THE EFFECTS OF STRESS AND POLLUTION ON MARINE ANIMALS

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PRAEGER

PRAEGER SPECIAL STUDIES • PRAEGER SCIENTIFIC

New York • Philadelphia • Eastbourne, UK
Toronto • Hong Kong • Tokyo • Sydney

Library of Congress Cataloging in Publication Data

Main entry under title:

The effects of stress and pollution on marine animals.

Bibliography: p.
Includes index.
1. Marine fauna—Effect of water pollution on.
2. Marine fauna—Physiology. 3. Stress (Physiology)
I. Bayne, B. L. (Brian Leicester)
QL121.E34 1985 591.5'2636 84-18145
ISBN 0-03-057019-0 (alk. paper)

Published in 1985 by Praeger Publishers
CBS Educational and Professional Publishing
a Division of CBS Inc.
521 Fifth Avenue, New York, NY 10175 USA

56789 052 987654321

Printed in the United States of America
on acid-free paper

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1970). The formation and sources of mutagenic compounds are now topics of some interest, and although petroleum hydrocarbons do contain small amounts of such compounds, a number of workers believe that they are unimportant as a source of mutagenic activity in the marine environment (King, 1977; Payne et al., 1979). More likely sources are suggested to be sewage, industrial waste and the products of petrol combustion (Payne et al., 1978; Payne et al., 1982). A correlation between induced MFO activities and increased mutagenicity has recently been observed in *Mugil cephalus* taken offshore from an industrial environment (Kurelec et al., 1979). The decision as to which of possibilities (1) or (2) may have occurred would be made on the basis of a consideration of the particular physiological, cytological and biochemical studies.

NADPH-producing Enzymes

The electron-donor for the hydroxylation reactions catalyzed by the MFO system is NADPH. The major enzymes thought to be responsible for the formation of NADPH for the MFO system are glucose-6-phosphate dehydrogenase (G6PDH) (E.C.1.1.149), phosphogluconate dehydrogenase (decarboxylating) (E.C.1.1.1.44), malic enzyme (E.C.1.1.1.38) and NADP-dependent isocitrate dehydrogenase (E.C.1.1.1.42). It is well established for mammalian tissues that treatment with compounds such as phenobarbital, which will result in an increase in MFO activity, will also lead to an increase in G6PDH activity and in the activities of other NADPH-producing enzymes (Altman, 1972). The results are less definitive for the effects of polycyclic hydrocarbons, with both increases (Koudstaal and Hardonk, 1972) and no change (Bresnick and Yang, 1964) in G6PDH activity being recorded. However, such differences may have arisen because the different techniques used by different workers either measure cytosolic or microsomal G6PDH activities or both; it is now thought that in vertebrates the two enzymes are genetically distinct with different properties and different functions, the cytosolic isoenzyme having a major role in the pentose phosphate pathway and the microsomal isoenzyme being important in MFO reactions (Kimura et al., 1979; Stegeman and Klotz, 1979). The latter enzyme is identical with the enzyme formerly known as glucose dehydrogenase (E.C.1.1.1. 47) and has now been retermed hexose-6-phosphate dehydrogenase (Hori and Takahashi, 1974). The possibility therefore exists that,

if the same systems operate in the tissues of marine organisms, G6PDH and other NADPH-producing enzymes may be responsive to the presence of organic xenobiotics and therefore offer potential as a specific index of sublethal stress.

Studies of *M. edulis* indicate that changes in G6PDH occur in response to aromatic hydrocarbons. However, the responses were variable and different from the mammalian system (Moore et al., 1980; Livingstone, unpublished data). The main observations were: (1) Specific activity (S.A.: activity per mg protein) in the blood cells increased with exposure of mussels to water-accommodated fraction of North Sea crude oil (WAF) and following injection of 2,3-dimethylnaphthalene but not with exposure of mussels to increased temperature. (2) The number of blood cells increased greatly (activity of G6PDH per ml increased up to ×6) but in response to both WAF and temperature. (3) The digestive gland contains cytosolic and microsomal G6PDH which increased in S.A. (activity per fresh weight) following injection of 2,3-dimethylnaphthalene; the combined fractions ("post-40,000 *g* supernatant") did not increase with the exposure of mussels to WAF. (4) The digestive gland cytosolic and microsomal enzymes have similar properties (substrate specificity, electrophoretic mobility) which are intermediate between those of the mammalian isoenzymes. The blood cells of *M. edulis* in particular appear to offer potential for future study. In addition to being important in MFO reactions they may be involved in other detoxication reactions which are critically dependent on NADPH, e.g. reactions involving reduced glutathione (Meister, 1975). Future studies of these and other tissues will need to examine the seasonal variability of G6PDH (Livingstone, 1981) and its functional relationship with the detoxication system. The latter will have a bearing on the second criterion of an index of sublethal stress, i.e. that the change in the biochemical process causes or is associated with decreased animal performance.

