A STUDY OF THE UPTAKE AND EXCRETION OF INORGANIC MERCURY, AND THE LONG TERM EFFECTS OF EXPOSURE TO LOW LEVELS OF THE METAL IN THE LOBSTER, HOMARUS GAMMARUS (L) WHITE 1874

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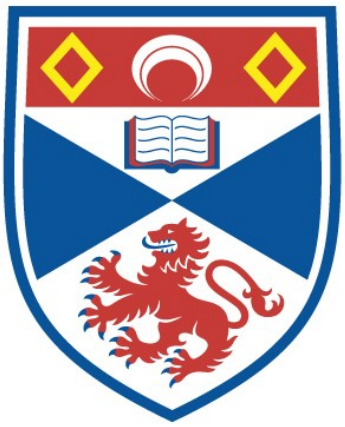

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A Study of the Uptake and Excretion of Inorganic Mercury, and the Long Term Effects of Exposure to Low Levels of the Metal in the Lobster, Homarus gammarus (L) White 1847

by

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Abstract

The aim of this work has been to make an overall study of the uptake accumulation, and excretion of inorganic mercury, and the effects of long term exposure to the metal, in the lobster, Homarus gammarus, using relatively simple techniques considered appropriate to pollution studies. In this way it was hoped to gain an understanding of the way in which the lobster deals with mercury and to be able to predict what organs were likely to be affected by low levels of mercury.

The initial approach was to measure background levels in the different organs of the lobster, and also of lobsters exposed to 100ppb. and 10ppb. mercury for varying periods of time, using neutron activation analysis. These results were amplified by radioisotope tracer experiments so that besides the sites of principal accumulation being identified the route of uptake could be determined. By the same means, the ability of lobsters to excrete mercury was investigated.

Long term studies of the effects of exposure to mercury on organs identified as being susceptible to damage because they were sites of uptake, major accumulation, or excretion were studied by histological techniques which were amplified in part by electron microscopical examination.

Since it was likely that the lobster larvae might be weak links in the life history, measurements of mercury uptake by the larvae was attempted using X-ray microanalysis. This was unsuccessful, and studies of the effects of mercury on the survival of the lobster larvae were inconclusive.

It was found by these varied approaches that uptake was mainly through the gills where highest levels of mercury accumulated, but it is probable that most of this mercury was bound to the cuticle or in the cells. A small proportion passes into the blood and is taken up and gradually excreted by the green glands, Long term damage is found in these organs but not in either the gills or the digestive gland. It is therefore suggested that damage due to mercury only occurs at sites of active control. The damage in the green glands due to exposure to levels even as low as 10ppb. is likely to lead to death if exposure is prolonged, but a more important finding was evidence of greater susceptibility at different stages of the moult cycle. This is worthy of more detailed investigation.

SUPERVISOR'S CERTIFICATE

I certify that Janet H. Brown has fulfilled the conditions laid down under Ordinance General No. 12 and Resolution of the University Court, 1967, No. 1, and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

DECLARATION

I declare that the work reported in this thesis is my own and has not previously been submitted for any other degree.

VITAE

I was educated at Sutton Coldfield Grammar School for Girls, and the University College of North Wales, Bangor, where I graduated with an honours degree in Zoology with Marine Zoology. The work described in this thesis was carried out between August 1973 and November 1975.

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Units of measurement

There is a wide choice of units for use in measuring metal concentrations in solution, and in animal or plant tissue.

In this study, all tissue and solution concentrations have been expressed as parts per million (ppm) or parts per billion (ppb). All units used in the literature have been converted to either ppm or ppb to enable comparisons to be made more easily.

1 ppm = 1 mg/kg ; 1 µg/g ; 1 mg/l

1 ppb = 1 µg/kg ; 1 ng/g ; 1 µg/l

Wet weight determinations have been used throughout although dry weight values have been determined for comaparison (see page 54)

General introduction

Pollution is not a modern problem but it has only come to be studied scientifically in fairly recent years. Its effects cannot be researched properly from the confines of narrow, conventional subject barriers but call for a multidisciplinary approach. The pollutant materials are chemicals whose behaviour and action alone, and in combination with other elements and compounds, can only be understood fully by a chemist; the interaction of these pollutant materials with the biosphere can only be interpreted by biologists. This is a study of a particular pollution problem viewed from a number of different standpoints. By using this multiple approach it is hoped to arrive at a clearer understanding of a specific pollutant chemical in a particular animal.

Mercury as a pollutant

The pollutant chemical in this study is inorganic mercury. Mercury is one of the more dangerous pollutant chemicals because its effects can be insidious. A severe neurological disease was recognised amongst people living in Minamata, Japan, in 1953 (Kurland, Faro and Siedler, 1960). Many died and others were seriously affected. By September 1971 forty eight deaths amongst 134 patients had occurred (Fujuki, 1972). Methyl mercury was not recognised as the cause of it until some time after the effects were observed. This methyl mercury

was produced by the local acetaldehyde and vinyl factory and was disposed of in Minamata Bay with the factory effluent. It thereafter accumulated in the fish and shellfish of the bay which were then consumed by the local inhabitants (Irukayama, 1967; Kurland, Faro and Siedler, 1960). A similar outbreak occurred in 1965 at Niigata in Japan where six people died and forty-one others suffered chronic poisoning (Holden, 1973).

In Sweden, increased mercury concentrations were found in birds and freshwater fish in the 1940's and 1950's while at the same time there was an increased use of alkyl mercury compounds as fungicidal seed dressings and a marked increase in the mortality of wild birds (Klein and Goldberg, 1970). From a comparison between museum specimens of birds collected between 1840 and 1940 and recently collected wild birds, it was found that there had been a twenty-fold increase in mercury content in the more recent samples (Berg et al, 1967).

The danger with mercury is that animal and plant life tend to accumulate it from even low concentrations in the environment to levels at which harm can be done to that animal or plant, or to animals higher up the food chain.

Occurrence of mercury

Mercury is a naturally occurring element, being found in the earth's crust at levels ranging from 0.01 ppm to 20 ppm (US Dept of the Interior, 1970). It is sometimes found in its elemental form, but the most common source is from deposits of cinnabar (mercuric sulphide, HgS). Mercury has exceptional abilities to form a variety of organic and

inorganic compounds. The inorganic forms are metallic mercury or salts of the metal, eg mercuric chloride or mercuric sulphide. The organic forms fall into three categories: alkyl mercuric compounds (eg methyl mercuric chloride), aryl mercuric compounds (eg phenyl mercuric acetate), and alkoxy-alkyl mercuric compounds (eg methoxy-ethyl mercuric acetate). The classification is further complicated by the fact that interconversions can occur between the different forms (eg inorganic mercury can be converted to methyl mercury; phenyl mercury can be broken down to the inorganic form, (Jernelov, 1968). There is a great variation in toxicity between these different compounds of mercury and in the modes of action of the more toxic forms. It is therefore important to distinguish between the different forms of mercury.

