

QUANTIFICATION AND CONFIRMATION OF GLUTATHIONE

This Project's research on detoxification mechanisms, utilized by marine animals in dealing with xenobiotic hydrocarbons, requires that we determine the metabolite loading capacity of the glutathione pool and correlate overloading with the onset of toxic effects. In order to determine the loading capacity, we need to measure tissue concentrations of both free and conjugated glutathione. Conjugation of xenobiotic hydrocarbon metabolites with the tripeptide glutathione (GSH) is a major detoxification mechanism (Jakoby 1978; Orrenius and Jones 1978; Chasseaud 1973) and, as such, warrants close inspection. This report describes the adaptation of the enzymatic recycling assay for free GSH to our particular experimental regimen and research goals and presents some preliminary data on GSH distribution in liver cytosol of control scorpionfish (*Scorpaena guttata*). Our findings indicate that GSH is found predominantly in the low molecular weight pool of the liver cytosol.

BACKGROUND

Glutathione is an ubiquitous tripeptide (L- γ -glutamyl-L-cysteinylglycine) found in many tissues of both mammals and fishes. Many, if not all, of its functions are associated with the sulfhydryl group of the central amino acid cysteine. Glutathione (GSH) accounts for approximately 90% of the intracellular nonprotein thiol. It is present in tissues predominantly as the reduced form GSH, the balance being present in the oxidized form, glutathione disulfide (GSSG). The enzyme glutathione reductase maintains this proportion, as it catalyzes the reduction of one molecule of GSSG to two molecules of GSH.

An ever increasing body of research implicates GSH in a multiplicity of important cellular functions. It is a major factor in xenobiotic hydrocarbon detoxification, where it has been shown to form GSH-hydrocarbon conjugates (in a reaction catalyzed by the GSH-S-transferases) which can then be converted to mercapturic acids and excreted from the organism (Jakoby 1978; Orrenius and Jones 1978; Chasseaud 1973). Glutathione can inhibit lipid peroxidation of biological membranes via reduction of toxic hydroperoxides (such as hydrogen peroxide, a normal product of cell metabolism) in a reaction catalyzed by glutathione peroxidase (Chance *et al.* 1978; Jocelyn 1978). Glutathione can function as a reservoir of cysteine for protein synthesis when the normal sources of cysteine are depleted (Tateishi and Higashi 1978). It functions as a coenzyme for the enzymes of prostaglandin metabolism, which are modulators of hormone activity (Christ-Hazelhof and Nugeteren 1978). Glutathione can detoxify methyl mercury via formation of GSH-MeHg conjugates in the bile, which can then be excreted in the

feces (Ballatori and Clarkson, 1982). It has been shown to facilitate the membrane transport of amino acids and sugars in kidney tissues (Leibach, *et al.*, 1978). It is a component of the cellular oxidation-reduction system, where it may act as a buffer against thiol oxidants, thereby protecting protein sulfhydryl groups from oxidation (Jocelyn, 1978; Siliprandi, *et al.*, 1978). Finally, it has been hypothesized to modulate protein activity via formation of GSH-protein disulfides (Mannervik and Axelsson, 1978).

METHODS

A suitable quantitative assay for GSH is one that can be integrated into our existing experimental regimen with a minimum of disruption, and not be compromised in the areas of accuracy, precision, reliability, and specificity as a result of this integration. An assay method that meets these requirements is the enzymatic recycling assay for GSH (Owens and Belcher, 1965; Tietze 1969; Griffith, 1980).

The mechanism of this assay is dependent upon the cyclic reduction of the thiol reagent DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] by GSH and the cyclic regeneration of GSH from GSSG (Figure 1). The specificity of this assay is a consequence of the high substrate specificity of GSH-reductase for GSSG, which effectively excludes GSH-protein and GSH-hydrocarbon conjugates from contributing to the assay (Mannervik and Axelsson, 1978). The sensitivity of this assay, which is greater than that expected from a simple stoichiometric reaction, is a consequence of the fact that GSH is being recycled, and is not being consumed, and therefore acts as a catalyst.

