USE OF THIOLATED AMINOETHYL CELLULOSE TO REMOVE MERCURY BOUND TO SOLUBILIZED FISH PROTEIN

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Abstract

Aminoethyl cellulose (AEC) was thiolated with S-acetylmercaptosuccinic anhydride. The S-acetyl protective groups were removed at pH 11.5 to yield thiolated aminoethyl cellulose (TAEC) preparations with 0.077 to 0.109 $\times$ 10^{-3} mole SH per g TAEC. TAEC bound approximately 0.1 $\times$ 10^{-3} mole of p-mercuribenzoate/g.

Mercury was removed from fish protein by stirring solubilized tuna fish protein concentrate with TAEC at pH values from 6 to 11. The fish protein was solubilized by either a high temperature, high pH process, or by succinylation. The amount of mercury removed was pH dependent, being maximal at pH values 6.4 and 9. Under reducing conditions, that is, TAEC treated with dithiothreitol, and the mercury removed under nitrogen, 80% removal from a 2% protein solution at pH 7 was achieved. Srafion NMRR, a commercial chelator for dissolved organic and inorganic mercuric salts, was ineffective in removing mercury from soluble fish protein concentrate.

Of all forms of mercury, methylmercury is best absorbed and most slowly excreted by man and animal *(3)*. Swedish investigators *(26)* reported that mercury in fish tissue from lakes in Sweden and Japan exists almost entirely as methylmercury. Smith et al. *(18)* reported the same to be true in North American fish. The hazards of mercury have been reviewed extensively by Miller, Berg and co-authors *(15)* and by Nelson et al. *(16)*. However, Ganther et el. *(6)* have indicated that the danger of mercury in tuna for humans and other animals may be less than anticipated, since selenium in tuna and, possibly other modifying factors, may reduce methylmercury toxicity.

High levels of mercury in tuna have been reported in museum specimens caught 62 to 93 years ago, and there is little difference in mercury concentration between the museum samples and samples caught recently *(14)*. Thus, the high mercury levels now being found in ocean fish are not the consequence of man-made pollution, but apparently are of natural origin.

Attempts to accelerate excretion of mercury by animals after ingestion or injection have been reported by Takahashi and Hirayama *(23)*, and by Trojanowska et al. *(25)*; however, ideally, the best approach is to prevent ingestion of mercury initially. Recently, Regier *(18)* used acidified isopropanol to extract mercury from fish protein concentrate. Four extractions with 2.6% concentrated HCl in 99% isopropanol were necessary to remove 93.1% of the mercury. Methylmercury in fish is protein bound, distributed in muscle evenly, and is excreted very slowly *(2, 13)*. A material, such as a sulfhydryl-containing resin, that has a capacity for binding methylmercury could be used to remove mercury from fish protein.

In this paper, we report the capacity of thiolated aminoethyl cellulose to remove mercury from a solution of tuna protein at pH values ranging from 6 to 11. In addition, a resin reported by Law *(11)* to have a high affinity for both methylmercury and inorganic mercury was tested.

Materials and Methods

Materials

Freeze-dried tuna, which contained levels of mercury > 0.5 ppm, was obtained from Dr. M. L. Sunde of the Department of Poultry Science, University of Wisconsin. Aminoethyl cellulose was purchased from Bio-Rad, whereas S-acylmercaptosuccinic anhydride, 5,5'-dithiobis (2-nitrobenzoic acid), and dithiothreitol were purchased from Calbiochem. Srafion NMRR resin was a product of Ayalon Water Conditioning Company of Haifa, Israel, 2,4,6-trinitrobenzenesulfonic acid was from Eastman Chemical, and p-chloromercuribenzoic acid (Na) was from Nutritional Biochemicals. All other chemicals were reagent grade.

Methods

Thiolation of aminoethyl cellulose. Aminoethyl cellulose (AEC) was thiosuccinylated by the method of Klotz and Heiney *(9)*, using S-acetylmercaptosuccinic anhydride at pH 7.0 in 0.1 M phosphate buffer. A 20-fold molar excess of anhydride over exchange groups on the AEC was employed. Acetyl protective groups were removed by base hydrolysis at pH 11.5 for 45 min. The thiolated aminoethyl cellulose (TAEC) was washed and freeze dried. The sulfhydryl content of the TAEC was determined by the method of Ellman *(5)*.

