Thiamine Modifying Properties of Heme Proteins from Skipjack Tuna, Pork, and Beef

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The thiamine modifying factors (TMF) of Skipjack tuna dark red muscle (blood channel) were concentrated in protein fractions precipitated at 70 and 80% saturation with $(NH_4)_2SO_4$ at pH 6.8. These fractions contained myoglobin and hemoglobin as the major constituents. The thiamine modifying property of the tuna myoglobin and hemoglobin was similar to that of commercial samples of these iron complexes. Beef and pork also contained TMF which was associated primarily with the 70 and 80% $(NH_4)_2SO_4$ saturation fractions while that of tuna light muscle was spread throughout the fractions from 30 to 80% saturation.

Extracts from various fish and crustacea have been found to destroy thiamine (Fujita, 1954; Green *et al.*, 1942; Yudkin, 1949). These investigations led to the postulation that an enzyme, thiaminase, was the active antithiamine agent. More recently an antithiamine factor, isolated from carp viscera, was shown to be thermostable and nonenzymatic in nature. The active factor was identified as hemin or a related compound (Kundig and Somogyi, 1967; Somogyi, 1966).

Work in this laboratory has shown that various Pacific fish contain thermostable antithiamine activity (Hilker and Peter, 1966, 1968).

The present study concerns thiamine-modifying properties of heme protein fractions of Skipjack tuna (*Katsuwonus pelamis*), beef, and pork.

EXPERIMENTAL SECTION

Isolation Procedure. The dark muscle (blood channel) from fresh Skipjack tuna was homogenized (1:2 w/v) with distilled H_2O for 3 min in a Waring blender. The homogenate was centrifuged at 10,400 × *g* for 10 min at 4° and the supernatant passed through several layers of cheesecloth. The pH was brought to 6.8 with 1 *N* NaOH and the requisite amount of solid $(NH_4)_2SO_4$ was added slowly with stirring at 4° to give 30% saturation according to the procedure described by Dollar *et al.* (1959) for the isolation of myoglobin. The system was centrifuged (27,300 × *g*, 15 min, 0°) and the precipitate collected. The precipitation and centrifugation were repeated with increments of 10% saturation until 80% saturation was reached. Each fraction was then dissolved in a minimal volume of distilled H_2O, dialyzed against distilled H_2O at 4° until free of SO_4^{2-}, centrifuged (36,400 × *g*, 20 min, 0°) and the supernatant was collected.

Dry weights were taken of each fraction for calculation of thiamine modifying activity (TMA). In later preparations the pH was adjusted to 6.8 and the system was immediately brought to 60 then 70 and to 80% $(NH_4)_2SO_4$ saturation omitting the 10% progression steps. Pork (loin) and beef (sirloin tip) were treated similarly.

DEAE Ion-Exchange Cellulose Chromatography. DEAE-cellulose (Calbiochem) was washed several times with distilled H_2O, then equilibrated with Tris buffer (0.05 *M*, pH 8.6). The cellulose was packed by gravity into a 2.0 × 60.0 cm column and the separation of the heme proteins was made according to Brown (1961).

Electrophoresis. The tuna dark muscle heme protein fractions from the $(NH_4)_2SO_4$ precipitation and chromatographic separation were analyzed by low voltage electrophoresis on cellulose polyacetate (Sepraphore III) strips at pH 8.8 using the buffer system, Tris 16.5 g, EDTA 1.52 g, and boric acid 0.92 g per 1000 ml. The protein concentration varied from 2 to 20 mg/ml. A Gelman electrophoresis apparatus was used with a voltage of 340 at 5 mA current. The protein was stained with 0.5% Poinceau S.

Molecular Gel Filtration. The hemoglobin and myoglobin bands from the DEAE-cellulose chromatography column were desalted by passage through a 4600 exclusion limit molecular gel column (Bio-Gel P-6, Bio-Rad Laboratories) and then lyophilized. The myoglobin sample was then placed on a molecular gel column (Biogel P-30), exclusion limit 30,000, and eluted with Tris buffer, pH 8.8, and collected in 1-ml samples at a flow rate of 40 ml/hr. The absorption of the eluted samples was read at 407 mμ in a Beckman DGB spectrophotometer to identify samples containing heme protein. Comparison was made with identical elution chromatograms run with commercial myoglobin (Calbiochem, mol wt 17,000).