As the cyclic reduction of DTNB by GSH is allowed to proceed, there will be an increase in the intensity of the yellow color in solution, due to accumulation of the indicator product 2-nitro-5-thiobenzoic acid. The rate of this color change is proportional to the concentration of total free glutathione, in both its reduced (GSH) and oxidized (GSSG) forms. The rate of color change can be monitored at 412 nM with a UV-VIS spectrophotometer.

Preparation of tissue for GSH analysis (Figure 2) is essentially that described by Jenkins, *et al.*, (1981) with the exception that column chromatography is performed at 4°C (instead of room temperature) to prevent extensive degradation of GSH during the six to seven hours required for completion of column chromatography. Compositing of sub-samples into the high molecular weight pool (enzyme containing pool), the metallothionein pool and the low molecular weight pool (glutathione containing pool) is based on the criteria of Jenkins, *et al.*, (1981). The pool designated as total pool is a composite of 500 μ l subsamples from all sixty fractions. The GSH assay *per se* is shown at the bottom of Figure 2. Volumes and concentrations of the assay components were adjusted to give final concentrations (in the assay volume of 2.0 ml) similar to those in the balanced assay system described by Tietze (1969).

DISCUSSION

To date, the majority of our research on GSH has been devoted to adapting the GSH assay to our particular needs and looking at those experimental parameters that affect the accuracy and reliability of the assay. In the course of optimizing the assay for our purposes, we have collected some preliminary data on free GSH distribution among the three liver cytosolic pools of scorpionfish (control specimen). As expected, on the basis of its molecular weight, we find most of the free GSH in the low molecular weight cytosolic pool (Figure 3).

Our glutathione research in the near future will be directed towards the following goals: (1) determination of the natural variation in tissue concentration of free GSH in liver, kidney, gills

