It has been previously reported by Stegeman (Fed. Proc. 36:941, 1977) that sexually mature male rainbow trout (Salmo gairdnerii) contain more hepatic microsomal cytochrome P-450 and have higher aminopyrine demethylase activities (about 3-fold) than do females during spawning. This suggested that spawning and age (sexual maturity) of the winter flounder might be related to the wide variations in 7-ERF and AHH activities that we observed in this fish population. Consequently, we compared AHH activity to the gonad wt/body wt. ratio in female fish. Relatively large variations were noted in gonad wt/body wt. ratios (0.8 to 2.3%), consistent with different levels of ovarian function in these fish. Hepatic AHH activities (in homogenate) varied from 0.1 to 7.5 Units/min/mg protein. However, the correlation coefficient for AHH activity vs. gonad wt/body wt. ratio was only 0.34 (N=72), indicating no meaningful relationship between the two parameters. Thus, it appeared that spawning and sexual maturity were making a minor contribution, at best, to the widely divergent AHH and 7-ERF activities, which is also consistent with induction of the hepatic monooxygenase system in these fish by a PAH-type environmental pollutant.

Future work will determine the chemical(s) responsible for the PAH-type enzyme induction observed in Maine winter flounder and the amount of the compound(s) required for minimal and maximal AHH induction. The support of this research by the U.S. Environmental Protection Agency through the Federal Interagency Energy/Environmental Research and Development Program is acknowledged.

PURIFICATION OF A GLUTATHIONE S-TRANSFERASE FROM LITTLE SKATE LIVER, PREPARATION OF AN ANTIBODY TO THIS PURIFIED ENZYME AND IMMUNOLOGICAL SIMILARITY OF THE PURIFIED ENZYME AND HEPATIC AND EXTRAHEPATIC GLUTATHIONE S-TRANSFERASES FROM SEVERAL MARINE SPECIES FROM MAINE

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The glutathione S-transferases (GS-T) are an important family of enzymes involved in the detoxication of electrophiles, including carcinogenic and mutagenic epoxides. We have previously shown these enzymes to be widely distributed in hepatic and extrahepatic tissues of marine vertebrate and invertebrate species (James et al., Chem. Biol. Interactions 25:321-344, 1979). Since little skate was efficient at catalyzing the reaction of benzo(a)pyrene 4,5-oxide, a polycyclic arene oxide, with glutathione (GSH), the GS-T of this species were purified. Antibodies were prepared to the major purified enzyme and were tested for cross-reactivity to cytosolic GS-T from several tissues of various marine vertebrate and invertebrate species to investigate the phylogenetic distribution of GS-T that would react with the antibodies to this particular GS-T.

The following procedure was used for the purification of hepatic GS-T from the little skate. An aliquot (130 ml) of microsomal supernatant fraction pooled from several (6-10) male little skate livers was eluted from a DEAE-cellulose column (29 x 5 cm) with 0-0.1 M KCl linear gradient in 2 liters of 0.01 M potassium phosphate buffer, pH 8.0. Five separate peaks of GS-T activity with 2,4-dinitrochlorobenzene (DNCB) as substrate were obtained, accounting for 82%-106% of the activity applied (Figure 1). After concentrating each eluted peak by ultrafilitration (Amicon, PM-10 membrane), they were applied individually to a column (21 x 1.5 cm) of epoxy-activated Sepharose 6B (Pharmacia) which had been reacted with GSH (0.6 μmol GSH/g Sepharose). After thorough washing with 0.01 M Tris buffer, pH 8.0, the GS-T activity was removed from this column by elution with 15 mM glutathione in 0.05 M Tris (100 ml). This eluate was then successively concentrated and diluted with 1 mM GSH in 0.01 M Tris, pH 8.0. Electrophoresis of these peaks showed that this process had purified 2 of the 5 enzymes, including the major enzyme (peak 4, Figure 1) which accounted for over 60% of the total catalytic activity with DNCB. The enzyme was further characterized using conventional techniques. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated a molecular weight in the range of 28,500 daltons; electrofocusing showed the protein to have a pI of 5.4.