The fact that it is a naturally occurring element makes its study as a pollutant particularly difficult. It has frequently been pointed out that the difference between background levels in the marine environment and levels that are harmful to the biosphere is very small (Hammond, 1971; Berlin 1972). It is also often difficult to distinguish between natural and man-made levels. This is in contrast to such pollutants as radioactive elements, or pesticides such as DDT when their source is much clearer. The sources of the man-made levels are:

a) the burning of fossil fuels which contributes to aerial contamination which can then be distributed and recycled through precipitation

b) from industrial processes such as the electrolytic production of chlorine and caustic soda, from the use of mercury in electrical appliances, and in the making of pharmaceuticals, paints and amalgams, dental preparations, and in general laboratory use. Mercury compounds have been used as slimicides in the pulp and paper industry, and as fungicides in agriculture.

Many of these uses have been restricted or curtailed because of the risk of environmental contamination. For example, the use of phenyl mercury as a slimicide in the making of paper was banned in Sweden in 1967. In agriculture, the use of methyl mercury compounds as fungicides was banned in Scandinavia in 1966, and in USA and Canada in 1970, and their use was discouraged in other countries. The use of alkoxy-alkyl and aryl mercuric compounds as fungicides is still permitted in all countries except Japan, since it is considered that there is much less degree of risk associated with these compounds and in any case no more effective fungicide has yet been found (Saito, 1972; Riessanen and Miettinen, 1972).

The mercury levels in oceanic waters are considered to be largely due to natural sources of mercury (Hammond 1971); the total amount of mercury present in the oceans being four order of magnitude greater than man's annual use of the metal. Man's annual usage of mercury is about 10^4 tons of which approximately half is recycled. The total content of the waters of the world is estimated at about 10^8 tons (Peakall and Lovett, 1972). The natural background level of mercury in the oceans is usually taken as being around 0.1 ppb (Hammond 1971). The levels in coastal waters on the other hand, vary according to the influence of local industrial activity, run-off from mercury rich areas, or waste disposal in the sea. Typical values for different marine areas are shown in Table A.

Mercury tends to be taken out of solution in the sea on to particles in the sediment on the sea floor (Dall'Aglio, 1968). There is an equilibrium between the mercury in the water and in the sediment varying with the type of sediment, but the levels of mercury in the sediments are always higher than in the water (Feick, Horne and Yeaple, 1972). The levels of mercury in the sediment therefore, may well be of significance to bottom dwellers (Klemmer, Luoma and Lau, 1973). Sediments in the

Adriatic and Tyrrhenian Seas showed levels from 50 to 1500 ppb (Selli et al, 1973). In the sediments of Minamata Bay, the mercury content was up to 2010 ppm at a site near the factory discharge pipe, dropping to 12 ppm on the far side of the Bay (Kurland, Faro and Siedler, 1960).

Alternatively, the sediments may act as a reservoir for mercury since Fujiki found that the levels of mercury in the mud of Minamata Bay did not drop in seven years, even after discharge into the Bay had ceased (Fujiki, 1972).

TABLE A Mercury Content of Coastal and Oceanic Waters

Coastal Waters in Britain		
Bristol Channel below Cardiff	0.05 ppb	Gardner & Riley 1973a
Central Bristol Channel	0.01 ppb	
Irish Sea	0.03-0.05ppb	Gardner & Riley 1973b
Irish Sea in region of sludge disposal	0.2 ppb	
Clyde estuary in region of sludge disposal	0.110 ppb	Halcrow et al 1973
North Sea	0.03 ppb	Stock & Cucuel 1934
Coastal Waters in Japan		
Minamata Bay	1.6-3.6 ppb 200-700 ppb	Hosohara 1961 Yoshida 1967 (Samples taken on 25.5.1960)
In region of Lamopo deep	0.1 ppb at surface 0.15-0.27 ppb at depth	Hosohara 1961
Ocean Water		
North eastern Atlantic	0.013-0.02	Leatherland et al 1972
North western Pacific	0.06-0.27	Hosohara 1961
North eastern Atlantic (at depth of 4030 m)	0.142 ppb	Leatherland et al 1973

The Lobster as experimental animal

The animal used in this research was the common lobster, Homarus gammarus (L) White 1847. This animal was convenient for this study as it is a suitable size for the measurement of metal concentrations in different tissues, and is very easy to keep under experimental conditions and readily available. Furthermore, its larvae can be reared under laboratory conditions. The lobster is also relevant to such a study as it is of economic importance as a source of food. Studies on the lobster will also be of use with reference to other crustacea, most of which play an important role in food chains. There are other reasons for investigating the lobster in particular with regard to mercury pollution. Concentrations of mercury in lobsters have been found to exceed the 0.5 ppm level set as the safe limit for human consumption (first established in 1970 by the Food and Drug Administration, USA). While marine fish are found to have mercury contents well below this level, the few measurements that have been made of the mercury content in lobster have found levels that are on average higher than this level (Holden and Topping, 1972; MAFF 1971, 1973; Freeman et al, 1974).

There are two possible explanations for this apparent tendency of the lobster to accumulate high levels of mercury, which are not mutually exclusive. Firstly, the lobster as a benthic animal may be exposed to special risks of accumulating mercury. Klemmer, Luoma and Lau (1973) found that benthic feeders accumulate more mercury than animals feeding above the water-sediment interface. Also, as an omnivore,

the lobster may ingest greater amounts of mercury. Klemmer et al found that the diet also affects uptake, and typical tissue concentrations of mercury that they found off the coast of Hawaii were 0.25 ppm for benthic omnivores, 0.12 ppm for grazing benthic herbivores, and 0.15 ppm for benthic carnivores. Hamilton (1971) found differences in accumulation between plankton feeders and detritus feeders, thelatter having the higher mercury values.

Secondly, the other explanation may simply be that as a species the lobster has a greater tendency to accumulate mercury than other species. Barber, Vijayakumar and Cross (1972) found evidence that there are tendencies in some species to accumulate mercury even in unpolluted sites. In this study of deep oceanic fish they found that certain species contained more than the 0.5 ppm level whilst others of similar feeding habits contained orders of magnitude less mercury. Hence the mercury content of the deep ocean (which is of natural origin) did not affect the mercury content of the fish; species specific factors and size were the determining factors.

