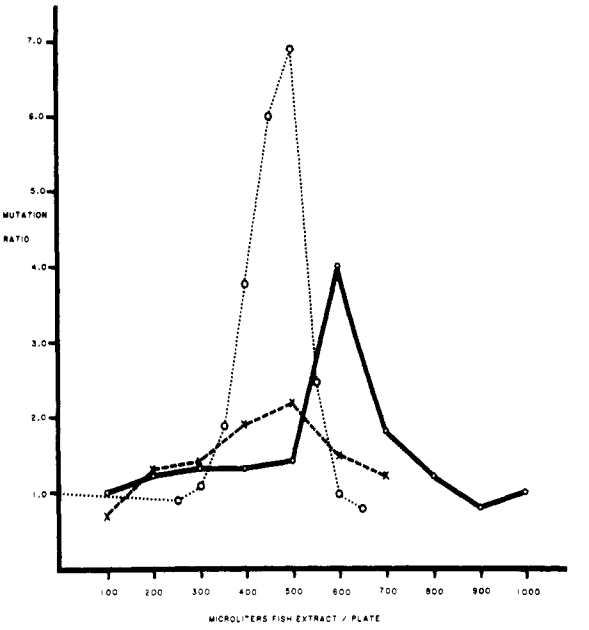

Figure 1. Dose–response curves of variable amounts of extracts of dried/salted preparations of three Hawaiian fishes in the Ames *Salmonella* assay using TA100 in the absence of rat liver microsomes. Extracts of salted/dried striped tuna (Aku) were prepared in Me_2SO as described under Materials and Methods and assayed in the Ames pour plate mutagen assay (TA100) in variable amounts as indicated on the abscissa. After 48-h incubation revertant colonies were counted on experimental and control plates and mutation ratios calculated and plotted along the ordinate. Control plates routinely contained 110–125 spontaneous revertants. No significant activity was observed with TA98, TA1537, *E. coli* WP-2, and *E. coli* rec⁻ assays under these conditions. Additions of arochlor-induced rat liver microsomes either had no effect or reduced the mutagenicity of the extract. (O–O) Extracts of Aku; (X---X) extracts of Papio; (O—O) extracts of Akule.

High-Pressure Liquid Chromatography. High-pressure liquid chromatography (HPLC) was carried out by dissolving the residues obtained from fish extracts in 50:50 methanol–H_2O to give solutions of 0.5–1.0 mL. Chromatography was carried out by injecting 0.1 mL of the methanol–H_2O mixtures on an Altex Custom Pack C-18, 18 μm, 10 mm × 25 cm column equilibrated with 50% methanol–H_2O at a flow rate of 2.0 mL/min. The eluate was monitored at 254 nm with the optical density trace displayed on a Hewlett-Packard Model 3390A integrator. Fractions containing UV-absorbing material were collected and evaporated under a stream of N_2 at room temperature. The residues were dissolved in Me_2SO and tested for mutagenic activity as described above.

RESULTS

Mutagens were not contained in either the hexane or aqueous buffer extracts of the fish products. Ethyl acetate extracts of each of the three fish products examined, however, contained mutagens detected by Ames strain TA100 in the absence of rat liver microsomes. These mutagens were absorbed on silica Sep-PAK columns and eluted from the columns by acetone. The dose–response curve of each of these Sep-PAK eluates is shown in Figure 1. A similar dose–response curve was obtained with each extract of dried striped tuna (Aku), purchased and tested at 1-month intervals over a period of 3 months. Storage of dried fish samples for 30–40 days at 0 °C did not result in loss of mutagenicity, and heating the fish product in an oven at 110–120 °C for 30 min did not decrease activity.