Metallothioneins

Many marine organisms have a large capacity for trace metal accumulation, the consequences of which may be deleterious to the organism (for a review of the uptake, fate and effects of trace metals in marine organisms, see Moore, 1981). However, as in

other organisms, potentially toxic trace metals can be detoxified intracellularly by partitioning into lysosomes (Moore, 1980) or by binding to the protein metallothionein.

Upon entering the cell many metal ions are bound by metallothioneins. The metallothioneins are a group of specific non-enzyme proteins that are increasingly being demonstrated to play a central role in metal metabolism. They were first discovered by Margoshes and Vallee (1957) and are now recognised as low molecular weight proteins [approximately 10,000 daltons (gel filtration studies: Kägi and Vallee, 1960, 1961); 6,000 to 7,000 daltons (amino acid composition: see Kojima and Kägi, 1978)] of unusual structure. Cysteine constitutes one-third of the amino acids, and there are approximately 24 cysteine residues per metallothionein molecule (Bremner and Davies, 1975; Winge et al., 1975). Each three cysteine residues bind 1 metal ion with a resultant 8 metal ions bound to each metallothionein molecule; metallothionein appears always to occur in the saturated state (Kägi and Vallee, 1960, 1961; Pulido et al., 1966; Bremner and Davies, 1975). Aromatic amino acids are absent or low, and sequencing studies indicate the preservation of the metal-binding residues in different species (Kojima and Kägi, 1978).

Metallothionein is apparently ubiquitous, having been described in mammals (Margoshes and Vallee, 1957), fish (Olafson and Thompson, 1974), bivalves (Noël-Lambert, 1976; Talbot and Magee, 1978; George et al., 1979), zooplankton (Brown and Parsons, 1978) and phytoplankton (Maclean et al., 1972). Metallothionein may exist to some level in most or all animal tissues since it has been found to occur in liver, kidney, gills, testes, intestine, muscle, plasma, erythrocytes, tissue cultured skin epithelial cells and urine (Jakubowski et al., 1970; Nordberg, 1972; Nordberg and Piscator, 1972; Bouquegneau et al., 1975; Rugstad and Norseth, 1975; Sugawara and Sugawara, 1975). In mammals and fish, metallothionein appears to be concentrated in liver and kidney tissue (Jakubowski et al., 1970; Nordberg, 1972), while in fish (Bouquegneau et al., 1975) and bivalves (Roesijadi, 1979) high levels of metallothionein are also found in gill tissue. Metallothionein is described as a cytoplasmic protein (e.g. Winge et al., 1975), although there is some evidence that an insoluble protein polymer of metallothionein-amino acid composition is found within lysosomes (Porter, 1974); this is consistent with the known partitioning of metals into lysosomes (Moore, 1977) and the apparent marked resistance of metallothionein to degradation by proteolytic enzymes (Webb, 1972).

Natural tissue levels of metallothionein can be greatly increased (up to 40 times: Piscator, 1964; Piotrowski et al., 1973) by exposure of the organisms to various trace metals, namely mercury, cadmium, copper, zinc, silver, and tin (Winge et al., 1975; Sabbioni and Marafante, 1975). The induction of metallothionein may occur at the translational level (increased synthesis of protein from a given mRNA) for low metal exposures, or at the transcriptional level (increased synthesis of mRNA) for higher metal exposures (Webb, 1972; Squibb and Cousins, 1974). The metallothionein binds the metal ions, so preventing them from exerting toxic effects through binding to enzymes or other sensitive sites (Goldman, 1970; Brown et al., 1977; Brown and Parsons, 1978; see also Moore, 1981). If, however, the rate of influx of metals into the cell exceeds the rate at which metallothionein can be synthesised, there may be a "spillover" of metals from metallothionein into the enzyme pool (Brown et al., 1977; Brown and Parsons, 1978; Engel and Fowler, 1979; Pruell and Engelhart, 1980; Roesijadi, 1979). Toxic effects can then be due to the displacement of essential metals from metalloenzymes* by non-essential metals (Friedberg, 1974). This displacement can change the conformational shape of the enzyme so that the substrate molecules no longer fit the binding sites in the enzymes, resulting in the loss of enzyme activity (Friedberg, 1974).

The metal composition in the naturally occurring metallothionein is variable and depends on the tissue of origin; e.g. Zn is the principal constituent in metallothionein from liver. This composition can change and will reflect the metals to which an organism has been exposed. It appears that increased tolerance to trace metals may be mediated by the production of metallothionein with a portion of binding sites occupied by a relatively nontoxic and readily displaceable metal. Leber (1974) found that rats, preinjected with Cd, synthesised metallothionein with Zn in approximately half of its binding sites. If a further dose of Cd was administered, then the Cd displaced Zn from the metallothionein. Similarly, pretreatment with Zn increased tolerance to subsequent

*Metalloenzymes are enzymes that require specific metal ions to be catalytically active. In such enzymes the metal ion may serve as (1) the primary catalytic centre; (2) a bridging group, to bind substrate and enzyme together; or (3) an agent stabilising the conformation of the enzyme in its catalytically active form.

exposure to cadmium since Cd could then displace Zn from Zn-containing metallothionein. This suggests that the increased tolerance in both cases results from the presence of high levels of Zn in metallothionein binding sites with subsequent displacement of Zn from metallothionein by Cd. Free cytoplasmic Zn is much less toxic than Cd as it is a natural component of over 70 metalloenzymes (Riordan, 1977). Similarly, exposure to Ag, Cu or Hg results in the synthesis of metallothionein containing approximately equimolar levels of Zn and the exposure metal (Winge et al., 1975). Therefore, it would appear that exposure to any one of Cd, Hg, Ag, Cu or Zn should result in increased tolerance to any of the other metals upon subsequent exposure. However, it is also important to realise that sometimes large concentration differences are required before one metal will displace another, due to the greater affinity of metallothionein for the original metal. Such concentration differences may not be environmentally realistic.