Methods of studying mercury

Mercury is a complex pollutant and the approaches to its understanding can also be diverse. Its action can be studied in the natural environment or under experimental conditions. Within these two categories there are various approaches that can be made, some of which are common to both the natural and the experimental situation. A review of the possible methods that can be used to study mercury will be given here. At the same time the information derived from these studies will be reviewed and the gaps

in this knowledge will be pointed out.

Studies on mercury under natural conditions

i) The first possible approach to the study of mercury pollution in the marine environment is to determine the levels of mercury already present in animals and plants. Stock and Cucuel (1934) were probably the first to do this. They measured the levels in the sea as 0.03 ppb and in fish as 25 - 180 ppb. Raeder and Snekvik (1941) measured concentrations of 0.04 - 0.16 ppm of mercury in fresh marine fish muscle. However industrial use of mercury has risen sharply since then. World production of mercury at the time of these analyses was about one quarter of that for 1972 (Peakall and Lovett, 1972).

This type of investigation has become much more common following the outbreaks of mercury poisoning in Japan and the finding of extremely high mercury levels in many Swedish lakes and rivers (Anon 1965;Westermark,1965; Johnels et al, 1967). As already mentioned, studies have been made of mercury levels in the nekton and benthos in which the lobster was the only species containing more than the 0.5 ppm level of mercury. In some restricted coastal localities however certain fish did contain an equally high level but these were attributable to the locality whereas the lobsters sampled were taken from areas considered free of pollution. For example, in the Mersey estuary which is heavily industrialised, the average mercury contents of dab, plaice, whiting and flounder were all above the 0.5 ppm level (Holden and Topping, 1972; MAFF 1971, 1973; Freeman et al, 1974). Plankton levels were examined by Windom, Taylor and Stickney (1973) who found that concentrations of mercury in the plankton were considerably lower

offshore (0.1 - 0.3 ppm dry weight) than in coastal waters of the North Atlantic, in the New York Bight, where the levels were as high as 5.3 ppm dry weight (approximately 1 ppm wet weight).

ii) A more detailed approach was made by Jones, Jones and Stewart (1972) who measured mercury levels in selected algae and invertebrates and in two species of vertebrates in the Tay Estuary, Scotland. [This estuary receives all the untreated sewage and industrial effluent of the city of Dundee, yet because of extremely good tidal mixing is considered one of the cleanest estuaries in Europe (Buller, McManus and William, 1971; Khayrallah and Jones, 1975).] They compared the results with levels from a site further north. Besides measuring total levels they extended the scope of such work by also measuring the accumulation in different tissues in the mussel, Mytilis edulis, the grey seal, Halichoerus grypus, and the eider duck, Somateria mollissima mollissima, so that the main sites of accumulation in the body could be identified. For the mussel, these were the gills and visceral mass, and for the two vertebrates the principal accumulation sites were the liver and the kidney. The level of mercury in the gills of the mussel was as high as 3.4 ppm while in the liver of the seal there was 66 ppm mercury.

These studies give baseline values for the mercury content in different species and different locations and at the same time highlight areas of risk or species with a particular tendency to accumulate the metal. The work by Jones et al is even more important, as it illustrates how the animal is dealing with the metal and may provide useful information on how this pattern of accumulation may affect the animals, and other species, and man, that may eat the animal.

iii) These natural levels studies can usefully be extended in another way by measuring the actual degree of accumulation found in a particular animal from a known concentration of mercury in a natural habitat. Johnels et al (1967) found that pike, Esox lucius, accumulates mercury by a factor of 3000. Matsunaga (1975) calculated concentration factors of 25,000 ± 2,100 for total mercury and 10,000 ± 2,600 for inorganic mercury in the crucian carp, Carassius carassius langsdorfii of the Hoono River, in Japan. From this he was able to suggest that waters should not contain total mercury levels higher than 0.016 or 0.04 ppb of inorganic mercury if they are to be fished for fish that have a concentration factor similar to that for crucian carp.

iv) Another approach is to use the study of the natural levels of mercury in plants and animals to determine whether mercury is passing via the food chain or whether it is absorbed direct from the water via the epithelial tissue as suggested by Wobeser et al (1970) in their studies on fish in the Saskatchewan River. Cocoros, Cahn and Siler (1973) measured the mercury content in three Western Atlantic estuaries. At the same time they measured the mercury content of the estuarine plankton and of the fish, the atlantic menhaden, Brevoortia tyrannus (Latrobe) which feeds on the plankton. They found no definite evidence of food chain enhancement. Although the fish contained twice as much mercury as the plankton, and this was mainly concentrated in the viscera rather than in the epithelial tissue (such as the gills) suggesting that it might have been taken up from the food, the zooplankton contained four times less mercury than the phytoplankton. There is not therefore a progression that might be expected through the food chain from phytoplankton, to zooplankton to the fish.

Experimental investigations

The approaches described above have been more commonly used under experimental laboratory conditions, where in theory it should be easier to get clear cut results.

i) The simplest approach to the experimental study of mercury pollution is again the straightforward measurement of accumulation or loss. This was done by Majori and Petronio (1972) who found that the mussel, Mytilis galloprovincialis Lmk. would accumulate mercury from concentrations of 5, 50 and 100 ppb at an increasing rate until levels of 40,000, 100,000 and 130,000 ppb respectively were reached. Getsova and Volkova (1964), measuring the ability of the larvae of some aquatic insects to concentrate various elements, determined the total body concentrations of mercury. The results however are presented as coefficients of accumulation, that is, as the ratio of the concentration resulting in the animal after exposure, to the initial concentration in the external medium. This concentration in the external medium, which was a solution of radioactively-labelled mercuric chloride, is only given as a measure of radioactivity present. This paper, therefore, only allows a comparison to be made between the different accumulation rates of the different elements. The coefficients of accumulation for mercury range from 173 after two days in one species to 8310 after sixteen days in another. There is however no reason to suppose that accumulation rates are constant and independent of the external concentration of the element. It would have been simple to determine the elemental concentration in the experimental solution and this would have made the paper very much more informative to a pollution scientist. (Getsova et al were actually making this enquiry to determine the geochemical roles

of living organisms in the biosphere.) However, the same omission occurs in another study. Seymour (1971) determined the rate of loss of mercury by the Pacific oyster Crassostrea gigas. Having said that the rate of loss is directly related to the concentration factor he proceeds to describe an experiment in which the initial concentration is not given because the specific activity (ie the degree of radioactivity in a known weight of radioactively-labelled element or compound) of the mercury is not known. It is a useful experiment in that the rate of loss may be one of the most relevant things to determine. If a food animal such as the oyster is exposed tc a pollutant, it is important to know how long the animal will take to purge itself of the metal so that it is once again fit for human consumption. Seymour does not relate his experimental levels to such factors, and there is no way of knowing if the oysters are dealing with exceptionally high levels of mercury or the converse. Loss might well proceed at a higher rate at high initial concentrations so that the rates of loss given are not as informative as they might be if it was known how they related to the concentration of mercury present in the animal.