For antibody production, mg quantities of purified GS-T 4 were emulsified (w/o) in Freund's complete adjuvant (the ratio of antigensolution [0.5-0.9 mg/ml] to adjuvant was 1:1.2). The emulsion was injected subcutaneously at

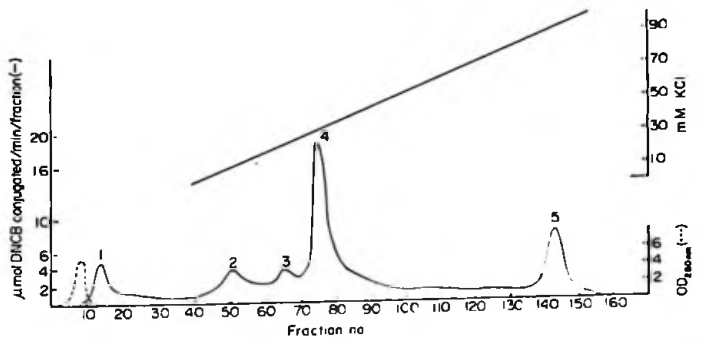

Figure 1. Elution of glutathione S-transferase activity (with DNCB as substrate) from DEAE-cellulose column with 0-0.1 M KCl gradient in 0.01 M phosphate buffer, pH 8.0, following application of little skate hepatic cytosol (fraction volumes ~ 13 ml).

multiple sites (0.1 ml each) along the midline of adult male New Zealand white rabbits according to the following schedule:

TIME (wks)	0	2	3.5	7	8
AMT (µg) of ANTIGEN/RABBIT	250	150	Serum collected	100	Serum collected

Blood was collected from the central ear artery. The serum (containing the antibody) was decanted from the clot, centrifuged and frozen until use.

Cross-reactivity reactions between the serum (antibodies) and antigens (GS-T enzymes) were determined by the double diffusion-precipitation technique of Ouchterlony using 0.9% agarose gels, pH 7.7, in the following manner: Tissue homogenates (33% w/v) were prepared in 1.15% KCl, pH 7.4, containing 0.001 M HEPES at 4°C using a motor-driven teflon pestle and a glass homogenizer. Microsomal supernatant fraction was prepared by ultracentrifugation (172,000xg for 45 min). Small amounts (3-10 µl) of the cytosolic fractions were placed in the perimeter wells of the agarose gels while various dilutions of sera were placed in the center well (Figure 2). Diffusion was allowed to proceed for at least 18 hours, after which the gels were immersed in saline for 3 days to remove unprecipitated protein.

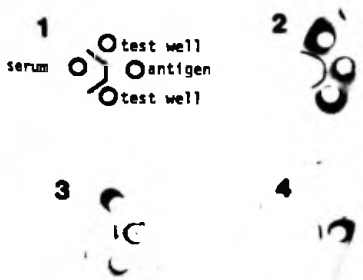

Figure 2. Ouchterlony precipitation patterns observed when antibodies prepared to a purified hepatic glutathione S-transferase from male little skate were challenged by microsomal supernatant fractions from hepatic and various extrahepatic tissues from several marine species. 1. Ouchterlony double diffusion test pattern, 2. Identity reaction -- test wells contain two concentrations of male little skate liver microsomal supernatant fraction, 3. Partial identity reaction -- test wells contain two concentrations of male large skate heart microsomal supernatant fraction, and 4. No cross-reactivity -- test wells contain two concentrations of flounder hepatic microsomal supernatant fraction. All reactions performed in 0.9% agarose gels, pH 7.7. Precipitation was allowed to proceed 18 hr; unprecipitated protein was salted out for an additional 72 hr. Gels were then stained in 0.115% Coomassie Brilliant blue and destained in 30% MeOH.

