(Appu Rao and Narasinga Rao, 1975). Although EDTA treatment reduced the binding of Ca(II), it had no effect on precipitation of the protein. If anything, the precipitation was higher. The mechanism of precipitation of the 11S protein by the two cations thus appears to be different.

Difference in the precipitation of the unfractionated soybean proteins by Ca(II) and Mg(II) was revealed by the following experiment. Soybean proteins were extracted with water at a meal to solvent ratio of 1 to 10. The carbohydrate residue was removed by centrifugation. To the supernatant Mg(II) was added in the cold to a concentration of 5×10^{-2} *M*. After standing in the cold for 6 hr the precipitate was separated and dissolved in 0.05 *M* phosphate buffer of pH 7.8 containing 5×10^{-3} *M* EDTA. It was then dialyzed extensively against 0.05 *M* phosphate buffer of pH 7.8 containing 0.5 *M* NaCl to remove EDTA and Mg(II). The sedimentation velocity pattern of this preparation consisted of a major fraction, 11 S, and two minor fractions, 15 S and 7 S (Figure 9A). The proportion of the minor fractions was 5–10% of the total. A similar experiment was conducted using Ca(II) for precipitation. The sedimentation velocity pattern consisted of 3 peaks, 11, 7, and 2 S (Figure 9B). The proportion of the 11S fraction was only 60% of the total. Thus precipitation with Mg(II) appeared to yield a more homogeneous 11S fraction and thus offers a single step procedure for the preparation of this protein in a fairly homogeneous form.

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Removal of Mercury from Fish Protein Concentrate by Sodium Borohydride Reduction

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Mercury removal from fish protein concentrate (FPC) can be accomplished by reduction with sodium borohydride in aqueous FPC slurries at a pH between 9 and 10. Investigation of the effects of contact time and temperature revealed that both inorganic and methylmercury were most effectively removed in 20 to 30 min at a temperature of 23 ± 2°. Studies relating sodium borohydride concentration to effectiveness of mercury removal indicated that for each part per million of mercury in 100 g of FPC, 1.2 g of sodium borohydride was required to obtain a final level of 0.5 ppm of mercury in the FPC. A 100% removal of mercury may be achieved with excess sodium borohydride. Metaborate ion, formed during $NaBH_4$ treatment, may be washed out of the FPC with water. A test of possible changes in the nutritional value of the treated FPC was made using chicks as the subject of a growth–response study which lasted 3 weeks. The growth of chicks fed a treated FPC diet was equal to that for chicks fed a diet employing untreated FPC.

There has been considerable recent interest in the removal of mercury from FPC. The methods described heretofore involve competitive complexation (Regier, 1972; Yannai and Saltzman, 1972; Spinelli et al., 1973; Lee and Richardson, 1973) or a chemical modification of the FPC (Archer et al., 1973). In the former cases, the extent of mercury removal is limited by the relatively small equilibrium constants possible for competitive complexation processes. On the other hand, removal of mercury coupled with extensive modification of the FPC does not always produce a product with the most desirable qualities.

Our research was initiated for two reasons. One was to find a method of removing mercury which could be tailored to any production process for FPC and could produce any desired level of mercury removal up to 100%. The second reason was to examine the possibility of using Lake Erie fish as the input for an FPC processing plant. Although the commercial catch of fish from Lake Erie over the past 150 years has exceeded that of the other four Great Lakes combined, a variety of factors has reduced the value of the fish caught in recent years even though there has been little, if any, fall-off in their abundance. Fish of low commercial value such as carp and sheepshead are now in such abundance that they make fishing for the more valuable species difficult. Use might be made of these fish to produce FPC (Finch, 1970). Because of heavy previous contamination of some of the Great Lakes with mercury (Pillay et al., 1971), consideration of the mercury removal problem comes into any plan for utilization.

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In the work to be presented, mercury is removed from FPC by reduction using sodium borohydride and mechanical washing to remove the metaborate anion formed. In addition to the determination of the optimum conditions for the reduction process, studies were carried out on the pathway of the reduction. Possible changes in the quality of the FPC due to treatment with sodium borohydride were monitored in a growth–response study.