It is clear, therefore, that it is important to know what levels of mercury are being dealt with both internally and externally; otherwise the advantage over studies in the uncontrolled environment are lost.

Kramer and Neidhart (1974) also did uptake experiments but extended the scope by using both inorganic and organic mercury. They used levels of mercury low enough to be of significance in environmental studies, with the experimental solutions ranging from 0.1 ppb to 20 ppb. They looked at the uptake of radioactively labelled mercuric nitrate and methyl mercuric chloride in the females of the fish, Poecilia reticulata. The

uptake was measured by the amount of radioactivity present in the whole body. Since this meant that measurements could be made on the live fish, the uptake and loss could be measured over a period of time for the same fish. The disadvantage of this method is that the accuracy cannot be very great because the variations in body size and geometry make the accurate counting of the radioactivity difficult. However Kramer and Neidhart were able to show that the uptake of the organic mercury was far greater than that of the inorganic. They also use the results to postulate that the inorganic mercury is converted to the methyl form in the body. This is based on the fact that there are two quite distinct rates of loss of inorganic mercury. The different patterns of loss could equally well be due to differential rates of loss from different organs. Without analysis there is no reason to suggest that methyl mercury is formed. This point does however illustrate the value of knowing the accumulation patterns in different tissues and will be referred to in more detail later.

From their work on the loss of inorganic mercury from the crab, Cancer magister, Sloan, Thompson and Larkin (1974) suggest that rates of loss vary as the mercury moves from organ to organ on its excretion pathway. Seymour (1971) found four different rates of loss of mercury from the Pacific oyster. Yoshida et al (1967) also measured the rate of loss from the tissues of the shellfish, Venerupis philippinarum and found quite disparate rates of loss from the different tissues at different times. Evidence for this phased loss is also given by Miettinen et al (1969). They looked at the uptake and

excretion of methyl mercury nitrate and phenyl mercury nitrate in four species of fish, the pike, Esox lucius, the perch, Perca fluviatilies, the flounder, Pleuronectes flesus, and the roach, Leuciscus rutilus. Again no measure of quantities of mercury administered are given; only the degree of radioactivity present, but they found that 10 - 50% of the administered dose was excreted within one to two days in the pike and perch, while the rest was lost more slowly with a biological half-life (time taken for half the administered dose to be lost) of 100 - 600 days for methyl mercury, and 60 - 150 days for phenyl mercury. Similarly with the roach and flounder this two phase excretion was found.

ii) As described under the studies in the environment, these uptake studies can be extended by measuring the actual distribution of the element within the tissues of the animal. Corner and Rigler (1958) did this, and also extended the scope of the experiment by comparing the uptake of organic and inorganic mercury. To do this they used radioactively-labelled mercuric chloride and n-amyl mercuric chloride. They chose levels that were poisonous to the larvae of Artemia salina and Elminius modestus within three hours (5 ppm, 1 ppm & 0.2 ppm). In their experiments with the prawn Leander serratus they used levels as high as 50 ppm. They measured the uptake in the whole larvae, but in the prawn they measured the distribution within the different tissues and were able to compare different patterns resulting from exposure to the different forms of mercury.

Yoshida, Kawabata and Matsue (1967) also measured the different patterns of accumulation resulting from exposure to organic and inorganic

forms of mercury. They studied the accumulation of ^{203}Hg labelled phenyl mercuric chloride and ^{203}Hg. labelled mercuric chloride in the bivalve, Venerupis philippinarum. Unfortunately their results are only expressed as a measure of gamma ray activity present in the different organs. The accumulation rates given are therefore only relative and again there is no way of knowing if these values represent the animal's action under conditions of acute poisoning, or of exposure to sub-lethal levels of the element. The fact that the experiment only lasted four days however may suggest a high initial level of mercury.

iii) Mercury uptake in the natural environment may be studied by its accumulation in the food chain and this can also be done under laboratory conditions. Yoshida et al (1967) investigated the uptake of mercury compounds through the food chain; from the initial uptake by the marine bacteria, Pseudomonas fluorescens to the brine shrimp, Artemia salina, and then to the shellfish, Venerupis philippinarum. Only 0.1% of the initial mercuric chloride reached as far as the shellfish and the distribution of the mercury in the tissues was found to be quite different from the pattern resulting from uptake from the external medium. From their results they considered that the transference of mercury occurs both directly via the sea water and through the food chain.

Rucker and Amend (1969) fed two year old chinook salmon fingerlings of the same species contaminated with up to 3 ppm mercury. The resulting mercury concentrations in the two year olds were as high

as 30 ppm in the liver and 1.9 ppm in the muscles. Similarly, sablefish reared for twenty eight months on a diet of equal amounts of herring and dogfish accumulated mercury and were found to have muscle levels as high as 1.29 ppm. The mercury concentrations in the dogfish and herring were 0.39 ppm and 0.05 ppm respectively. In a later experiment sablefish kept on a diet of dogfish only built up mercury concentrations of 1.77 ppm after thirty one months and after thirty seven months on the mixed diet have mercury levels of 1.14 ppm (Kennedy and Smith, 1972, 1973). It is clear from these studies that the diet is an extremely important factor in determining mercury levels in the body and is therefore of great relevance in aquaculture. Feeding the cultured animals on food that has even quite low levels of mercury may result in unmarketable fish.

iv) The organ in which the animal accumulates mercury, and to what extent, and whether it can excrete it or not, is very important in environmental studies. However the effect of the accumulation may not be as important for its effect in the food chain as for its direct action on the animal itself. It is therefore essential to see how the metal acts within the animal.

Corner (1959) made a study of the physiological effects of toxic levels of mercuric chloride and n-amyl mercuric chloride on Maia squinado (spider crab). He was studying the accumulation and loss via the gills and excretory organs, and at the same time looking for possible physiological effects on the animal. He did this by comparing the patterns of amino acids and sulphates in the blood and urine of the animals, and how these patterns were affected by the mercury.