As discussed previously, the toxic effects of trace metals usually occur only when the binding capacity of the metallothionein has been exceeded and there is a resultant interaction of the toxic trace metals with the enzyme pool. However, in addition to this, high levels of toxic trace metals may occur in the enzyme pool before metallothionein becomes saturated if there is a deficiency of an essential trace metal in the enzyme pool. For example, it has been demonstrated by Brown and Chatel (1978), for duck liver and kidney tissues, that when the enzyme pool was replete with Zn, over 75% of cytoplasmic Cd was bound to metallothionein. However, when the enzyme pool was apparently Zn deficient, only 25% of the Cd was bound to metallothionein with 75% occurring in the enzyme pool. This increase of the portion of cytoplasmic Cd in the enzyme pool of Zn-deficient ducks was attributed to an increased ability of Cd to compete for binding sites in the enzyme pool. Conversely, if the enzyme pool was Zn-replete, then Cd would be out-competed for the enzyme binding sites. In the latter situation, the Cd would be more likely to bind to mRNA coded for metallothionein, resulting in translational induction of metallothionein (Webb, 1972).

In the light of the types of metal interactions described above, it is becoming apparent that a proper understanding of metal metabolism will be possible only when several metals have been examined simultaneously in any given study. Furthermore, understanding of the interactions of metals with metallothionein will be clear only if the relative levels of competing metals in the metalloenzyme pool are examined. Zn appears to play a particularly important role in toxic metal metabolism and should always be considered.

Metallothionein as a Specific Index of Sublethal Stress

The role of metallothionein in detoxifying metals has been identified as a promising area for the development of a stress index specific for metals as a pollutant (Bayne et al., 1980b; Lee et al., 1980). The types of information that such a study might provide are discussed later. There are a few examples of the application of metallothionein studies in the field, but a relationship between metallothionein and pollution has been demonstrated for mussels. For example, in *M. edulis*, Brown et al. (1977) found that the ratio of total metal (Cd, Cu and Zn) in the metallothionein pool to total metal in the enzyme-containing pool increased with increased levels of pollution; similarly, Brown (1978; also see Bayne et al., 1980b) demonstrated that the levels of metallothionein-bound Cd, Cu and Zn increased with exposure to increasing levels of metals in the vicinity of a sewer outfall, and that at the site of highest exposure (the mussel population here was sparse) there was a dramatic increase in the amount of Cd, Cu and Zn in the enzyme-containing pool. Viarengo et al. (1982) found that the level of metallothionein-like Cu-binding protein was three times higher in the digestive gland of *M. galloprovincialis* from a polluted environment than in the tissue of those from a clean environment; also, the rates of protein and RNA synthesis and amino acid uptake were reduced in the animals from the polluted environment. Induction of metallothionein in the tissues of mussels has also been indicated in laboratory studies, namely for *M. edulis* in response to Cd (whole tissues: Nöel-Lambert, 1976; digestive diverticula: George et al., 1979) and to a mixture of Cd, Cu and Zn (whole tissues: Brown, 1978), and for *M. galloprovincialis* in response to Cu (gills: Viarengo et al., 1980; mantle and hepatopancreas: Viarengo et al., 1979). These studies indicate that several Cd-binding proteins exist in *M. edulis* and can be isolated as monomers [molecular weight of between 10,000 (Nöel-Lambert, 1976) and 13,000 (Talbot and Magee, 1978)] or dimers [molecular weight of 25,000 ± 5,000 (George et al., 1979)]. The most complete analysis to date of these proteins indicates that they have many properties in common with mammalian metallothionein (George et al., 1979).