MATERIALS AND METHODS

Fish. Carp (*C. carpio*) and sheepshead (*A. probatocephalus*) were caught commercially and obtained frozen from the Great Lakes Laboratory, State University of New York College at Buffalo.

Reagents. Sodium borohydride was the 98% pure powder, purchased from Ventron Company, Beverly, Mass.

Apparatus. A mercometer (Anti-pollution Technology Corporation, Model 2006-1) was used as the flameless atomic absorption detector in the analysis of the samples for mercury.

Preparation of FPC and Mercury Removal. The aqueous phosphate method described by Spinelli et al. (1971) was used in the preparation of FPC. Mercury removal was accomplished by adding sodium borohydride at some point in the experimental procedure, usually to the water wash immediately following the isopropyl alcohol extractions. As mentioned below, the effect of placement of the sodium borohydride treatment was also investigated.

When the addition of $NaBH_4$ was made to the water wash, the crude FPC was first slurried 1:10 v/v with water containing the desired amount of sodium borohydride. In a separate measurement, it was determined that the pH of the FPC–water slurry in the absence of $NaBH_4$ was 5.8 to 6.8. The presence of the borohydride increased the pH to between 9 and 10. It is in this pH range that the reduction proceeds. The reducing properties of sodium borohydride can be destroyed and a reaction involving $NaBH_4$ thereby quenched by adding 6 *M* HCl to the slurry. The acid catalyzes the hydrolysis of sodium borohydride to metaborate ion and hydrogen (Sullivan, 1973). Addition of acid reduces the pH of the slurry to between 4 and 5.

Studies of the effect of contact time were carried out using time periods ranging from 2 to 40 min. Sodium borohydride concentrations in these experiments ranged between 1 and 5 g/100 g of FPC while the temperature was 23 ± 2°. After quenching as discussed above, the mixture was then centrifuged and the FPC taken off and lyophilized prior to analysis.

Two series of experiments were done to determine the effect of temperature on the removal of mercury from FPC. In one series, temperatures of 25, 50, and 70° were employed using an $NaBH_4$ concentration of 1 g/100 g of FPC and a contact time of 20 min. In another study, the temperatures were 0, 25, and 50° with a 30-min contact time and a sodium borohydride concentration of 2.2 g/100 g of FPC. In each series, the temperatures were controlled to ±0.5° using a water bath.

Several other experiments were carried out to establish optimum conditions for the removal of mercury by this method. In one experiment, the effect of sodium borohydride concentration on the removal of total mercury, methyl mercury, and inorganic mercury was studied at 23 ± 2° using a contact time of 30 min. In another series, the best position for the treatment of FPC with sodium borohydride during the aqueous phosphate process was studied by adding, in separate experiments, sodium borohydride to the last water wash before the isopropyl alcohol extractions, to the last isopropyl alcohol extraction, and to the water wash directly following the isopropyl alcohol extractions.

The fate of the mercury removed by the borohydride treatment was investigated by the analysis for mercury of the supernatant wash liquid and acid used to wash the walls of various containing vessels. In addition, a direct analysis for mercury was made of the gases evolved during the reduction process.

Analytical Methods. Analyses for inorganic mercury as well as for methylmercury were accomplished using the Magos (1972) method. Some of the methylmercury measurements were confirmed using the Westöö (1968) method. Boron analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., using the curcumin lake method.

Growth–Response Study. Five groups of day-old chicks were fed different diets for a period of 3 weeks. The diets were (1) Agway Chicken Diet, a standard commercial feed, (2) a casein diet with vitamins and minerals added in proportions described in the literature (Miller and Kifer, 1970), (3) FPC prepared as mentioned above but without sodium borohydride treatment, (4) FPC treated using sodium borohydride and washed once with water, (5) FPC treated using sodium borohydride and washed four times with water after treatment. Appropriate vitamins and minerals were added to the three FPC diets. As in previous investigations (Ousterhout and Snyder, 1962; Miller and Kifer, 1970) involving growth–response studies with fishmeal, the total protein content of the diets containing FPC was 15%. The casein diets were also made up to contain 15% protein. On the other hand, the commercial diet contained 21% protein.