The rationale for this was that it was already known that Maia squinado excreted a urine with lower amino nitrogen and a higher sulphate content than that of its blood. Evidence of impaired renal function might therefore have been obtained by measuring these parameters. He found that with inorganic mercury (10 ppm) the level of amino nitrogen rose with respect to the blood and the sulphate level was unchanged. With organic mercury the amino nitrogen level rose in both the blood and the urine.

Renfro et al (1974) made an investigation of the effect of mercuric chloride and methyl mercuric chloride on selected physiological events in a variety of different fishes. They combined this study with uptake experiments. They studied the effect of the two mercury forms on sodium transport in the two osmoregulatory organs of the fish, the gill epithelium, and the urinary bladder. They also looked at the effect on the enzyme adenosine tri-phosphatase involved in sodium transport and determined whether the ionic regulation within the intracellular compartments was affected by mercury accumulation. They found considerable difference in the action of the two mercury forms. The effect of the mercuric chloride was to stop sodium transport in the gill and bladder completely, whereas methyl mercuric chloride only had a transient effect on these functions. A study of the patterns of accumulation showed how the different effects might come about. They found that the mercuric chloride went initially to the gills and remained at the highest concentration in the body there until up to seventy two hours after the cessation of exposure, and thereafter redistributed gradually to the liver and kidney. The methyl form on

the other hand also went to the gills initially but was quickly redistributed to result in higher concentrations of mercury in the kidneys and liver within forty eight hours. Also by using methyl mercuric chloride labelled with both ^{203}Hg and ^{14}C Renfro et al. were able to show that the ^{203}Hg stayed longer in the gills than the ^{14}C. They suggested therefore that methyl mercuric chloride is broken down to inorganic mercury. This is a completely different idea to that of Kramer and Neidhart (1974), (see page 14), but with much more substantial evidence for the hypothesis.

A similar physiological approach was made by D'Amelio, Russo and Ferraro (1974) who investigated the effect of heavy metals on protein synthesis in crustaceans and fish. So far work has only been done on lead, where they have shown that changes occur in the polyribosomes after exposure to the metal. They have investigated three parameters; cell density using Ficoll density gradient analysis; haemoglobin synthesis and aminolevilinic acid dehydrase activity.

The study of pollution is an applied science, and it is arguable, if not necessarily true, that studies that are not comprehensible to those people concerned with pollution in general are largely wasted effort. Thus such complex investigations as that of D'Amelio et al, while of great relevance medically, may not be of much use to pollution studies. In any case, the simpler approach to the study of the effect on the animal may provide more straightforward yet more compelling results.

v) Such an approach is that of simple histological examination. Rucker and Amend (1969) looked at uptake of ethyl mercury phosphate in rainbow trout. The trout were exposed on eleven occasions for periods of one hour to a 2 ppm solution of Timsan, a commercial form

of mercury compound containing 6.25% ethyl mercury phosphate which is used to control the external bacteria and parasitic infections of fish. The pattern of accumulation was studied and the histological effect of these exposures on the main sites of accumulation, ie, the gills, the red blood cells, liver and kidney. They also did a longer term experiment using twelve weekly one hour exposures. The only histological changes observed were of hyperplasia of the gill epithelia.

This type of study has not been made with inorganic mercury but its effectiveness as a research method is clearly illustrated by the work of Hubschman (1967) and Aiken and Byard (1972). By using the simplest experimental technique they were able to show damage to the cells. Hubschman studied the long term effects of copper on the crayfish, Orconectes rusticus (Girard). He found that the antennal glands removed from adult crayfish killed in a copper concentration of 1 ppm completely lacked histological organisation, and that the glands of many animals that survived exposure to 0.5 ppm copper for thirty days were found to be severely damaged. Aiken and Byard examined the effect of yellow phosphorus on the American lobster, Homarus americanus. They demonstrated that there was serious cell damage in the digestive gland, and to a lesser extent in the green gland, although the levels of phosphorus in the exposure solutions or the duration of exposure are not given.

Crandall and Goodnight (1963) made a very much more detailed and informative study of the effect of exposure to lead nitrate, zinc sulphate, and sodium pentachlorophenate, on the organs of the common

guppy, Lebistes reticulatus (Peters). Tissues were examined after different exposure times of up to 183 days. Various histological changes were found; much of it in renal tissue, and also a retardation of gonadal development. More serious damage was found at higher concentrations.

With inorganic mercury the only comparable approach is that of Thiriot Quiévreux (1966). She studied the effect of mercuric chloride on the green gland of the crab, Carcinus maenas. The mercury was however administered by injection into the heart such as to give a subsequent body concentration of 5 ppm. The response observed is therefore an acute one; and changes were also observed through a hole cut in the carapace to expose the green gland, as well as by more conventional histological techniques. It is not consequently of such value to pollution studies while being of interest from other points of view.

Light microscopy may only be capable of showing the grosser changes in cell structure while sub-lethal effects pass unnoticed. Trump, Jones and Sahaphong (1975), in their study of the effect of mercury on the kidney tubules of the goldfish point out that cell damage goes through five distinct stages. Only the last two are irreversible, but the sub-lethal effects on the cell in the earlier stages may well be of significance in the future life history of the animal. These earlier stages may not be seen with the light microscope and even the final stage of complete necrosis may not be visible with the light microscope for up to eight hours after it has occurred. These more subtle changes must therefore be studied with the electron microscope.

Such studies are not numerous, especially in invertebrates. Ware, Chang and Burkholder (1974) looked for ultrastructural evidence of foetal liver injury in rats from in utero exposure to small doses of methyl mercury. Doses that caused no effect in the mother produced lesions in hepatic tissue of the foetus. Although cellular changes could be seen by light microscopy, additional and significant changes were seen with the electron microscope. Fowler (1972) demonstrated changes in the proximal tubules of rat kidney induced by low doses of methyl mercuric chloride. Large numbers of spherical masses were visible with light microscopy, but electron microscopy was necessary to show them to be mainly consisting of smooth endoplasmic reticulum. This was consistent with the hypothesis that the methyl mercury was broken down to inorganic mercury in the tubules, because of the known association of the smooth endoplasmic reticulum with detoxification activity.

vi) Another approach similarly simple in its concept for the investigation of the effect of a particular pollutant on an animal is that of toxicity testing. This is often the starting point of an investigation. It is of value to studies on the environment involving acute conditions such as those described by Zitko et al (1970) when an industrial discharge of yellow phosphorus killed fish and crustacea in Long Harbour, Placentia Bay, Newfoundland in 1969. It can also allow different susceptibilities in different species, or different stages in the life history of a species, to be identified. It may also allow identification of different modes of poisoning, or different toxicities between different forms of the same pollutant.