From the studies described above it is clear that metallothionein fulfills the first criterion of an index of sublethal stress, namely that the changes in the profile of metal binding are a response to change in an environmental factor, i.e. metals. It is a *primary biochemical response* that is likely to be specific to this environmental stressor. The specificity with respect to the metal (in the sense of identifying the particular metal that is being bound and eliciting a response) is theoretically absolute as the metallothionein is identified by its molecular weight range and by the identified bound metal. Changes in the metal composition of the metallothionein pool presumably will identify the environmental metal that is eliciting the response (Leber, 1974). The limited information on the time-course and sensitivity of the metallothionein response in marine organisms includes the following points: (1) The response time appears to be short; although most studies have only examined the changes after several weeks of exposure, Viarengo et al. (1980) recorded a change in the metal binding of the metallothionein pool of the gills of *M. galloprovincialis* between 24 and 48 hours after exposure of the mussels to Cu. (2) Although knowledge of the effective concentrations of metals in the marine environment is limited because of the problems of bioavailability (see Moore, 1981), the indications are that the levels of metals used in laboratory experiments to elicit a response are environmentally realistic; e.g. Cu: 0.015 ppm (Viarengo et al., 1979); a mixture of Cd, Cu and Zn: 0.001, 0.09 and 0.07 ppm, respectively (total 0.161 ppm) (Brown, 1978). (3) The amount of induction of metallothionein may be difficult to estimate because it is unlikely that an increase of the relevant protein will be detected and because an increase in binding of a specific metal could represent displacement of another metal.* However, a measure of induction can be obtained from the increase in the amount of total metal bound to metallothionein. In this case, the induction observed for mussels (increase in bound Cd, Cu and Zn) varies from ×1.3 (Brown, 1978) to ×10 or greater (Nöel-Lambert, 1976). Another more exacting and precise method for the determination of the level of induction is the measurement of the incorporation of radio-labelled amino acids into the metallothionein pool

*The displacement of one metal by another requires time, and it may be that, given sufficient background knowledge, changes in the binding of a single metal may be employable as a measure of induction.

(Premakumar et al., 1975; Olafson et al., 1979; Viarengo et al., 1980). With this method, Viarengo et al. (1980) recorded a 7- to 10-fold increase in the incorporation of ^{35}S-cysteine into the 12,000 molecular weight fraction from the gills of copper-exposed mussels. No information appears to be available on the time-course of the induced changes, i.e. on the formation of a new steady state.

The second criterion for a biochemical response to be acceptable as an index of sublethal stress is that it must lead to or be associated with decreased animal performance.* Metallothionein is a detoxication system that functions initially by binding the toxic metal. Continuing or pre-exposure to the metal can lead to increased synthesis of metallothionein and therefore to an acquired tolerance to increasing metal levels (e.g. Bouquegneau, 1979). However, at a particular high concentration of a metal, according to the "spillover" theory, there will be toxic effects when the binding capacity of metallothionein is exceeded and the metal then interacts with the enzyme-containing pool or other sensitive sites. This movement of metal into the high molecular weight pool has been observed for a number of species including bivalves (Brown, 1978) and appears to coincide with the appearance of pathological effects (Winge et al., 1973; Brown, 1977; Brown et al., 1977; Irons and Smith, 1976). Therefore, the metallothionein-associated changes can eventually result in decreased animal performance. Although the decreased performance will have to be assessed by other criteria (e.g. cytological, physiological), observing changes in the metal-binding characteristics of the protein profile would appear to offer a very strong tool for the detection of events (metal-mediated) leading up to and resulting in decreased animal performance. Three stages are potentially discernable in the pattern of events: (1) displacement of "endogenous" bound metal from metallothionein by the foreign metal, (2) induction of metallothionein (increase in total bound metal) and (3) spillover of foreign metal into the high molecular weight pool (and resultant decreased animal performance). Application of metallothionein studies to the field will presumably require information on possible sex differences and seasonal changes. Similarly, the choice of tissue may

*There have been a few studies which suggest that induced levels of metallothionein may themselves have deleterious consequences for an animal; e.g. injection of isolated Cd-metallothionein into rats resulted in necrosis of kidney proximal renal tubular cells (Cherian et al., 1976).

require preliminary studies. Interactions of the metals with other pollutants are also possible and may lead to different patterns of binding. For example, it has been suggested that Cd in tumor-bearing flounders (*Parophrys vetulus*) may be alkylated by "bioactivated" organic carcinogens to form an alkyl Cd which is then no longer bound by metallothionein but interacts with the high molecular weight proteins (Brown, 1977).

5

Bioassay

RATIONALE FOR THE BIOASSAY APPROACH

Water quality is often assessed chemically in terms of the concentrations of known toxic contaminants. This method may be satisfactory when there are a limited number of contaminants whose biological effects are well known and predictable, but effluents are often extremely complex and may contain numerous synthetic organic compounds, for example, besides the better known contaminants like metals and ammonia. Effluents from a modern industrial complex entering a bay or an estuary may include thousands of individual elements and compounds, making it impractical to define and monitor water quality by chemical analysis alone. Furthermore, it is well known that many factors influence the toxicity of contaminants on entering seawater (Bryan, 1976). Some may degrade quickly into harmless products; indeed, increasing numbers of consumer products are designed to break down quickly in seawater. Some toxicants may become bound by organic matter or particulates, with consequent changes in their biological availability, and some may interact chemically to become more, or less, toxic in combination than separately.