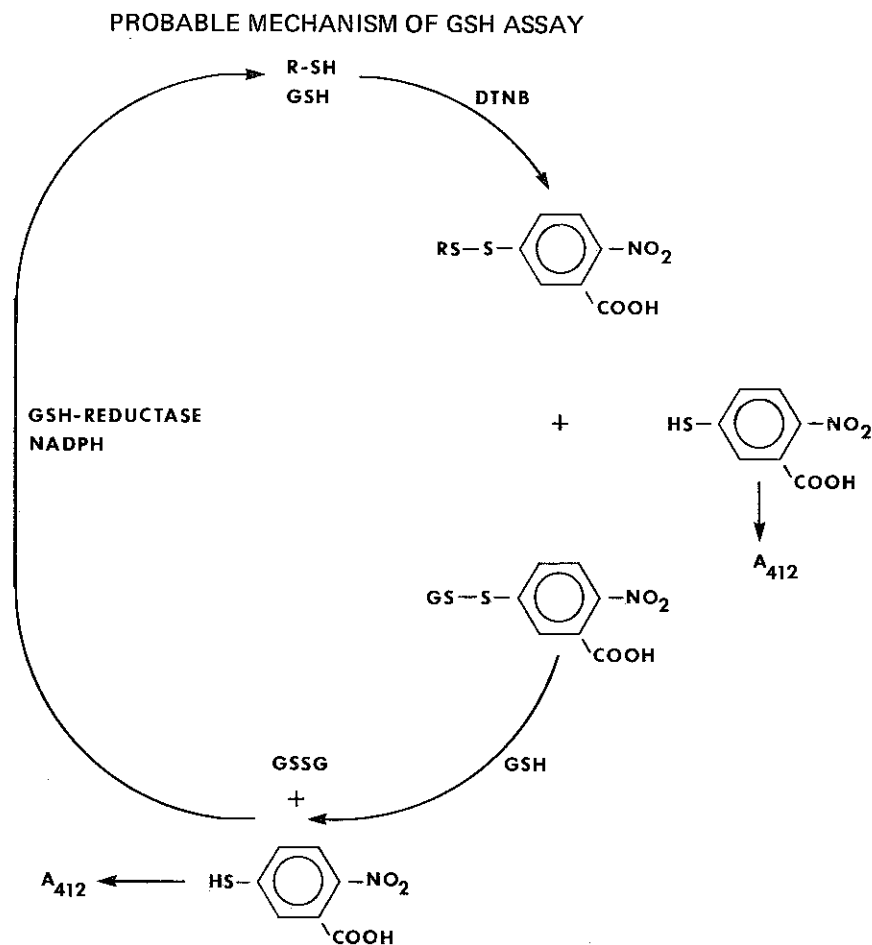
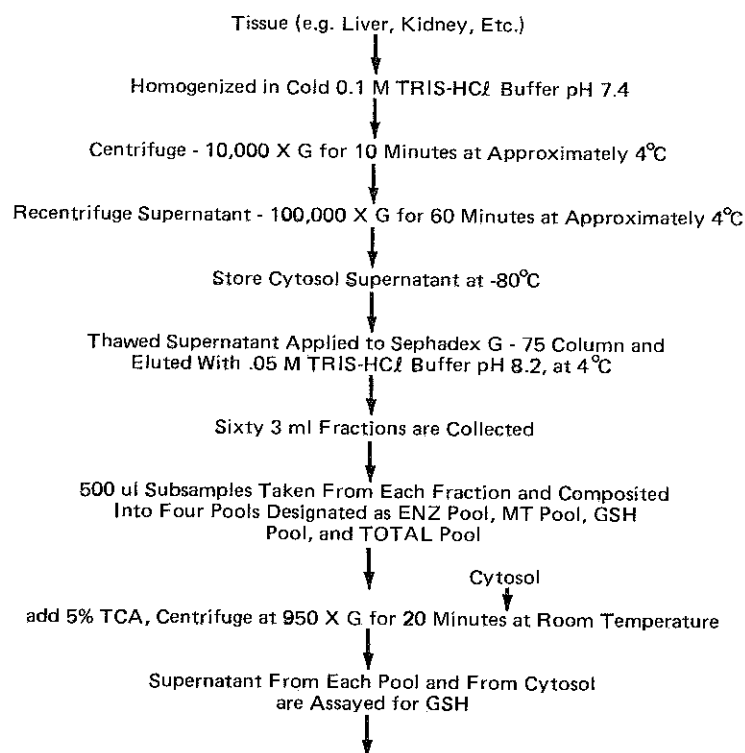


Figure 1. For every cycle, 2 molecules of indicator product (2-nitro-5-thiobenzoic acid) are formed per molecule of GSH. After the initial reaction of all thiols with DTNB, GSH is the only sulfhydryl containing compound remaining to react with DTNB.

and, possibly, other organs, (2) development of technique for measuring tissue concentrations of GSH-hydrocarbon conjugates, and (3) development of technique for measuring tissue concentrations of glucuronic acid-hydrocarbon conjugates (formation of glucuronic acid conjugates is an alternate mechanism for detoxification of xenobiotic hydrocarbons). Armed with a knowledge of the normal tissue concentrations of free GSH and the techniques for measuring free GSH, conjugated GSH, and conjugated glucuronic acid, we can determine the correlations among tissue concentration of free GSH, tissue concentration of GSH-hydrocarbon conjugates, tissue concentration of glucuronic acid-hydrocarbon conjugates, tissue concentration of xenobiotic hydrocarbon metabolites, tissue concentration of toxic metals such as Cd, tissue concentration of the amino acids cysteine and methionine (the rate limiting precursors of GSH biosynthesis), histopathological data and observations, and enzyme activities (for enzymes such as lactate dehydrogenase, catalase, carbonic anhydrase and GSH-S-transferase).