To initiate the study, the chicks were tagged and weighed. Thirteen chicks were fed on each diet. The chicks were weighed once a week for 3 weeks. In accordance with accepted practice, the weights of the eight heaviest chicks in each group were used in preparing the final average weights given below.

RESULTS

Mercury in FPC. Mercury levels in FPC made from Lake Erie fish varied widely. Sheepshead FPC averaged around 2 ppm with the general range between 0.6 and 3.0 ppm. One sample has as much as 4.8 ppm. On the other hand, carp FPC levels were much lower averaging 0.4 ppm. Since it is known that mercury is concentrated by about a factor of five in going from raw fish to FPC (Gasiewicz and Dinan, 1972), much of the starting material in this work was probably under the 0.50-ppm FDA guideline. Although this level refers to the fish from which the FPC is made and not the FPC itself (Stillings, 1974), the desirability of providing improved, flexible methods of removal, applicable to mercury levels, present and future, as well as to possible downward revision of the guideline, is obvious.

General Observations. The hydrolysis of the borohydride during the initial reaction period led to a large evolution of gas. The presence of extensive bubbles in the solution and the nature of the FPC–water slurry made it imperative to provide vigorous stirring in order to optimize the distribution of the sodium borohydride. The yield of FPC was unaffected by $NaBH_4$ treatment.

The dried, untreated FPC and the treated FPC were nearly identical on visual examination. Both were a fine light tan powder, with no odor or taste, although the treated FPC had a more grainy texture. The pH of an aqueous slurry of the treated, washed FPC ranged between 5.8 and 6.8, identical with the untreated material.

Dependence on Reaction Conditions. *Time.* The results of studies on the time necessary to effect removal of mercury from FPC using sodium borohydride reduction are shown in Table I for various sodium borohydride concentrations. In all cases, the optimum time of exposure of FPC to sodium borohydride was found to be between 20 and 30 min. During the first 10 min, the concentration of inorganic mercury (Hg^{2+} ion and Hg metal) appears to increase to 300% of its original concentration (data not shown). This increase is probably due to a rapid direct reduction of

Table I. Time Dependence of the Removal of Mercury from Sheepshead FPC by Sodium Borohydride at 23 ± 2°

	Sodium borohydride added, g/100 g of FPC									
	1		2		3		4		5	
Time, min	ppm of Hg	% removal	ppm of Hg	% removal	ppm of Hg	% removal	ppm of Hg	% removal	ppm of Hg	% removal
0	2.03	0	2.03	0	2.03	0	2.03	0	2.03	0
2	1.40	31			0.94	54	0.80	61	0.96	53
5	1.56	23	1.44	29	0.92	55	0.82	60	0.56	72
10			1.14	45	0.52	74	0.84	59	0.66	68
20	1.54	24	0.92	55	0.58	71	0.48	76	0.30	85
40	1.42	30	1.24	39	0.63	68	0.52	74	0.32	84

Table II. Dependence of the Removal of Various Forms of Mercury from Sheepshead FPC on the Sodium Borohydride Concentration at 23 ± 2°

$NaBH_4$, g/100 g of FPC	Mercury concn, ppm	% removal
	Total Mercury	
0.00	1.63 ± 0.23	0
0.75	1.02 ± 0.08	37
1.70	0.66	60
2.90	0.48 ± 0.22	69
3.50	0.10 ± 0.02	94
4.60	0.08 ± 0.06	95
	Inorganic–Metallic Mercury	
0.00	0.21	0
0.75	0.23	−5
1.70	0.42	−100
2.90	0.32	−53
3.50	0.00	100
4.60	0.07	67
	Methylmercury	
0.00	1.42	0
0.75	0.79	44
1.70	0.24	83
2.90	0.15	89
3.50	0.10	93
4.60	0.01	99*

Table III. Retention of Boron in FPC after Treatment with Sodium Borohydride

Sample	Boron, ppm
FPC (U)[a]	60
FPC (W0X)[a, b]	10,450
FPC (W1X)[a]	400
FPC (W3X)[a]	84
FPC (W4X)[a]	49