Too often, however, toxicity testing appears to be a painful elaboration of the obvious. Kulczyki (1968) studied the effects of solutions of mercuric nitrate in concentrations from 10,000 ppm to 50,000 ppm on Daphnia pulex (de Geer). All animals died within four hours and he compared the amounts of mercury incorporated into the animals under the different conditions. He could say that size, sex, and physiological state were factors important in determining the moment of death. Knapik (1969) similarly investigated the action of mercuric nitrate on four crustacean species, Gammarus locusta (Sars), Neomysis vulgaris (Thompson), Palaemonetes varians (Leach) and Rhithropanopeus harrisi tridentatus (Mtl). Concentrations in this case were from 10 ppm to 500 ppm. In these experiments the animals lasted up to fifteen hours in the lowest concentration and six hours in the others. Knapik uses this experiment to describe the animal's behaviour in dying.

Toxicity studies can be made to yield much useful information if properly designed. Corner and Sparrow (1956, 1957) were able to show that different species had different susceptibilities to mercury and that different mercury compounds had different toxicities.

The method of toxicity testing usually employed determines either the time taken, or the concentration required, for fifty per cent of the test animals to die. Portmann (1968) determined the lethal dose for 50% (LD_{50}) to investigate the toxicity of mercury, copper, zinc, nickel and phenol on the pink shrimp, Pandalus montagui, the brown shrimp, Crangon crangon, the shore crab, Carcinus maenas, and the cockle, Cardium edule. Although the experiments were only run for

forty eight hours, Portmann obtained a great deal of information. He found mercury to be the most toxic of the metals and that the degree of toxicity fell with the solubility of the metal in sea water. The order of toxicity was therefore mercury, copper, zinc and nickel. He also investigated the effects of temperature, starvation, and age on the toxicity of selected metals. A fall in temperature from 20^0C to 5^0C was sufficient to increase the tolerance of the brown shrimp to copper and mercury by a factor of five. For cockles the factor of increased tolerance was 80 and 130 for copper and mercury respectively. Starvation was found to reduce the tolerance of the animals to the toxin. The LD_{50} was reduced by a half for starved brown shrimp and by one third for cockles. The effect of age was only investigated in the brown shrimp. It was found that the larger shrimps were the most tolerant having an LD_{50} of mercury double that of the smallest size tested. Thus such variables are of importance when planning investigations on invertebrates.

This technique of the LD_{50} can be made more relevant to the natural conditions by using less acute levels and longer exposure times. Wilson and Connor (1972) were able to show toxic effects on the brown shrimp, Crangon crangon, after 1000 hours exposure to either cadmium or mercury in solutions as low as 10 ppb mercury and 100 ppm cadmium. They also showed that there was an even greater susceptibility to the metals when the animal was moulting.

The scope of toxicity testing can also be increased by investigation of the effects on the larval forms of a species. De Coursey and Vernberg (1972) studied the effects of mercury concentrations as low as from 0.018 ppb to 180 ppb on the activity, metabolic rates

and survival of zooeal stages of the fiddler crab, Uca pugilator. 180 ppb was lethal to the larvae within twenty hour hours whilst adults will survive this level of mercury exposure for two to three months (Vernberg and Vernberg, 1972; Vernberg and O'Hara, 1972). At the lower concentrations there were marked effects on the metabolic rates and swimming capabilities. Since these lowest levels are equivalent to levels of mercury in coastal waters such findings could be serious. Differences in susceptibilities were also found between different stages of larvae; Stage V larvae were more susceptible than Stage I larvae. A similar greater degree of susceptibility in larval forms was found in the case of oysters, shrimps and lobsters. In LC_{50} tests of up to 64 hours the larvae of these species were shown to be from 14 to 1000 times more susceptible than adults to copper, mercury and lead, in concentrations ranging from 0.0033 ppm to 10 ppm (Connor 1972). It is therefore of great importance to include different stages of a life history when investigating the effect of a pollutant on a particular species.

Toxicity testing can also be related to variable environmental factors. Roesijadi et al (1974) performed toxicity tests using the LD_{50} technique on the porcelain crab, Petrolisthes armatus, over a period of ninety six hours, and investigated the effect of the mercury in different salinities. He found that the 96 hour LD_{50} varied for this animal between 50 - 64 ppb depending on the test salinities. Such work is only of significance to animals living in changeable environments such as estuaries, but as industrial activity is often sited on estuaries such estuarine animals may be at most risk.

vii) This fact is the rationale behind work relating laboratory studies to variable environmental factors. This has been done with respect to such factors as temperature and salinity. A concentration of mercury (18 ppb) which was sub-lethal to the adult crab, Uca pugilator, under optimum conditions of temperature and salinity, greatly reduced survival times under conditions of temperature and salinity stress (Vernberg and Vernberg, 1972). Vernberg and Vernberg also found that the presence of mercury caused a drop in metabolic rate and that this drop was greater in the animals under the additional temperature and salinity stress. Vernberg and O'Hara (1972) determined uptake of ^{203}Hg into the gills and hepatopancreas of Uca pugilator held under six different salinity-temperature regimes. The total mercury taken up was found to be similar, but the proportions of mercury distributed in the tissues differed markedly. They suggested that at lower temperatures transport of mercury from the gill to the hepatopancreas is less effective than at higher temperatures. They also found that the survival time of two to three months under optimal conditions was decreased under the less than optimal conditions.

Vernberg, De Coursey and Padgett (1973) did similar work on the larvae of the fiddler crab, Uca pugilator. The mercury concentration used was 1.8 ppb and a variety of different temperatures and salinities were used. The survival rate, oxygen consumption, and phototactic response were all measured as indicators of larval response. The different regimes all affected the response parameters and the presence of mercury compounded the effects. This is further evidence for the far greater susceptibility of the larval stages.

Other heavy metals

The introduction has so far centred mainly on mercury, but the action of other heavy metals in the biosphere may also be relevant to the study of mercury pollution. Mercury is closely related to zinc, cadmium, and other metals which have a tendency to form complex ions and covalent bonds. Mercury is the least reactive of this group but is much more reactive than copper which is in the lower group of the Periodic Table. However, the important difference between mercury and these other heavy metals and one that should be kept in mind, is that mercury has no physiological role in living organisms whatsoever. Other metals such as zinc, cadmium, copper and lead have roles in enzyme systems (zinc, cadmium and lead) and respiratory pigments (copper in most crustacea, iron) (see Vernberg 1960). This important difference may result in different modes of action in response to mercury than to other metals, which makes a comparison valuable.