Mercury binding capacities of AEC and TAEC were determined at pH 7 by stirring a known weight of dry cellulose with a solution of p-chloromercuribenzoic acid, sodium salt (pCMB), in 0.1 M phosphate buffer under nitrogen. At various intervals a 1-ml aliquot was read at 231 nm in a Beckman DU-2 spectrophotometer. The amount of pCMB remaining in solution was determined from a standard curve.

Solubilization of tuna protein. Fish protein concentrate (FPC) was prepared from tuna according to the isopropanol extraction method of Power *(17)*. The FPC was solubilized by either of the two following procedures: I. The high temperature, high pH method described by Tannenbaum et al. *(22)*. II. Succinylation of the FPC with succinic anhydride at

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pH 7.0 (Chen and Richardson, *unpublished*).

In both instances, protein was recovered by precipitation at pH 4.8 (preparation I) and 4.0 (preparation II), respectively, where maximum precipitation occurred. The protein precipitates were washed several times with deionized water and freeze dried. Preparation I was readily soluble at pH 8 or above, and preparation II at pH 6 or above. The approximate degree of succinylation of preparation II was determined by labeling the epsilon amino groups of lysine and N-terminal amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS) according to the method of Habeeb *(7)*. The degree of succinylation was approximated from the number of free amino groups in preparation II compared with its total lysine content, determined by amino acid analysis with a Phoenix model M-7800 amino acid analyzer, according to the method of Spackman et al. *(20)*.

Removal of mercury from fish protein. A 1-g sample of preparation I or II was dissolved in 50 ml of either 0.1 M phosphate buffer (pH 6 and 7) or 0.1 M Tris buffer (pH 8, 9, and 11). Then 30 ml of the solution was stirred with 0.5 g TAEC for 2 hr. The level of sulfhydryl groups in the TAEC was in 3,000-fold molar excess over the mercury in the sample. The mixture was centrifuged to remove the TAEC, the protein was precipitated at the appropriate pH, then taken up in deionized water and titrated with 1 N NaOH until completely dissolved. The solution was made up to 25 ml with deionized water, and aliquots were taken for mercury and protein analyses. The remaining 20 ml of the protein solution was used as a control and was treated exactly as the sample, except that it was not stirred with TAEC.

Srafion NMRR resin was also stirred with fish protein solution. Amount of resin used was 2 g/g protein, and the subsequent procedures were the same as those for TAEC.

Determination of mercury. A 5-ml aliquot of fish protein solution was digested as described by the AOAC *(1)*. The digested mixture was diluted to 50 ml with deionized water, and mercury was measured by the method of Uthe et al. *(26)*.

Determination of protein. Freeze dried preparation II, its nitrogen content determined by semi-micro Kjeldahl, was used as a standard to determine the protein content of various fish protein solutions by the method of Lowry et al. *(12)*.

Results and Discussion

Thiolation of aminoethyl cellulose

Optimum conditions for removal of acetyl protecting groups after thiosuccinylation, and for analysis of sulfhydryl groups on cellulose are given in Fig. 1. It is evident that about 45 min at pH 11.5 is sufficient to yield maximum sulfhydryl content. From curves B and C, the optimum conditions for sulfhydryl analysis were established at 0.08 ml of standard Ellman's solution *(5)* with a reaction time of 2 hr.

Sulfhydryl contents and mercury binding capacities of TAEC and AEC are reported in Table 1 and Fig. 2. The exchange capacity of AEC was 0.27×10^{-3} mole/g cellulose, and by using a 20-fold molar excess of S-acetylmercaptosuccinic anhydride, we were able to thiolate 30 to 40% of the amino groups. Furthermore, when a 60-fold molar excess of anhydride was used, the degree of thiolation was the same. It is evident from Table 1 and Fig. 2 that TAEC binds pCMB very effectively whereas AEC has a negligible affinity for the mercurial. In each instance, the cellulose derivative binds more pCMB than anticipated from its "sulfhydryl" content. Presumably this results from nonspecific binding of pCMB by the cellulose derivatives.