The kinds of problem that arise in the estimation of biological water quality from a knowledge of chemical constituents alone are

Interpretation of Results

The use of MFO activities as measurements of response to chemical pollutants requires a quantitative change to be measurable above the normal variability in populations. Evidence of induction or depression of the system is found in different enzyme kinetics (change in V_{max} or K_m) or substrate turnover rates. MFO induction in fish has been demonstrated to result from exposure to specific drugs like phenylbutazone (Burns, 1976a) and 5,6-benzo-flavone (Chevion et al., 1977) and organic pollutants such as aromatic hydrocarbons (Bend et al., 1977b; Pedersen et al., 1976; Chevion et al., 1977), some halogenated hydrocarbons (Poland and Glover, 1977; Gruger et al., 1977), and some complex mixtures of petroleum hydrocarbons (Payne and Penrose, 1975; Burns, 1976a; Kurelec et al., 1977). Conversely, Ahokas et al. (1976a) showed a depression of MFO activity in fish exposed to pulp mill effluents. Thus, at least for fish populations, changes in MFO activity may be useful as a semi-specific indicator of stress from certain classes of organic pollutants. However, to date there is little evidence of induction of MFO activity in marine invertebrates exposed to organic xenobiotics, and therefore the techniques can only be recommended at present for use with fish.

METALLOTHIONEINS

Many reports of trace metal levels in organisms from polluted areas exist in the literature, but little can be inferred from these data as to the actual biological significance to the exposed organism. The following procedures describe a biochemically meaningful assay based upon the actual toxicology of the trace metals; it measures the levels of those metals that are bound by the trace metal detoxifying protein metallothionein, and those that are free to exert toxic effects by binding enzymes in the high molecular weight protein pool. These procedures are modifications of these described by Webb (1972), Shaikh and Lucis (1971) and Olafson and Thompson (1974) and were used in studies by Brown et al. (1977), Brown (1977), Brown and Chatel (1978), Brown and Parsons (1978), and Brown (1978).

Sample Preparation and Measurement

Equipment and Chemicals: Dissection instruments, motorised homogeniser with teflon pestle, centrifuge (maximum *g* required: 27,000), heater waterbath (70°C), ultracentrifuge (not essential), Pharmacia columns packed with Sephadex G-75 gel, fraction collector, UV spectrophotometer, atomic absorption spectrophotometer; 0.9% NaCl, 0.01 *M* NH_4HCO_3.

Liver (digestive gland), kidney and gills should be excised and analysed where possible, since metallothionein is particularly concentrated in these tissues (Nordberg, 1972; Bouquegneau et al., 1975; Roesijadi, 1979). Whole tissue of phytoplankton, zooplankton or bivalves can also be processed with elution of significant metallothionein peaks (Noël-Lambert, 1976; Talbot and Magee, 1978; Brown, 1978; Brown and Parsons, 1978).

A cytosolic extract is prepared by homogenizing tissue in 0.9% NaCl for exactly 3 min at a standard speed on a laboratory motor equipped with a teflon pestle (Fig. 10-6, steps 1-3). Sample sizes and volumes of sodium chloride used for different tissue types are given in Table 10-6. Homogenates are centrifuged at 27,000 *g* for exactly 10 min and the supernatant is collected (steps 4-5). An extraction of pellet can be done to increase the portion of metallothionein and other proteins extracted from tissue (steps 6-9). The homogenization and centrifugation settings are the same as for the first extraction. Volumes of sodium chloride used for different sample sizes and types are given in Table 10-6. For phytoplankton, zooplankton and smaller bivalves, minimal sample size may be available; in order that readily measurable levels of metals are eluted (step 15) a second extraction of pellet is not done (steps 6-9) as this would result in a dilution of tissue metal levels with resultant dilution of metal levels in fractions collected (step 16). Similar procedures are followed for very small samples of liver, kidney or gill tissue (Table 10-6).

Supernatants are heated in a 70°C water bath for 5 min (step 11, Fig 10-6) and then centrifuged to clear the tissue extract of cellular debris (step 12). The supernatant is collected (step 13) and the pellet discarded. An alternative to heating supernatants to remove cellular debris (step 11) is to centrifuge the supernatant at 105,000 *g* for 60 min. This latter procedure avoids losses of heat-coagulable enzymes from the high molecular weight protein pool. The ultracentrifugation procedure is particularly preferable if enzyme activities are to be measured in the high molecular weight