[a] U, untreated FPC; W0X, treated FPC not washed with H_2O; W1X, treated FPC washed once with H_2O; W3X, treated FPC washed three times with H_2O; W4X, treated FPC washed four times with H_2O. [b] This sample was treated with 0.75 g/100 g of FPC of sodium borohydride. All of the other samples were treated with 2.49 g/100 g of FPC of sodium borohydride.

methylmercury to metallic mercury followed by a slower (perhaps rate determining) step in which the metallic mercury is carried away from the FPC by the hydrogen produced by the hydrolysis of additional sodium borohydride. Direct analysis for methylmercury suggests that the sequence of events is probably correct. Removal of methylmercury reaches its maximum only 5 min after the introduction of sodium borohydride.

Temperature. The removal was found to be unaffected by temperature between 0 and 30°. Removal was about 25% less efficient at 50° and about 35% less efficient at 70° than at the lower temperatures. Sullivan (1973) has pointed out that stability of $NaBH_4$ solutions decreases with increasing temperature. The optimum reaction temperature was thereby selected as ambient, 23 ± 2°.

Sodium Borohydride Concentration. The results of studies of the dependence of mercury removal on the concentration of sodium borohydride are given in Table II. The uncertainties reported for the total mercury removal are mean deviations of two or more determinations. Replicate analyses for methylmercury were not carried out by the Magos method. However, analysis for methylmercury by the Westöö method confirmed these results.

A general correlation was obtained from these and additional data not shown. For each part per million of mercury in 100 g of FPC, 1.2 g of sodium borohydride is required to obtain a final level of, e.g., 0.5 ppm of mercury in the FPC. This relation holds up to concentrations of sodium borohydride of 3 g/100 g of FPC. Within this range, such linear behavior allows for the removal of any desired amount of mercury. Outside of this range, essentially 100% removal of mercury can be effected by the use of excess sodium borohydride.

The negative percentages observed for inorganic–metallic mercury removal at intermediate sodium borohydride concentrations probably reflect the incomplete purging of reduced metallic mercury out of the FPC matrix by the hydrogen evolved in the borohydride treatment.

Position of the Sodium Borohydride Treatment in the Preparation Sequence for FPC. An examination of the placement of the sodium borohydride treatment was attempted by the addition, in separate runs, of sodium borohydride to (1) the last isopropyl alcohol extraction in the preparation scheme (Spinelli et al., 1971) and (2) to the last water wash before the isopropyl alcohol extractions. These variations were compared to the "normal" treatment in which the sodium borohydride is added to the first water wash after the isopropyl alcohol extractions are completed. With the sodium borohydride treatment as in 1 above, approximately the same concentration of sodium borohydride was required to remove a given quantity of mercury as in the "normal" treatment. Using a placement as in 2 above, about three times as much sodium borohydride was required to achieve the same mercury removal. Two conclusions can be drawn from these experiments. First, the nature of the solvent medium does not appear to affect the removal of mercury at least for this limited group of solvents.

Table IV. Average Weight Gain of Chicks Fed Various Protein Diets

Diet	Gain in wt of chicks, g,[a] at no. of weeks			
	0	1	2	3
Casein	0.0 ± 1.6	7.8 ± 1.7	15.4 ± 2.1	26.8 ± 4.2
Agway	0.0 ± 0.8	19.4 ± 1.7	63.3 ± 4.8	128.9 ± 7.7
Agway × 15/21	0.0 ± 0.6	13.8 ± 1.2	45.2 ± 3.4	92.0 ± 5.5
FPC (U)[b]	0.0 ± 1.2	14.3 ± 1.0	45.5 ± 1.9	75.1 ± 2.7
FPC (W1X)[b]	0.0 ± 0.9	7.1 ± 1.7	16.5 ± 2.5	27.9 ± 3.4
FPC (W4X)[b]	0.0 ± 0.8	16.0 ± 1.2	52.5 ± 3.5	83.4 ± 4.5

[a] The error is reported as the standard deviation of the mean. [b] U, untreated FPC; W1X, treated FPC washed once with H_2O; W4X, treated FPC washed four times with H_2O.

Second, removal of oils must be carried out prior to sodium borohydride treatment. Otherwise, the procedure consumes an excessive amount of sodium borohydride.