An example of this difference between the metals is shown in the patterns of accumulation resulting from exposure to various heavy metals. Uca pugilator, the fiddler crab, accumulates mercury chiefly in the gills with lesser but more or less equal amounts in the digestive gland and green glands (Vernberg and Vernberg, 1972). In Leander serratus, the prawn, mercury accumulates mainly in the gills and then the green glands have considerably more than the hepatopancreas (Corner and Rigler, 1958). Cadmium, on the other hand, accumulates chiefly in the gills of the crab, Callinectes sapidus, but then only slightly less goes to the digestive gland while almost none is found in the green gland (Hutcheson, 1975). However, Hutcheson cast some doubt on the veracity of his results for the green gland and O'Hara (1973b) found the greatest accumulation of

cadmium occurred in the green gland of fiddler crabs and not the gills. Zinc has been shown to have a different distribution pattern still. In Carcinus maenas the greatest accumulation occurred in the digestive gland and then the gills, and Bryan (1966) related this to the fact that zinc can be stored in the digestive gland and excreted across the gills. Lobsters exposed to lethal levels of yellow phosphorus accumulated considerably more phosphorus in the digestive gland than in any other tissue, with the ovary containing the second largest concentration. Very little was found in the gill but the green gland was not analysed. This distribution was considered to correlate very well with the lipid content of the respective organs (Fletcher 1971).

Different species may react differently to the same metal. For example, the crab, Carcinus meanas deals with exposure to raised levels of zinc in a different manner to the lobster, Homarus gammarus. The lobster loses the excess zinc through the green gland while the crab mainly excretes it through the gills (Bryan 1966). It is possible that these differences may apply to the animals' treatment of other elements, so comparisons between different heavy metals can be useful.

Work on the combined actions of various metals may also be fruitful. Some metals are known to work synergistically, such as copper and mercury (Corner and Sparrow, 1956; Hunter, 1949). These studies of Corner and Sparrow, and Hunter were done using high levels of the metals, and more realistic levels would be relevant to environmental studies since industrial effluent is commonly a mixture of heavy metals.

The study of such two-metal systems has certainly proved fruitful. Ganther et al (1972) found that quail given 20 ppm of mercury as methyl mercury in diets containing 17% by weight of tuna fish, survived

longer than quail given the same dose of mercury in a corn/soya diet. Tuna contains a relatively high proportion of selenium. When rats were given an equivalent dose of selenium to that found in tuna fish their resistance to methyl mercury increased. Koeman et al (1973) found that selenium might have a protective effect against mercury poisoning in marine animals, but no such correlation between selenium and mercury content was found in the inhabitants of Minamata (Nishigaki and Harada, 1975). Preserved samples of umbilical cord were analysed for these two elements and although rises in mercury concentration were found to correspond to increased production of acetaldehyde from the local factory responsible for the methyl mercury in Minamata Bay, no such variations in selenium content were found.

This survey covers the possible approaches that can be taken to the study of mercury or to any heavy metal pollution. Mercury is a special case in that it can occur in a variety of chemical forms in the environment, with different effects and toxicities.

It is methyl mercury that has received most attention in both the scientific and the popular press but inorganic mercury is the subject of this study. The reasons behind the greater attention being paid to the former, and for the study in this report of the latter, should perhaps be discussed here.

Methyl mercury

Of the different forms of mercury that occur, methyl mercury is generally regarded as the most dangerous. It was directly responsible

for Minamata disease (Irukayama, 1961, 1962) and for the drastic drop in wild bird populations in Sweden in the 1950's due to the birds eating seeds that had been treated with methyl mercury as a fungicide (Abelson, 1970). These however are two cases in which methyl mercury can be directly implicated.

Since it is now known that inorganic mercury can be converted to methyl mercury (Wood, 1968; Jensen and Jernelov, 1969) by the action of bacteria, interest has become even greater in methyl mercury. This conversion was in fact first hypothesised by Fujiki in 1960. He suggested that inorganic mercury could be methylated by the plankton and other marine life and that this would account for the presence of thio-methyl mercury in the shellfish of Minamata Bay (Fujiki 1963). This hypothesis was rejected when it was found that the methyl mercury was formed from acetaldehyde and inorganic mercury used as the catalyst in the local acetaldehyde plant (Irukayama et al, 1961, 1962). This discovery that such a conversion could take place led many people to assume that such a conversion was necessarily always occurring.

The distribution of methyl mercury has been widely measured since then, but the results are extremely variable; the importance of the methyl form as part of the total mercury content of any particular species, or of animals in a particular locality varies greatly. The literature appears to be full of conflicting evidence as to exactly how relevant this conversion process may be, and how extensive the distribution of methyl mercury is in nature, so it is necessary to review the literature on this topic in some detail in order to clarify this matter as far as possible.

Environmental levels of methyl mercury

There are many studies of natural levels of mercury in fish and other aquatic animals that show that methyl mercury forms the greater part of the total mercury content. Leatherland et al (1973), measuring trace metal content of pelagic organisms in the north east Atlantic Ocean, found the methyl mercury levels in two species above 90% of the total mercury content. This total was however at a maximum of 0.38 ppm dry weight (<0.1 ppm wet weight).

In the United States, Smith et al (1971) investigated the mercury content of some fresh and canned fish of North America. Total mercury levels ranged from 0.2 - 1.6 ppm and this was almost entirely in the form of methyl mercury, since the inorganic mercury content never exceeded 5%.

Uthe et al (1972), also in North America, found that all fish samples had a greater proportion of methyl mercury except for the eel, Anguilla rostrata, species of crabs, seals, and beluga whale muscle; all of which had very little methyl mercury present.

In Canada, Zitko et al (1971) measured the methyl mercury content of freshwater fish from New Brunswick and marine fishes from the Bay of Fundy, and found that the average percentage of the methylated form was 86% but in one species, the level of methyl mercury had not changed in forty-six years. This was determined by comparing values of the freshly caught eels from a New Brunswick lake with those of museum specimens taken from the same lake forty-six years before.

In Japan, Suzuki et al (1973) measured the mercury content in the muscles, liver, brain and kidneys of thirteen species of marine fish, and in each tissue the mercury content was more than 50% methylated.