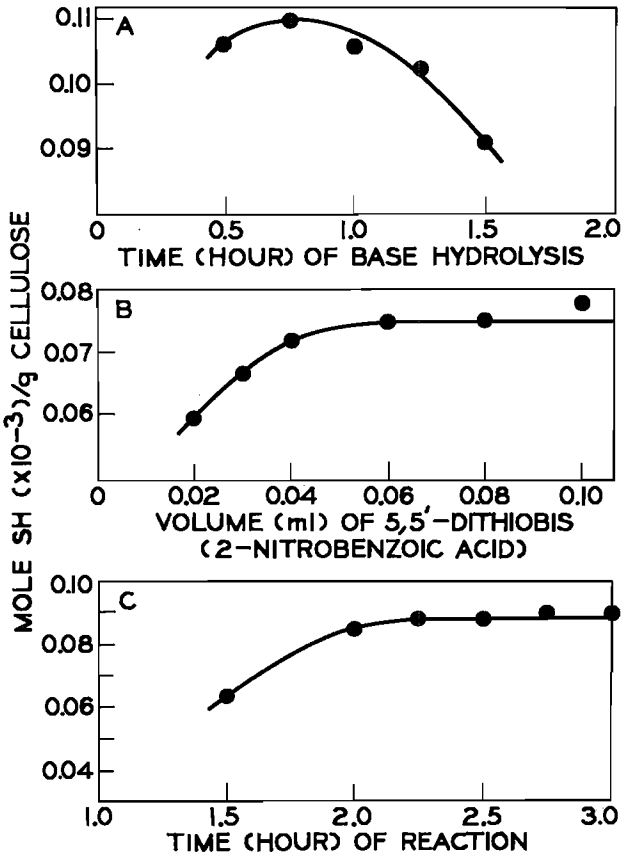

Figure 1. Optimum conditions for preparation and analysis of thiolated aminoethyl cellulose. A. Time of base hydrolysis (pH 11.5) of the acetyl thiosuccinylated aminoethyl cellulose to remove acetyl groups. B. Amount of standard 5,5'-dithiobis (2-nitrobenzoic acid) solution used to determine sulfhydryl groups. C. Time of reaction of TAEC with 5,5'-dithiobis-(2-nitrobenzoic acid) to determine the sulfhydryl contents.

Table 1. Sulfhydryl content and mercury binding capacity of thiolated aminoethyl cellulose (TAEC) and aminoethyl cellulose (AEC)

	Mole SH/g cellulose	Mole Hg[1]/g cellulose[2]
TAEC	0.109×10^{-3}	—
	0.077×10^{-3}	0.115×10^{-3}
AEC	0.09×10^{-3}	0.175×10^{-3}

[1]As p-chloromercuribenzoate
[2]Calculated from Fig. 2

Mercury content of tuna protein

Canned tuna was processed through several steps to yield a soluble protein, and, as shown in Table 2, the protein concentration increased at each stage due to elimination of water, lipid, and other nonprotein

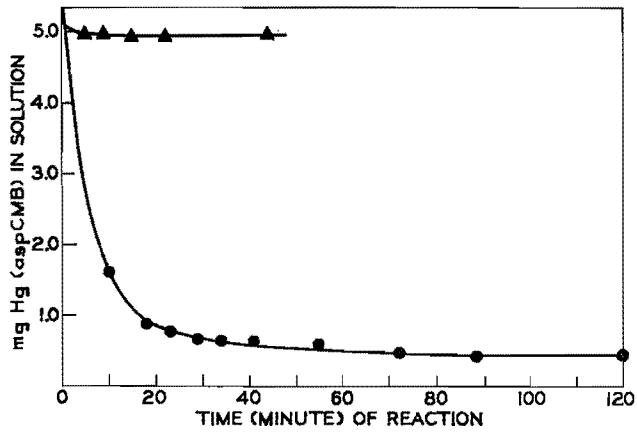

Figure 2. Binding of mercury (as p-chloromercuribenzoate) by thiolated aminoethyl cellulose (closed circles) and aminoethyl cellulose (closed triangles) as a function of time. The amount of cellulose used in each instance was 0.2 g.

substances. The level of mercury remains essentially constant at about 5 ppm based on protein indicating the mercury is associated with the protein. Data in Table 2 indicate there is no significant change in mercury levels due to heating and freeze drying. Westoo *(28)* reported that frying or boiling of fish did not remove methylmercury.

Removal of mercury from tuna protein

Preparations I and II treated with TAEC revealed a decrease in mercury level, as shown in Table 3. The mean removal varied from 49% to 70% with a high of 78%. It is evident that elimination of mercury from fish was pH dependent, having maxima at about 6.4 and 9. In studies using cysteine, penicillamine, glutathione, mercaptoacetic acid and related compounds to remove mercury from hemoglobin, Sugiura et al. *(21)* reported that there was a linear correlation between percent ethylmercuric chloride removed and dissociation constants of chelator sulfhydryl groups. The dissociation constants of sulfhydryl groups on TAEC and the protein-methyl-mercury complex are not known, but they could be responsible for the slightly increased removal at these two pH values. Further studies on this matter are needed.