Thiamine Modifying Factors. The effectiveness of the thiamine modifying factors (TMF) from the $(NH_4)_2SO_4$ fractions was determined by the addition of known amounts of the protein solution to a stock thiamine solution (0.12 μg/ml). The remaining thiamine was determined immediately by a modified thiochrome method (Hilker *et al.*, 1971). The fluorescence was measured with a Turner Fluorometer equipped with a 365 mμ primary and 450 mμ secondary filters.

The TMF determinations on the tuna light muscle, pork, and beef were run as recoveries with known quantities of thiamine added to the homogenized samples and carried through the analytical procedure along with thiamine standards.

The difference between the analyzed and calculated thiamine was considered to have interacted with protein and is designated as modified thiamine (MT).

The thiamine content of the tuna, pork, and beef was determined according to the standard fluorometric method (Strobecker and Henning, 1965). Thiamine was extracted by homogenizing the sample with 0.001 *N* HCl (1:2, w/v) in a Waring blender for 3 min at high speed. The mixture was heated 1 hr with occasional shaking on a boiling water bath, cooled to room temperature, shaken vigorously, centrifuged, and filtered. The filtrate was adjusted to pH 2.5 with 1 *N* HCl and incubated with pepsin overnight at 45°, cooled to room temperature, then the pH was adjusted to pH 4.5 with 10% sodium acetate and diastase added to achieve liberation of the phosphoric acid esters. The extracted sample was purified by passage through Permutit (Matheson, Coleman and Bell) and eluted with hot acidified KCl. The eluate was used for the thiamine analysis as described above. In preliminary tests, thiamine determinations were made on the original filtrate, the extract after incubation with pepsin and diastase, and on the eluate from the Permutit column. No increase in thiamine content was detected after enzyme hydrolysis. Therefore, the latter two steps were eliminated in the determination of thiamine content in the fish, pork, and beef.

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Table I. Thiamine-Modifying Activities of Commercial and Tuna Heme Proteins

Compound	Thiamine-modifying activity, μg/mg dry weight[a]
Myoglobin (tuna, isolated)	18.8
Myoglobin (equine heart, Calbiochem)	18.5
Hemoglobin (tuna, isolated)	20.0
Hemoglobin (bovine, Calbiochem)	18.8

[a] Experimental values are an average of three or more determinations. Values refer to thiamine (μg) modified per mg of heme protein.

RESULTS AND DISCUSSION

Previous reports from this and other laboratories have used the term antithiamine activity in referring to the decrease in thiamine after incubation with tissue extracts or compounds. Since this term is potentially confusing in that the biological significance and reaction products are unknown, a new term "thiamine modifying activity" (TMA) is being introduced.

Initial studies of the TMA of tuna protein showed that the majority of the activity resided in the dark muscle; *i.e.*, blood channel. Fractionation of this dark muscle extract by $(NH_4)_2SO_4$ precipitation showed that the 70 and 80% saturation fraction contained the highest TMA (Figure 1). These protein solutions had an intense reddish-brown color and exhibited a Soret band maximum at 407 mμ, indicating the presence of hemoglobin-type protein.

Heme proteins from the 70% fraction were further separated into a minor band identified as myoglobin, a major hemoglobin, and a nonmobile band by DEAE column chromatography. The two fractions were desalted and the tuna myoglobin was identified by elution on a gel permeation column to give a homogeneous band with an approximate molecular weight of 17,000, similar to commercial myoglobin. The 80% fraction was also separated into a major myoglobin component, which was further resolved into two myoglobins migrating 14 and 18 mm, respectively, under low voltage electrophoresis.

The TMA of the freshly isolated myoglobin and hemoglobin was identical with the activity of commercially available bovine hemoglobin and equine myoglobin under identical test conditions (Table I). Establishment of TMA in tuna heme proteins confirms similar activity referred to as "antithiamine activity" found by Kundig and Somogyi (1967) in carp viscera proteins.

Since heme proteins, hemoglobin, and myoglobin were shown to possess TMA, it would seem probable that any meat containing these compounds would have thiamine modifying activity as determined by the thiochrome procedure. For further comparison of food heme protein sources, pork, beef, and the light flesh of Skipjack tuna were tested for thiamine content and also TM content. These analyses are reported in Table II. The values for thiamine and TM refer to the analyzed thiamine (apparent thiamine, AT) and nonrecovered thiamine (modified thiamine, MT) in μg per 100 g of (fresh) tissue. It is the practice in some food laboratories to apply a correction factor based on recoveries to their analytical values. If such calculations were made for our analyses, the thiamine content would be 158, 676, and 196 μg/100 g for the beef, pork, and fish, respectively. Which value (AT or AT+ MT) more accurately represents biologically available thiamine is not apparent from present information. In agreement with published values (Church and Church, 1970), pork showed a higher thiamine content than beef. No thiamine values were reported for Skipjack tuna.