Fate of Mercury Released from the FPC. We considered three routes possible for the final disposition of the atomic mercury which was held by the FPC after sodium borohydride reduction: (1) the elution of mercury into the supernatant during separation of the FPC by centrifugation after sodium borohydride treatment, (2) adsorption of mercury on the walls of the centrifuge tube during centrifugation, and (3) volatilization or mechanical carrying away of the mercury by evolution of gases during the reduction step. Little or no mercury was found in the supernatant after centrifugation of the treated FPC, thereby eliminating route 1. Route 2 is eliminated by the evidence that no mercury was found in an acid solution used to extract it from the walls of the centrifuge tube. However, mercury was detected in the gas stream which was produced when FPC was treated directly with excess sodium borohydride favoring route 3 for the removal process.

Residual Boron in the Treated FPC. Table III gives the quantity of boron found in untreated FPC and the residual concentration after treatment alone or treatment plus a given number of water washings. Samples washed three times or more contain a boron concentration less than 350 ppm, the postulated safe level (Weir and Fisher, 1972).

Growth-Response Studies. The results of the growth-response study are reported in Table IV as the average weight gain for chicks fed various diets. Two listings are given for the diet of commercial (Agway) feed—one, the set of experimental data and the other, the same data multiplied by a factor of $^{15}/_{21}$ to adjust the data to the same percent protein in the other diets. While this procedure may appear to be arbitrary, Miller and Kifer (1970) have found that growth of chicks fed fishmeal is a linear function of protein concentration in the diet when the protein concentration is between 12 and 15%. We are assuming that an extrapolation outside of this range is possible with small error. The adjusted commercial feed diet, the untreated FPC diet, and the diet made using FPC treated to remove mercury and washed four times with water thereafter gave measures of growth which were nearly the same within the statistical uncertainty. On the other hand, the casein diet and the diet made using treated FPC which was washed only with water led to much poorer growth. Although the series employing the casein diet was repeated with a different batch of casein with identical results, no conclusions should be drawn regarding the quality of the FPC relative to casein. It is possible that unknown factors contributed to the poor results using casein.

We infer from these data that the nutritional quality of the FPC treated to remove mercury is comparable to the untreated FPC. The material must be washed sufficiently to reduce the metaborate ion concentration to acceptable levels.

DISCUSSION

The sodium borohydride reduction of mercury bound to fish protein should be applicable to most methods of FPC processing. Its versatility allows for as much mercury removal as required with no apparent changes in protein quality. Although the price of treatment seemed to be a problem when this work was started, the method may become economical if some further attention is paid to process detail. We estimate the cost of $NaBH_4$ used in this treatment on the basis of the carload price, $8.00/lb (Littlehale, 1973). The average FPC prepared from sheepshead contains 1–1.5 ppm of mercury. Reduction of the mercury content to below, e.g., 0.5 ppm would cost between 9.5 and 14.5 cents for the $NaBH_4$ per pound of FPC. This figure would be in addition to the normal preparation cost of FPC and does not include labor, water for washing, etc. The cost would be proportionately less as the mercury content of the starting material approaches 0.5 ppm.

Possible reduction in the treatment cost could be brought about in several ways. For one thing, the method of mechanically separating the mercury from FPC once reduction to Hg metal has taken place could be improved. The use of an inert gas such as nitrogen rather than relying on the hydrogen released during the sodium borohydride treatment to disengage the mercury from the FPC matrix might be effective here. Another possibility is to adjust the pH of the slurry initially with NaOH. This would avoid excessive hydrolysis of the borohydride before reduction has taken place.

The utility of sodium borohydride reductions for many applications including those involving food is only now being exploited. FDA approval has been obtained recently for a process involving sodium borohydride in which the shelf life of beverages is improved (Jula, 1975). Its selectivity as a reductant ensures that attack on protein components is minimal (Sullivan, 1973). The ease of removal of the decomposition product, BO_2^-, also should be noted. Finally, there may be an extra advantage which occurs by using sodium borohydride for treatment of FPC. Sodium borohydride has been shown to reduce elements such as Se, Cd, Pb, and As from a positive to a zero valence state in aqueous solution (Sullivan, 1973). These elements may also be reduced in fish and possibly be removed in the same manner as mercury. While this speculation requires verification, conceivably sodium borohydride could serve as a general purifier for natural materials.