This was followed by staining in 0.115% Coomassie brilliant blue and destaining in 30% methanol. Three different categories of precipitin patterns were observed as shown in Figure 2. All diffusion patterns were compared to that obtained with freshly prepared male little skate hepatic cytosol.

The inhibition of GS-T activity by antibodies to the purified skate GS-T was studied in cytosolic fractions of liver from various skate species. In these experiments the cytosolic fraction was mixed with serum containing antibody (in

1 ml H_2O) for at least 10 min prior to the addition of GSH (5 mM) and DNCB (1 mM). The final reaction mixture also contained 0.1 M potassium phosphate buffer (pH 6.5) in a final volume of 3.0 ml. Control reactions were run identically except that the antibody containing serum was replaced by an identical amount of preimmune serum. Enzyme rates were corrected for the non-enzymatic contribution and for the contribution of serum activity (even though the latter was very low).

Based on the identity precipitin-reaction (Figure 2.2), the hepatic GS-T from female little skates was immunologically identical to that from male little skates (Table 1). Similarly, hepatic cytosol from the thorny skate (Raja radiata) and the

Table 1. Ouchterlony precipitin reactions occurring between hepatic microsomal supernatant fractions from various species and antibodies prepared against a purified hepatic glutathione S-transferase (GS-T) from the little skate, Raja erinacea

Species	Precipitin Reaction[a]
Little skate	I (9; 7 males, 2 females)
Big skate	I (3; 1 male, 2 females)
Thorny skate	I (2 females)
Dogfish shark	N (2; 1 male, 1 female)
Flounder	N (2)
Sculpin	N (1)
Gull	N (9)
Rat	N (3)

[a] I = identity reaction compared to male little skate hepatic microsomal supernatant fraction (no. of animals); N = no cross-reactivity.

big skate (Raja ocellata) gave identity reactions with the antibody prepared to purified GS-T 4 from little skate liver. By contrast, hepatic cytosol from the dogfish shark (Squalus acanthias), a member of the same subclass as these skates, showed no cross-reactivity with the antibody to the skate GS-T.

Hepatic cytosol from other classes including osteichthyes (winter flounder and sculpin), aves (gull) and mammalia (rat) likewise showed no cross-reactivity to the little skate antibody, although all species do have GS-T activity. These studies demonstrate that in only very closely related species are the hepatic GS-T (or more accurately one or more of the GS-T) immunologically similar.

There was also considerable reactivity between the various extrahepatic tissues of the three skate species and the antibody prepared to the skate hepatic GS-T. Identity and partial identity reactions were found to predominate (Table 2, Figure 2). Partial identity can result from an imbalance in the quantity of the reactants (for example, where there is insufficient antigen in the tissue cytosolic fraction to precipitate and block the diffusion of serum antibodies) or it can result from the absence of certain sites on the antigen, which would normally be recognized by the antibody. In the present situation the partial identity reactions could well be related to very low GS-T concentrations in the cycosolic fractions of the extrahepatic tissues. In any case, the immunological similarity between the hepatic and extrahepatic GS-T of these three skate species is quite obvious.

The double diffusion-precipitation technique of Ouchterlony provides qualitative data only; the amount of cross-reactivity between the antigen and antibody is not indicated. However, direct titration of enzyme activity against the antibody can provide some measure of the interaction between antigen and antibody. The results of such experiments using livers of different sexes and species of skates, which had shown identity reactions to the antibody, are shown in

Table 2. Ouchterlony precipitin reactions of hepatic and extrahepatic cytosolic fractions from three Main skate species with antibodies prepared to purified hepatic glutathione S-transferase 4 from male little skate liver

Tissue	Little Skate (male, 2)	Little Skate (female, 2)	Big Skate (female, 1)	Thorny Skate (male, 1)	Thorny Skate (female, 2)
Liver	I[a]	I	I	I	I
Pancreas	PI	PI	I	I	PI
Rectal Gland	PI	N.D.	I	PI	PI
Gonad	PI	N.D.	I	I	N
Stomach	PI	I	I	PI	PI
Kidney	I	I	I	PI	I
Heart	N	N	PI	N	N
Spiral Valve	I	I	I	I	I
Spleen	PI	N.D.	PI	PI	N.D.