Table II. Comparison of Thiamine and Modified Thiamine Contents of Beef, Pork, and Skipjack Tuna

	Beef	Pork, μg/100 g	Fish, fresh tissue
Thiamine (apparent, AT)	119[a]	613	33
Modified thiamine (MT)	38	63	163

[a] Values are an average of two to three determinations. Values are based on 100 g of fresh tissue.

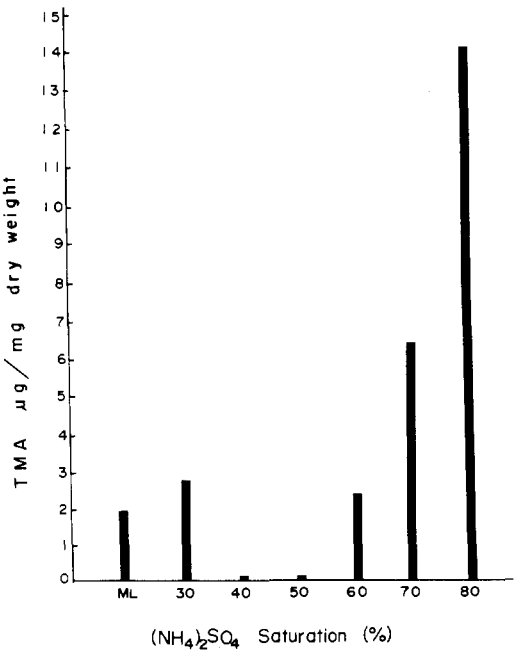

Figure 1. Thiamine-modifying activity of various $(NH_4)_2SO_4$ fractions of Skipjack tuna dark muscle. ML refers to mother liquor. The TMA values refer to μg of thiamine modified per mg of dry solids from the various $(NH_4)_2SO_4$ fractions after dialysis.

The MT was highest in fish, lowest in beef, and intermediate in pork. The MT of these tissues was determined by measuring the unrecovered thiamine when thiamine was added to the homogenate and carried through the extraction and analytical procedure. The unrecovered thiamine was assumed to be changed (chemically) so that thiochrome formation was prevented. While the end product(s) of the reaction of thiamine with hemoglobin and myoglobin has not as yet been identified, our studies involving chromatography of thiamine and heme proteins (Hilker, 1972) indicate that thiamine is not split at the methylene bridge as in the case of thiaminase (Fijita,

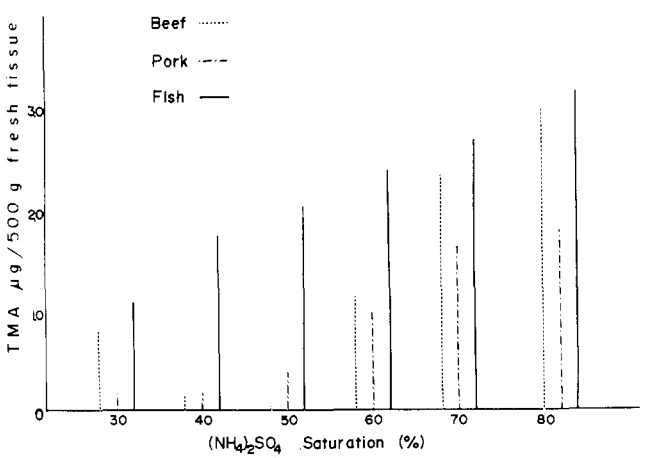

Figure 2. Thiamine-modifying activity of $(NH_4)_2SO_4$ fractions of beef, pork, and light muscle of Skipjack tuna. The TMA values refer to μg of thiamine modified per 500 g of wet tissue from the various $(NH_4)_2SO_4$ fractions after dialysis.

1954), nor is it bound to the heme protein. These studies indicate that the coexistence of thiamine and thiamine-modifying compounds in foods such as fish limits the usefulness of present methodology for the accurate analysis of thiamine in food. Thiamine exists in tissues as the coenzyme thiamine pyrophosphate thiamine monophosphate and is also bound to protein. It is therefore unlikely that reaction between thiamine compounds and heme protein would take place in tissues. Free thiamine is formed during the analytical procedure and could react with hemin and heme protein during this process. The relationship of thiamine-modifying compounds and biological availability of thiamine needs further study.