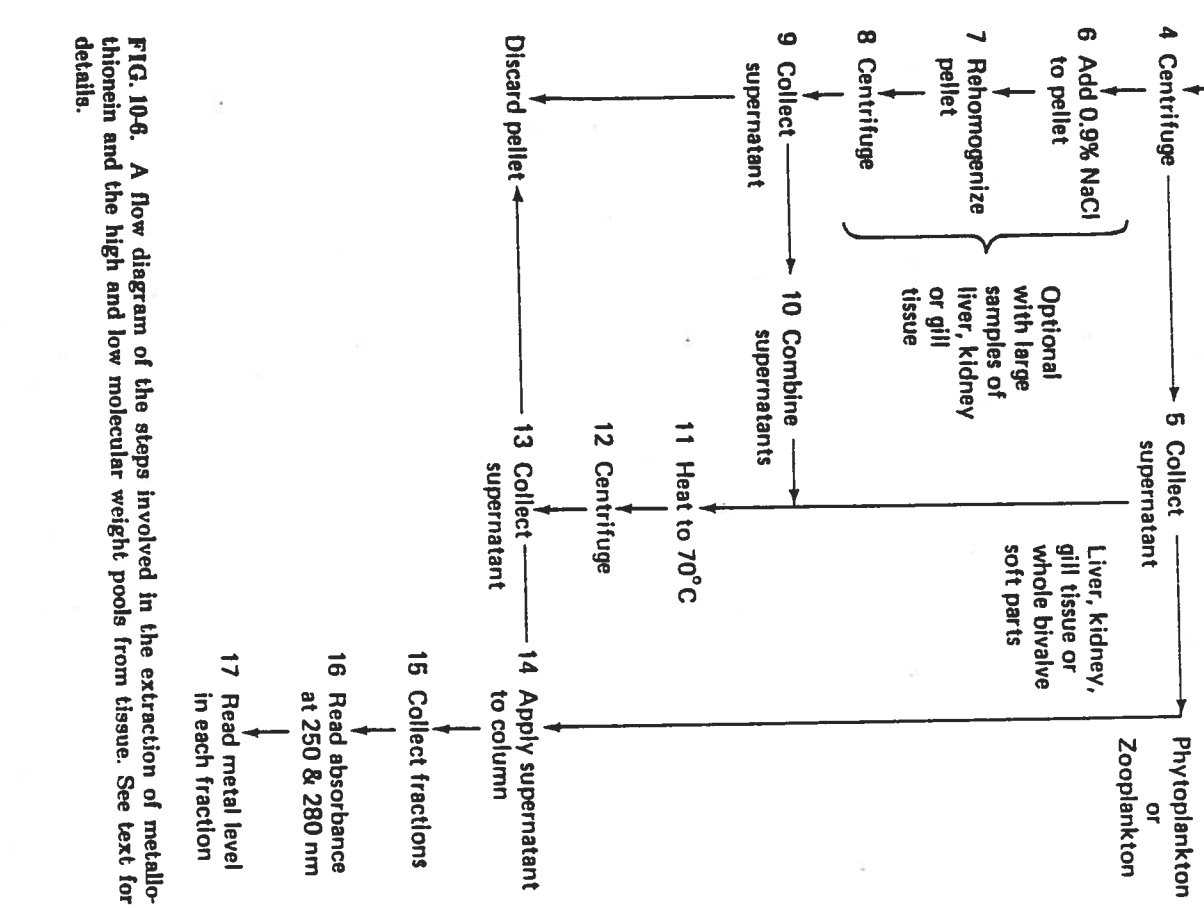

FIG. 10-6. A flow diagram of the steps involved in the extraction of metallothionein and the high and low molecular weight pools from tissue. See text for details.

TABLE 10–6. Recommended Procedures for Various Tissue Types

Tissue type	Sample size (1)[a] (g)	First homogenization (3) 0.9% NaCl volume (ml)	Second homogenization (7) 0.9% NaCl volume (ml)	Time to heat at 70°C (11) (min)	Volume to apply to column (14) (ml)	Column type[b] and size used	Fraction size[c] to collect (15) (ml)
Phytoplankton (whole tissue)	1	3	not done	not done	2	K.9/60	1.5
Zooplankton (whole tissue)	1	3	not done	not done	2	K.9/60	1.5
Bivalves (soft parts)	1	3	not done	5	2	K.9/60	1.5
Liver, kidney or gill tissue	0.1	1.5	not done	5	1.0	K.9/60	0.8
	0.5	2.0	not done	5	1.5	K.9/60	1.0
	1	2.5	1.5	5	2	K.9/60	1.5
	2	4.5	2.5	5	5	K1.6/100	6
	3	9	6	5	14	K2.5/100	15

[a]Number in brackets refers to the step number in Fig. 10–6.
[b]Pharmacia brand; column type (K) and diameter/length (cm).
[c]To produce about 30 fractions per profile.

TABLE 10-7. The Specifications for Various Sizes of Pharmacia Columns when Packed with Sephadex G-75 Gel

Column type, diameter/length (cm)	Bed volume (ml)	Min/max sample size (ml)	Maximum flow rate (ml)	Void volume (ml)	Time for first macromolecules to elute (h)
K.9/60	38	0.4/10	17	13	0.8
K1.6/100	200	2/50	44	70	1.6
K2.5/100	485	5/120	114	168	1.5

From Pharmacia technical literature.

pool, as many enzymes are irreversibly denatured by heat. It is not necessary to heat phytoplankton and zooplankton supernatants (Table 10-6) as these appear clear (free of cellular debris) after one extraction and centrifugation (steps 1-5) Resulting supernatants are applied to a column packed with Sephadex G-75 gel (step 14) and eluted with 0.01 M NH_4HCO_3; maximal flow rates are given in Table 10-7.

The size of the column employed (Table 10-7) will depend mainly upon the sample size used (Table 10-6). A narrower column is used for smaller sample sizes to prevent sample dilution. A longer column is preferable to a shorter column as it provides a better resolution of peaks. Better resolution of peaks can also be obtained by collecting smaller fraction sizes as eluant from the column (step 15). Resolution also improves with reductions of sample size down down 1-2% of the bed volume (Table 10-7). Sample sizes up to 25-40% of the bed volume give less-diluted cytoplasmic pools, but with less resolution.