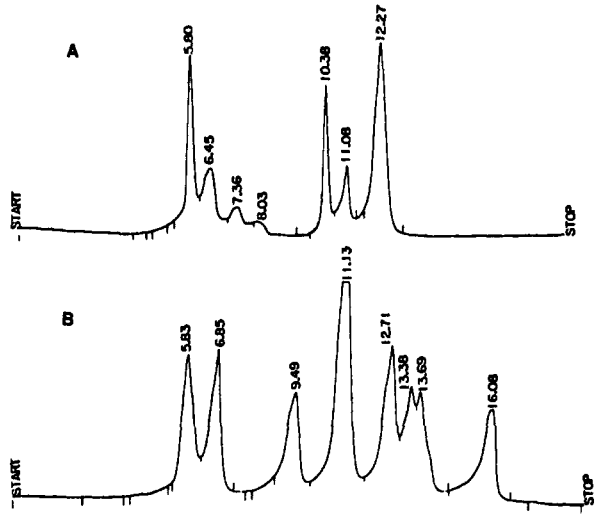

Figure 2. HPLC chromatogram of extracts of salted/dried preparations of Aku and Akule. Extracts of dried/salted preparations of Akule (A) and Aku (B) were prepared in 50% CH_3OH–50% H_2O, and 0.1 mL was chromatographed as described under Materials and Methods. The optical density of the column effluent at 254 nm was recorded on a Hewlett-Packard 3390A electronic integrator. Increasing optical density is indicated by vertical peaks that are indentified by the retention time (RT) recorded by the integrator at the maximum of each absorption peak.

No activity was observed with the other Ames strains or by the *E. coli* test systems described under Materials and Methods in the presence or absence of rate liver microsomes. Extracts of freshly caught raw fish or salted/dried fresh fish were void of mutagens.

Silica Sep-PAK eluates of striped tuna (Aku) product chromatographed by HPLC gave the chromatogram shown in Figure 2. Each of the peaks shown in the chromatogram was individually collected and tested for mutagenicity. Peaks at retention times of 9.49 and 11.13 min showed mutagenicity when tested with Ames strain TA100 in the absence of rat liver microsomes. The other peaks contained no mutagenic activity. Rechromatography of either of the mutagenic fractions showed that UV-absorbing material and mutagenic activity were contained in a single symmetrical peak eluting with a retention time within 0.1 s of the same material in the chromatogram of the original extract. The two mutagen peaks were found in all samples of dried Aku tested. These chromatograms vary somewhat in the presence and relative amounts of the nonmutagenic component but are relatively invariant in the mutagen peaks. Chromatography of extracts of the big-eyed scad (Akule) product showed only a single mutagenic component at RT = 10.38 min (Figure 2).

DISCUSSION

The mutagens that we have discovered in three species of Hawaiian salted/dried fish all have similar properties. Each is detected by Ames tested strains sensitive to point mutagens, and each one does not cause revertant colony formation with the frame-shift sensitive tester strains. None requires metabolic activation. Each is extracted from the fish in ethyl acetate and is insoluble in hexane. Each is absorbed from the ethyl acetate onto a silica Sep-PAK column and is eluted from the column by acetone. On HPLC using a C-18 reverse-phase column, each of the mutagens was eluted with retention times of 9–12 min in a 50% methanol eluting solvent.

These results contrast with the work of Ho et al. (1978), who have shown that a variety of mutagens can be found in Chinese salted/dried fish. Extracts of these fish contain mutagens that are detected by either TA98 or TA100 and often are enhanced by metabolic activation with rat liver microsomes.

The sources of the mutagens in the group of Hawaiian fish products are unknown. In Hawaii the fresh fish or fresh fish that is dried and salted soon after being caught contain no mutagens. Mutagens are present only in those fish products produced from fish frozen and stored while at sea or fish chilled for the fresh fish market but unsold after 6–8 days. Fish treated in these ways develop an "off-flavor" and undergo a characteristic change in color, both of which are masked by the salting and drying process. Presumably these changes are brought about by the growth of microorganisms during storage, which leads to mutagen formation and accumulation.

In addition to our work with Aku reported here, other workers using similar dried preparations of this fish have shown that alkylating agents (Yano, 1981) and nitrosatable methylurea precursors (Mirvish et al., 1980) are present in the fish product.

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Received for review October 2, 1981. Revised manuscript received April 26, 1982. Accepted June 1, 1982. This work was financially supported by the American Cancer Society (ACS-PDT 136A) and the Japan Hawaii Cancer Study (National Institutes of Health Grant N01-CP-33216).