The TMA of the various $(NH_4)_2SO_4$ fractions of beef, pork, and the light flesh of Skipjack tuna is shown in Figure 2. The TMA of the fish was spread throughout the fractions, while that of the beef and pork was more concentrated in the 70 and 80% saturation fractions. The solutions of lower $(NH_4)_2SO_4$ saturation fractions of the fish had a pink color, indicating the presence of hemoglobin-type proteins, while the equivalent fractions of the beef and pork were colorless. This would indicate that the physical properties of the Skipjack tuna heme proteins differ from the beef and pork heme proteins and are precipitated to some extent at lower $(NH_4)_2SO_4$ saturation levels. The lack of correlation between the TMA of the 70–80% $(NH_4)_2SO_4$ myoglobin precipitations and the TMA of the fish, beef, and pork suggests that TMA factors other than heme protein may be involved.

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Structure Activity Correlations of Biodegradability of DDT Analogs

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Methoxy-methiochlor [2-(*p*-methoxyphenyl) - 2-(*p*-methylthiophenyl) - 1,1,1-trichloroethane], methyl-ethoxychlor [2-(*p*-methylphenyl) - 2-(*p*-ethoxyphenyl) - 1,1,1-trichloroethane], and chloro-methylchlor [2-(*p*-chlorophenyl) - 2-(*p*-methylphenyl) - 1,1,1-trichloroethane] were studied for metabolic pathways in mice and insects and for biodegradability in a model ecosystem. Methoxy-methiochlor and methyl-ethoxychlor were good substrates for multifunction oxidases and showed biodegradability indices of 2.75 and 1.20, respectively. Chloro-methylchlor, with only a single degradophore on the aromatic ring, was also a satisfactory substrate and showed a biodegradability index of 3.43, compared to 0.015 for DDT.

Recent reports from this laboratory have described the principles of biodegradability of DDT-type molecules (Hirwe *et al.*, 1972; Kapoor *et al.*, 1970, 1972; Metcalf *et al.*, 1971b). Here we report the comparative metabolism and biodegradability of three asymmetrical biodegradable DDT molecules: methoxy-methiochlor or 2-(*p*-methoxyphenyl)-2-(*p*-methylthiophenyl) - 1,1,1-trichloroethane; methyl-ethoxychlor or 2-(*p*-methylphenyl)-2-(*p*-ethoxyphenyl) - 1,1,1-trichloroethane; and chloro-methylchlor or 2-(*p*-chlorophenyl)-2-(*p*-methylphenyl) -1,1,1-trichloroethane. The insecticidal properties of these compounds were described by Metcalf *et al.* (1971a). These data, together with comparable information on symmetrical DDT analogs (Kapoor *et al.*, 1970, 1972), permit discussion of the relationships of the chemical structure of the DDT analogs to biodegradability.

MATERIALS AND METHODS

Radiolabeled Compounds. ^{3}H-Ring-substituted methoxy-methiochlor and methyl-ethoxychlor were synthesized by the method of Hilton and O'Brien (1964) and purified by column chromatography on silica gel using 2% diethyl ether in petroleum ether (60–68°). The products had a radiopurity of 99.9+%, evaluated by thin-layer chromatography (tlc) using solvent system P.B. (Table I), with specific activities of 9.3 and 3.35 mCi/m*M*, respectively.

^{14}C-Ring-labeled chloro-methylchlor was prepared in 72.8% yield by condensation of 100 μCi (5 mCi/m*M*) of ^{14}C-ring labeled chlorobenzene diluted to 10 μl with 30 mg of *p*-methylphenyl trichloromethyl carbinol in 5 vol of H_2SO_4. The product was purified by column chromatography on silica gel with petroleum ether (60–68°) as the eluent and had a radiochemical purity of 99%, with a specific activity of 0.43 mCi/m*M*.

Radioassay. Tritium-labeled compounds were assayed by scintillation counting in 10 ml of ^{3}H cocktail (200 g of naphthalene, 10 g of PPO, and 0.25 g of POPOP in dioxane to make 1 l.). The ^{14}C-labeled compounds were similarly assayed in 10 ml of cocktail D (7 g of PPO, 100 g of naphthalene in dioxane to make 1 l.).

Chromatographic and Chromogenic Techniques. Thin-layer chromatography was performed in the usual manner using 0.25 mm silica gel with a fluorescent indicator coat-

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