[a] I = identity reaction; PI = partial identity reaction; N = no cross-reactivity, and N.D. = not determined.

Figure 3. GS-T activity (with DNCB as substrate) of pooled male little skate hepatic cytosol was inhibited by about

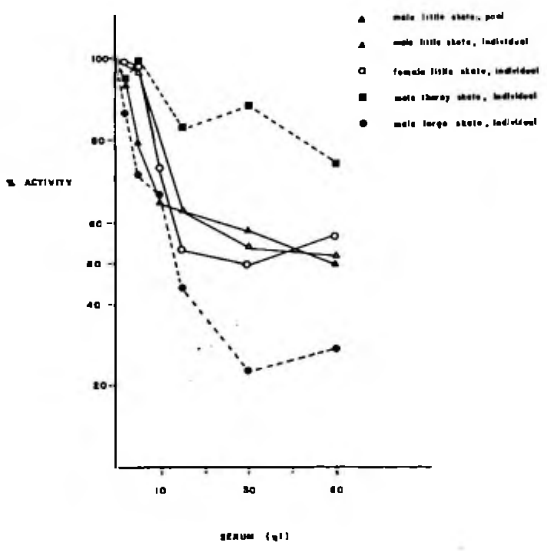

Figure 3. Inhibition of glutathione S-transferase activity with DNCB as substrate in hepatic cytosol from various species of skates by antibodies (rabbit) made to a purified hepatic glutathione S-transferase from male little skate, Raja erinacea. Enzyme assay conditions: Enzyme source (hepatic microsomal supernatant fraction) was mixed with serum-containing antibodies for 10 min in 1 ml H_2O at 37°C. Cofactor, buffer and substrate were then added (in final volume of 3 ml) at the following concentrations: 5 mM glutathione, 0.1 M KPO_4 (pH 6.6), and 1 mM 2,4-dinitrochlorobenzene. The inhibition of glutathione S-transferase activity was determined by comparison to a concomitantly run incubation mixture containing preimmune serum instead of antibodies.

50% in the presence of serum-containing antibodies as were GS-T activities in hepatic cytosolic fractions from individual male and female little skates. The inhibition titration curves obtained with the different little skate liver preparations were very similar. In contrast, approximately 75% of the GS-T activity in big skate hepatic cytosol was inhibitied by the antibody whereas only about 30% of the GS-T activity in thorny skate liver was inhibited upon titration with the antibody. Since it is well known that a number of closely related GS-T enzymes are normally present in the liver of vertebrate species, the antibody inhibition experiments are consistent with a differential distribution of GS-T enzymes in the livers of the three skate species, although each species contains at least one enzyme that is immunologically

identical to the GS-T enzyme purified from little skate liver. However, it is also possible that minor steric or compositional differences near the active site of the "immunologically identical" GS-T enzymes are responsible for the different inhibition titration curves in each species.

In summary, the major hepatic glutathione S-transferase enzyme from little skate has been highly purified and the protein is of considerable interest because of the relative efficiency with which it catalyzes reactions between polycyclic arene oxides and glutathione. An immunologically similar protein(s) was demonstrated in hepatic and several extrahepatic tissues of two other skate species indigenous to Maine, and in extrahepatic tissues of the little skate.

Minus Low tides - Summer 1980

June 11 Wed	4:06 AM	-1.3
12 Thurs	4:59 AM	-1.5
13 Fri	5:50 AM	-1.5
14 Sat	6:38 AM	-1.4
15 Sun	7:25 AM	-1.1
July 11 Fri	4:47 AM	-1.1
12 Sat	5:36 AM	-1.1
Aug 26 Tues	5:21 AM	-1.3
27 Wed	6:07 AM	-1.6
28 Thurs	6:55 AM	-1.7
29 Fri	7:43 AM	-1.5
30 Sat	8:34 AM	-1.2