TABLE 2. PROTEIN AND MERCURY LEVELS OF TUNA AT VARIOUS STAGES OF PROCESSING

	% Protein	ug Hg/g protein
Lyophilized canned tuna	57.12	5.10
Tuna FPC	83.46	5.32
Preparation I[1]	99.30	5.05
Preparation II[2]	98.90	5.05

[1]Protein solubilized by the method of Tannenbaum et al. *(22)*
[2]Protein solubilized by succinylation

With the TNBS reagent, the number of amino groups in preparation II was calculated to be 0.28×10^{-3} mole/g protein, using 1.09×10^{4} as the molar extinction coefficient of one trinitrophenylamino group *(8)*. From amino acid analysis, the total lysine content for this preparation was 0.60×10^{-3} mole/g protein. The difference between the above two values should reflect the amount of succinylated lysine. The degree of succinylation thus calculated was 53%. Presumably, most of the N-terminal amino groups were succinylated because of the lower pK of the alpha amino group; however, the uncertainty as to the extent of this reaction renders the above value only an approximation.

Because succinylated preparation II would possess a large net negative charge compared to preparation I, it was thought that the protein molecules might be more "open" as a result of charge repulsion, and protein bound mercury would be removed more easily. But Table 3 shows that mercury removal was similar, indicating that succinylation probably did not have any effect on the removal process.

Sulfhydryl groups on TAEC were quite stable to oxidation when the cellulose was stored in the dry state. In one preparation, the amount of these groups was 0.109×10^{-3} mole/g TAEC immediately after thiolation, and 0.102×10^{-3} mole/g after two months' storage at 4 C as a powder. However, during stirring with the protein solutions, air was incorporated into the solution, and sulfhydryl groups might be oxidized

TABLE 3. REMOVAL OF MERCURY FROM FISH PROTEIN WITH THIOLATED AMINOETHYL CELLULOSE (TAEC) AS A FUNCTION OF pH

pH	Preparation I		Preparation II	
	Untreated	Treated	Untreated	Treated
	(ug Hg/g protein)[1]			
6.0	5.1 ± 0.1	2.6 ± 0.1	—	—
6.4	5.2 ± 0.1	1.8 ± 0.1	—	—
7.4	5.1 ± 0.2	2.3 ± 0.2	—	—
8.0	—	—	5.17 ± 0.05	2.1 ± 0.1
8.4	5.1 ± 0.1	2.1 ± 0.2	—	—
9.0	—	—	5.1 ± 0.2	1.54 ± 0.08
11.2	—	—	5.2 ± 0.1	1.66 ± 0.06

[1]Mean ± S.D. of mean (n = 4)

to perhaps lose mercury binding capacity. This possibility was studied by first treating 0.5 g TAEC (0.077×10^{-3} mole SH/g) with a dithiothreitol solution under nitrogen for 1 hr to maximize the sulfhydryl content of TAEC. Ratio of sulfhydryl groups on TAEC and dithiothreitol was 1:10. The mixture was then centrifuged, and the TAEC was washed several times with deionized water (under nitrogen). The reduced TAEC was stirred with a 2% solution of preparation II at pH 7 for 2 hr, with nitrogen bubbling through the solution during the course of reaction. Mercury removal was 74%, compared with 65% obtained by using 0.5 g untreated TAEC (0.109×10^{-3} mole SH/g) in air.

The effect of protein concentration (preparation II) on mercury removal was also studied. All samples were stirred with reduced TAEC under nitrogen at pH 7. Low protein concentrations showed a slight but insignificant tendency toward higher mercury removal. Protein solutions greater than 4% were difficult to prepare because of solubility limits, and the higher concentrations were quite viscous to work with. Recovery of protein after treatment with TAEC was between 80 and 100%.

Since AEC did not bind mercury in the form of pCMB (Table 1), it was assumed that it also would not remove mercury from fish. This was further demonstrated by the failure of 0.5 g AEC when stirred with 30 ml of 2% preparation I at pH 9 to remove any mercury.

The ability of Srafion NMRR resin to remove mercury from fish was also tested. This product is a chelating resin for noble metals, and, as shown by Law *(11)*, has a high affinity for methylmercury and inorganic mercuric salts. The resin contains positively charged amidine groups on a styrene-divinylbenzene copolymer matrix, and its properties and selectivities were reported by Koster and Schmuckler *(10)*, and Law *(11)*. However, this resin was totally ineffective in removing mercury from preparation I at pH 9, and from preparation II at pH 6. Possibly, the strong positive charges on the resin bound the protein which masked the chelating groups.