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N-Nitrosodimethylamine in Cold-Smoked Sablefish

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Sablefish was treated with nitrite levels ranging from 0 to 1300 ppm prior to being cold smoked. The flesh was analyzed for the presence of *N*-nitroso compounds by gas–liquid chromatography immediately after processing and again after 2 weeks storage at 40°F. Trace amounts of *N*-nitrosodimethylamine (<10 ppb) were detected in samples with nitrite levels ranging from 0 to 550 ppm. This value did not increase with higher nitrite levels. Storage at 40°F did reflect a slight decrease in concentrations. The identity of the isolated compound was confirmed by gas–liquid chromatography as the nitramine derivative and by GC–MS.

Howard et al. (1970) have reported finding *N*-nitrosodimethylamine (DMNA) in smoked chub. Gas chromatographic determination and mass spectrometric confirmation of DMNA ranging from 4 to 26 ppb in samples of raw, smoked, and smoked nitrite and/or nitrate treated sable, salmon, and shad have been described by Fazio et al. (1971a,b). High amounts of DMNA (0.12–0.45 ppm) were also reported by Sen et al. (1972) in fish meal which had been implicated in the liver disease of mink in Canada. Studies by Crosby et al. (1972) showed volatile nitrosamines and nitrite levels in cured and fresh fish. Kawabata et al. (1973) found no DMNA in salted roe products prepared with less than 0.09 m*M* nitrite, while DMNA was detectable when higher concentrations were used.

Concern about the possibility of DMNA in smoked, nitrite-treated fishery products prompted the National Marine Fisheries Service to investigate their possible occurrence. Sablefish was selected as a target species because, in preliminary studies conducted at this Center, smoked sable was found from among several smoked-processed fishery products examined to contain the highest level of DMNA (22 ppb). We have attempted in this investigation to correlate DMNA concentration to nitrite level in stored and unstored samples of cold-smoked sablefish. The results of these findings are presented in this article.

EXPERIMENTAL SECTION

Materials. The solvents, methylene chloride, pentane, methanol, and ethyl ether, were purified by distillation. Solvents, silica gel, and Celite 545 were tested prior to use to assure the absence of interfering peaks.

Gas Chromatographic Conditions. A Victoreen Model 4000 gas chromatograph equipped with a Coulson electrolytic conductivity detector and an Autolab System IV computing integrator was employed in the analysis of sablefish extracts. A 6 ft × 4 mm i.d. glass column coated with 10% Carbowax 1540 + 3% KOH on 100–120 mesh Gas-Chrom Q support was used. The following parameters were maintained throughout all analyses: temperature of injector block, 190°; carrier gas (helium) flow rate, 70 ml/min; GC oven temperature, 120° from 0 to 300 sec; 120–180° at a program rate of 5°/min.

Conditions of Coulson detector operated in reductive mode were: hydrogen flow rate, 83 ml/min; venting helium flow, 70 ml/min; furnace temperature, 820°; venting block temperature, 200°; conductivity bridge, 30 V; attenuation, 1.

GC–MS Apparatus. A 3200F–6103 Finnigan (Quadrupole) Automated GC–MS system was used in conjunction with a 5 ft × 2 mm i.d. glass column coated with 3% SP-2340 on 80–100 mesh Supelcoport. GLC parameters were: carrier gas flow rate, 20 ml/min; injector temperature, 200°; oven, 80° from 0 to 300 sec; 80–180° at a programmed rate of 5°/min. Separator and transfer lines were maintained at 200°. Ionization was by electron impact at 70 eV and by chemical ionization using methane as the reagent gas.

Analytical Procedures. For this investigation, the multidetection method of Fazio et al. (1971a,b) was used to determine volatile *N*-nitrosamines. Briefly, this procedure involved digestion of the sample in methanolic KOH, liquid–liquid extraction of an aliquot equivalent to 25 g with methylene chloride, and distillation of the nitrosamines

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