We expect to show that in marine organisms, chronic depletion of free intracellular GSH, via conjugation with xenobiotic hydrocarbon metabolites, will lead to a variety of detrimental cellular and biochemical effects. These detrimental effects may come about because less free GSH will be available for detoxification of xenobiotic hydrocarbons, allowing these hydrocarbons to react with sensitive cellular macromolecules, and also because less free GSH will be available for other important GSH dependent cellular functions, such as maintenance of cell membrane integrity.



Assay Components - In Order of Addition	Volume ul
Phosphate-EDTA Buffer pH 7.5 (125 mM Na-Phosphate/6.3 mM EDTA)	750
1.2 mM DTNB	1000
Sample	100
(Allow Non-Enzymatic Reaction of DTNB W/Sample)	
GSH-Reductase (23.2 U/ml)	50
4.0 mM NADPH	100
Monitor change in absorbance at 412 nM for four minutes, at one minute intervals, at temperature of 30°C.	
The rate of change is proportional to the concentration of free GSH.	

Figure 2.

FREE GSH IN LIVER CYTOSOLIC POOLS
OF CONTROL SCORPIONFISH

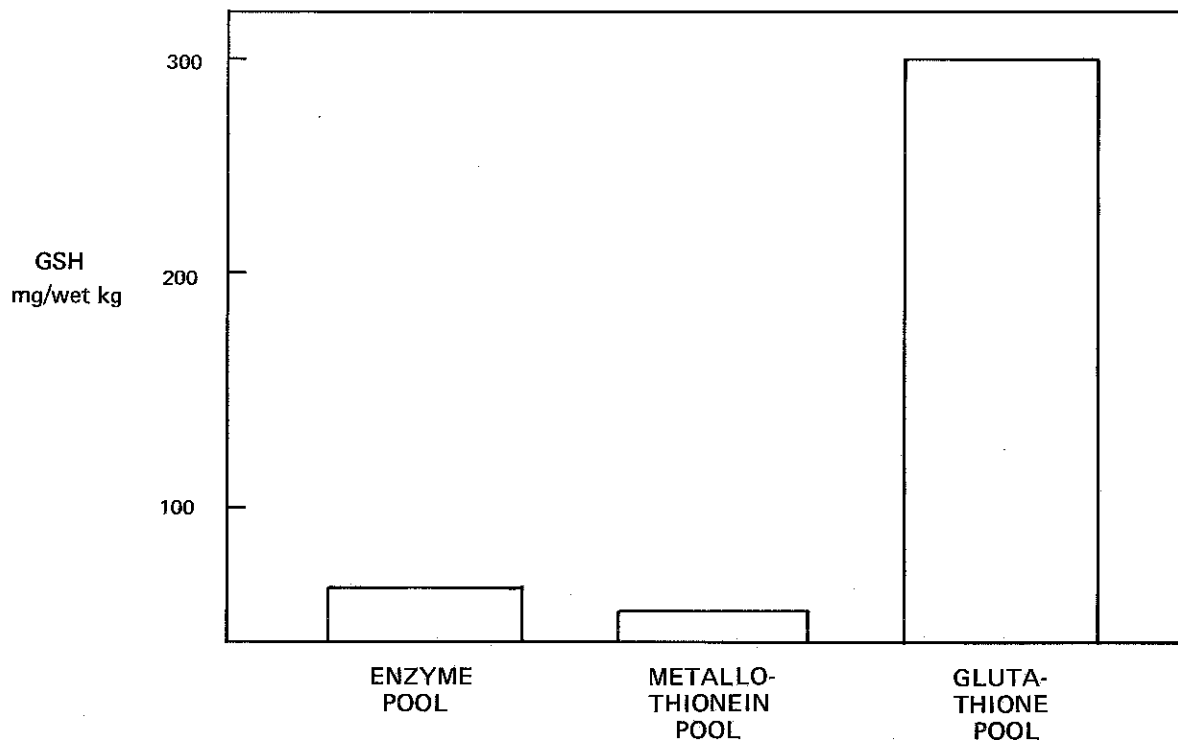


Figure 3.

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