The elutant will be collected as fractions using a standard fraction collector. The high molecular weight protein pool will be eluted as the first peak, with a shoulder or separate peak following due to the presence of haemoglobin, if applicable (Fig. 10-7). The metallothionein peak will elute next, followed by a double-peaked low molecular weight cytoplasmic pool (Shaikh and Lucis, 1971; Olafson and Thompson, 1974; Irons and Smith, 1976; Marafante, 1976; Noël-Lambert, 1976; Talbot and Magee, 1978). The position of peaks is determined initially by reading absorbances at 250 and 280 nm (step 16). The high and low molecular weight pools have high absorbance at 250 and 280 nm (Brown et al., 1977) whereas metallothionein absorbance is usually very low at 280 nm due to the absence of aromatic amino acids (Kagi and Vallee, 1961);

metallothionein may have a high absorbance at 250 nm due to the presence of sulfhydryl-Cd bonds (Kagi and Vallee, 1961). Each absorbance peak will correspond to a peak of various metals (Fig. 10-7). The high molecular weight protein pool usually contains Cu and Zn due to the presence of metalloenzymes (Brown et al., 1977). Metallothionein binds and thereby detoxifies the metals Hg, Cd, Ag and Sn, and also stores and/or detoxifies excesses of Cu and Zn above levels required for metalloenzymes (Brown and Parsons, 1978; Brown and Chatel, 1978). The low molecular weight cytoplasmic pools bind a small portion of metals in most organisms, but very high portions in phytoplankton and zooplankton (Brown, 1978).

In a gel elution profile comprising 45 fractions, the peaks will be tentatively identified as the high molecular weight protein pool (tubes 1-15), metallothionein (tubes 16-25), and the low molecular

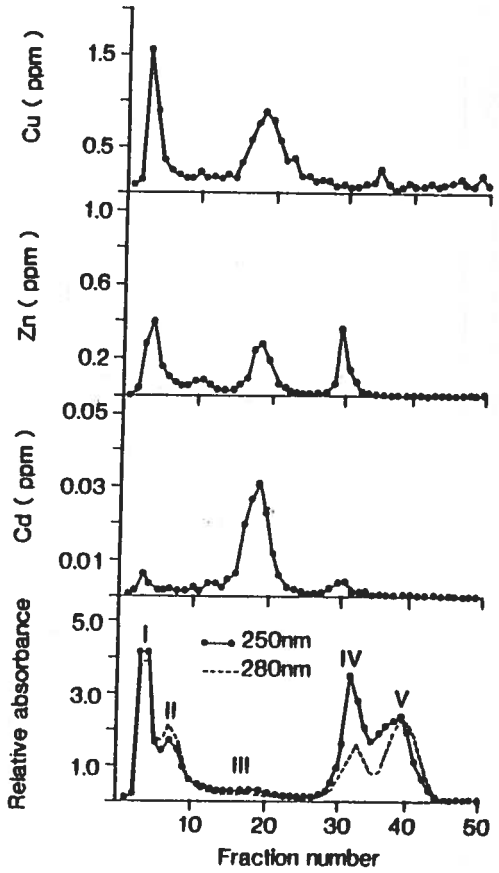

FIG. 10-7. Amounts of copper, zinc and cadmium in five cytoplasmic pools isolated from the liver of a Greater Scaup duck, using a Sephadex column and absorbance at 250 and 280 nm to identify the positions of the main protein peaks. Peak I corresponds to the high molecular weight protein pool which includes enzymes, peak II to haemoglobin, peak III to metallothionein, and peaks IV and V to low molecular weight cytoplasmic materials, such as amino acids, nucleic acids, and ATP. (After Brown et al., 1977.)

weight cytoplasmic pool (tubes 26-45) on the basis of elution position (Fig. 10-7). In other gel elution profiles from a variety of organisms, the high molecular weight protein pool consistently comprised the first 15/45 (0.33) tubes of the profile, metallothionein the next 11/45 (0.24) tubes of the profile, and the low molecular weight cytoplasmic pool the last 20/45 (0.44) tubes of the profile (Brown, 1978). The composition of the high molecular weight pool and metallothionein pool may be confirmed by procedures described later.

Metal levels in each fraction are then analysed using an atomic absorption spectrophotometer equipped with deuterium arc background correction and flame burner or graphite furnace as necessitated by the concentration of metal.