Westoo *(28)* postulated that methylmercury was attached to protein probably in the form of R-S-Hg-CH_3. Sugiura et al. *(21)* identified a ternary Hb-Hg-cysteine complex by gel filtration as an intermediate in removing mercury from hemoglobin, using sulfhydryl containing reagents, but the mechanism of TAEC in removing mercury from fish protein needs further investigation.

There are several binding sites for methylmercury in fish organs and muscles *(16)*, but these sites have not been identified. It is possible that some sites bind mercury more strongly than others, and mercury will probably bind to those strong sites first, and consequently be removed last. Clarkson and Magos*(4)* reported that in rat liver and kidney homogenates, two classes of mercury-binding sites were observed, one class having a chemical affinity for mercury 100-fold greater than the other. So the higher the mercury level in fish, the easier it probably will be to remove a large percent of it, only the amount that binds to the stronger sites will be difficult to eliminate. In the present studies, failure of TAEC to remove all mercury from tuna protein may reflect a stronger affinity of mercury for protein, or it may result from steric factors preventing the interaction of bound mercury with TAEC.

The necessity for treating soluble proteins with insoluble TAEC tends to limit the practical application of this system in processing relatively insoluble fish proteins. Consequently, thiolated polymers of this type may be more useful when fed with contaminated fish to prevent absorption of mercury, by poultry for example. Presumably, mercury would be bound to the undigestible thiolated cellulose in the gastrointestinal tract and be excreted with the thiolated cellulose.

Digestion of fish proteins in the intestinal tract tends to obviate any steric factors preventing interaction of bound mercury with TAEC. Thus, TAEC might be more effective in binding mercury in fish protein as part of the feeding regimen. Although the insoluble nature and undigestibility of cellulose derivatives by monogastric animals seems to render TAEC innocuous, the safety of feeding such compounds remains to be determined.

ACKNOWLEDGMENTS

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REPORT OF COMMITTEE ON FOOD EQUIPMENT

(Continued from Page 266)

of a specified size was presented by the Foundation staff. After considerable discussion, the recommendation of the Task Committee to delete the provisions of Item 4.404 of NSF Standard No. 4, was not approved; thus, the requirements of Item 4.404 will remain as issued. The public health representatives were not satisfied with either the Committee's recommendation or with the specifications to remove paricles 10μ or larger, and they indicated more stringent requirements should be adopted as soon as technology would permit the manufacturing and evaluating of such equipment.

Standard No. 5—Hot water generating equipment

The Foundation staff next presented the problems encountered in evaluating equipment and the reaction of industry relating to elimination of the 40° temperature rise in the listing of hot water generating equipment under the provisions of NSF Standard No. 5. They also noted that a decision was necessary as regards the efficiency ratings to be utilized in determining recovery capabilities of hot water generating equipment. After a brief discussion, the Joint Committee agreed that the 40° temperature rise would be acceptable and that the following efficiency should be utilized in determining recovery rates of such equipment: electric, 100%; and gas, 70%.

Standard No. 7—Food service refrigerators and freezers

The Foundation staff reviewed the activities of the Standards Task Committee for Alternate Methods of Effecting Coves in Walk-ins. According to one member of the Joint Committee, there was no possible method by which coves could be applied in the field and comply with provisions of the Standard requiring smooth continuous radii. The Foundation staff then outlined the numerous existing requirements relative to radii in 2 and 3 plane intersections of interior liners of refrigerators under various NSF Standards No. 1, 2, and 7 which indicated a lack of uniformity of specifications.

After a brief discussion, the Joint Committee recommended that the current Task Committee or another NSF Standards Task Committee be established to review this issue and recommend appropriate revisions in applicable NSF Standards to provide for uniformity; and it was requested that the Task Committee also be instructed to review the feasibility of drains in both walk-in and reach-in refrigerators and to recommend appropriate revisions in Items 4.154 and 5.01.

Standard No. 8—Commercial powered food preparation equipment

Item 4.01 of Standard No. 8 on cleanability of food contact surfaces, which was used as a guide in amending the same item in Basic Criteria C-2 and Standard No. 4, was amended as noted above under C-2.

The proposed extensive revisions in NSF Standard No. 8,

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