Calculation of results

Once metal levels have been determined in each fraction (step 17, Fig. 10-6), these can be added in each cytoplasmic pool and then expressed as a concentration of metal in each cytoplasmic pool per gram of tissue (wet weight). For instance, in a high molecular weight pool comprising 15 fractions (Fig. 10-7), the concentrations of Zn in each fraction (as mg Zn liter^{-1}) are added, correcting for the volume of each fraction (in this case, 10.2 ml):

$$\left(\frac{X_1 \text{ mg Zn}}{1{,}000 \text{ ml}} \times 10.2 \text{ ml}\right) + \left(\frac{X_2 \text{ mg Zn}}{1{,}000 \text{ ml}} \times 10.2 \text{ ml}\right) + \cdots + \left(\frac{X_{15} \text{ mg Zn}}{1{,}000 \text{ ml}} \times 10.2 \text{ ml}\right)$$

or

$$\frac{X_1 + X_2 + \cdots + X_{15} \text{ mg Zn}}{1{,}000 \text{ ml}} \times 10.2 \text{ ml}$$

A correction is applied for the wet weight of tissue initially homogenized (in this case, 3 g liver, wet weight):

$$\times \frac{1}{3 \text{ g liver (wet wt)}}$$

A further correction is made for the fact that not all supernatant (step 13, Fig. 10-6) may be applied to the column (step 14). For instance, a 3-g liver sample comprised of approximately 2.4 ml of water and soluble substances is homogenized initially in 9 ml of 0.9% NaCl, and the pellet extracted in 6 ml of solution. Of this 17.4 ml of tissue extract, 14 ml are applied to the column (Table 10-6). Therefore a correction factor is needed for discarded tissue extract:

$$\times \frac{17.4}{14}$$

A further correction is made since data is converted into μmol so that competition between metals can be evaluated in terms of the relative numbers of molecules of each metal present:

$$\times \frac{1 \text{ mmol Zn}}{65.4 \text{ mg Zn}} \times \frac{1{,}000 \text{ μmol}}{\text{mmol}}$$

The complete calculation is:

$$\frac{(X_1 + X_2 + \cdots + X_{15}) \text{ mg Zn}}{1{,}000 \text{ ml}} \times 10.2 \text{ ml} \times \frac{1}{3 \text{ g liver (wet wt)}} \times \frac{17.4}{14} \times \frac{1 \text{ mmol Zn}}{65.4 \text{ mg Zn}} \times \frac{1{,}000 \text{ μmol}}{\text{mmol}}$$

Calculations from a typical gel elution profile (Fig. 10-7) are:

$X_1 + X_2 + \cdots + X_{15}$ = 1.515 mg Zn = 0.098 μmol Zn g^{-1} liver (wet wt) in the high molecular weight protein pool

$X_{16} + \cdots + X_{25}$ = 1.035 mg Zn = 0.067 μmol Zn g^{-1} liver (wet wt) bound to metallothionein

$X_{26} + \cdots + X_{45}$ = 0.690 mg Zn = 0.045 μmol Zn in the low molecular weight cytoplasmic pool

These procedures and calculations have been used in studies to determine the loading capacity of metallothionein (Brown and Parsons, 1978); spillover from metallothionein to the enzyme-containing pool (Brown and Parsons, 1978; Roesijadi, 1979); the normal metal component of the enzyme-containing pool (Brown and Chatel, 1978); displacement interactions between metals in,

and between, cytoplasmic pools (Brown and Chatel, 1978); reductions in metallothionein synthesis as a result of exposure to organic carcinogens (Brown 1977, 1978); and reductions in the metal components of the enzyme pool as a result of exposure to organic carcinogens (Brown, 1978).

Confirmation of Metallothionein

The fractions corresponding to the usual elution position for metallothionein are pooled, and metallothionein, if present, is separated into its two charge separable forms on a diethylaminoethyl (DEAE) A25 Sephadex column (40 × 1.5 cm). All fractions are then analysed for Cd, Cu and Zn content. If the metal-binding proteins are metallothionein, they will separate out into at least two forms: MT1 and MT2.

The various forms can then be analyzed for their amino acid content. Metallothionein is confirmed if approximately one-third of the amino acids of MT1 and MT2 are cysteine and if no aromatic amino acids are present (Kagi and Vallee, 1960, 1961).

In addition, a technique using isolectric focusing followed by gel electrophoresis can be used to determine the number of different proteins that occur in the partially purified metallothionein pool eluted from the Sephadex G-75 column. The proteins can then be analyzed for metal content and, where possible, isolates should be subjected to amino acid analysis. This procedure may be necessary because there is some evidence to suggest that metal-binding proteins other than metallothionein, which also may have detoxification capabilities, occur in the same molecular weight pool as metallothionein. For example, Premakumar et al. (1975) have described a copper-binding protein called Cu-chelatin which is distinct in properties from metallothionein.

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The Use of Hydroids in Toxicology

METHODOLOGY

Introduction

Hydroids have been used in experimental studies by many people since the work of Rees and Russell (1937), but it was not until *Artemia salina* eggs became commercially available that the large-scale culture of hydroids became feasible for those without access to freshly caught plankton. Since then, many have used hydroids for studies of regeneration (Tardent, 1963), morphogenesis (Braverman, 1974) and other fundamental processes. Their size, sessile habit, tolerance and mode of asexual reproduction make them ideal experimental organisms for many purposes. As they generally reproduce by budding, a laboratory population can be built up from a single explant, providing a genetically homogenous source of experimental material. This is one of the main reasons for using hydroids for toxicology, because the use of a single genotype reduces variability and improves precision.

Culture Technique

When beginning this work several different hydroid species were isolated and cultured, but for a number of reasons none proved