In the previous accounts the total mercury contents are on the whole fairly low, but in Sweden, the problem with methyl mercury has been much greater. In freshwater fish the levels of mercury found have been quite high. For example, in pike levels of 5 - 8 ppm have been measured and 1% of the lakes and rivers have been declared too highly polluted for the fish in them to be fit for human consumption. [This is on the basis of sampling five pike, and if the levels found in the pike are above 1 ppm that particular river or lake is blacklisted. The public are advised to restrict to once a week consumption of fish from waters where mercury levels in the pike are between 0.5 and 1 ppm.] Most of this mercury is in the form of methyl mercury (Anon 1971; Westoo 1966; Westoo and Rydalv 1969, 1971.)

On the other hand there are reports of much lower methyl mercury proportions of the total mercury. Ueda et al (1971) did a nationwide survey of freshwater and marine fish in Japan. Of all the levels of methyl mercury measured the highest percentage was 65% but the average value was closer to 33%, with levels as low as 4%.

Berglund et al (1971) report that Japanese levels of methyl mercury were far lower than Swedish values; they ranged from 0 - 75% with a mean of about 25% (Kitamura. Pers comm to Berglund et al. 1971).

In Canada, Bache et al (1971) found that the content of mercury in lake trout, Salvelinus namaycush increased with age as did the methyl mercury proportion of the total content. The methyl mercury was less than 50% until the trout were three years old.

Amongst certain pelagic and inshore fish caught for human consumption around Hawaii, the Pacific Blue Marlin, Makaira ampla, had the highest mercury content at 4.78 ppm. Only 0.93 ppm was organic mercury (Rivers, Pearson and Schultz, 1972). This high content was

thought to be due to local volcanic activity.

Gaskin, Ishida and Frank (1972) found high levels of inorganic mercury in the liver, but with a higher proportion of the mercury in the muscles as methyl mercury in the harbour porpoise, Phocaena phocaena in the Bay of Fundy region of Canada. The levels varied widely: muscle values ranged from 0.21 - 2.58 ppm (100% methylated) and liver values were from 0.89 - 91.30 ppm (7.4 - 41% methylated). The lowest percentage of the methyl form was found in the highest total mercury contents. Similar findings were made in the short finned pilot whale, Globicephala macrorhyncha and the long snouted dolphin, Stenella (longirostris?)(sic) (Gaskin et al, 1974). Total mercury levels were high ranging from 1.33 - 5.36 ppm in muscle (42 - 100% methylated), 2.28 - 14.00 ppm in kidney (14% methylated) and 13.00 - 157.00 ppm in the liver (2 - 17% methylated).

Noren and Westoo (1967) found high levels of methylated mercury in freshwater fish in Sweden, but only very small amounts in marine fish.

Sources of methyl mercury

In view of the variability of the findings when methyl mercury content is measured, it is important to know what the source of methyl mercury might be. In Sweden the problem of high methyl mercury contents is particularly pronounced and also where most research on the problem has been done. The sources of the methyl mercury pollution in Sweden can therefore be usefully considered here.

i) Methyl mercury and ethyl mercury compounds have been used since

the 1930's as fungicides for treating agricultural crops (Riessanen and Miettinen, 1972). Therefore one possible source of methyl mercury is from run-off from agricultural land. This is not a generally accepted view since it has been calculated that Swedish soil receives about 1.2 g Hg/ha annually due to rainfall, and this is approximately the same amount as that contributed by agriculture; this contribution then being negligible in comparison to the amount of mercury contributed by sewage effluent (Andersson, 1967). However, Johnels et al (1967) found higher mercury levels in pike, Esox lucius, from agricultural areas (100 - 300 ppb) than in areas believed to be uninfluenced by human activities involving mercury (60 - 140 ppb). Industrial areas gave much higher levels (450 - 2500 ppb). Since methyl mercury has such a long half-life in fish, eg for flounder, pike and eel the excretion rate of methyl mercury ranged from $0.5X10^{-3}$ to $1.1X10^{-3}$ day^{-1} (Jarvenpaa et al, 1970) and has a far greater concentration factor than inorganic mercury (Hannerz, 1968) the agricultural source of methyl mercury might account for the high levels found in fish. Methyl mercury is also more stable in the environment (Berglund et al, 1971).

ii) Another source of methyl mercury could be from industry as at Minamata in Japan. There appears to be no evidence however for such a possibility in Sweden.

iii) This does not exclude industrial sources as an indirect cause since the other possible origin of the methyl mercury is from bacterial conversion of inorganic mercury to the methyl form. The most common source of mercury in any form in Sweden is from the pulp mills which from 1946 to 1966 used phenyl mercury as a slimicide in the process of making

paper. The phenyl-mercury bond unlike the methyl-mercury bond, is easily broken down to inorganic mercury and can then be converted to organic mercury by bacterial action. The use of both methyl mercury as a fungicide and phenyl mercury as a slimicide was banned in 1966.

Since there is an element of doubt as to whether the source of methyl mercury in fresh water is from agricultural uses of methyl mercury or from the conversion of inorganic mercury (derived probably from phenyl mercury) into methyl mercury by bacterial action, evidence for this conversion should perhaps be sought in environments where these two possibilities do not arise. Such an environment would be the sea. However evidence for this conversion derives mainly from experimental work.

Conversion of inorganic mercury to methyl mercury

Jensen and Jernelov (1967, 1969) showed that biological methylation occurred in organic sediments from aquaria, freshwater and coastal water of Sweden. The amounts of methyl mercury produced under the experimental conditions were not large. In sediments to which 100 ppm inorganic mercury were added 0.018 ppm - 0.440 ppm of methylated mercury were produced within ten days. Wood, Kennedy and Rosen (1968) showed that cell free extracts of a methanogenic bacteria could convert inorganic divalent mercury to methyl mercury in the presence of methylcobalamin (B_{12}-CH_3) and ATP, in a mild reducing atmosphere.

Evidence that this conversion can occur without the presence of bacteria and in metazoans has been sought.

Kramer and Neidhart (1974) suggest that methyl mercury is formed

from inorganic mercury in the fish, Poecilia reticulata. Their reasoning behind this is that administered inorganic mercury is released from the fish in two phases: one with a half-life of 4.2 ± 0.3 days and the other with a half-life of 67.7 days ± 1.8 days. The half-life of methyl mercury in the fish was 69.1 ± 2.4 days. They therefore suggest that the inorganic mercury must be converted to methyl mercury. Alternative explanations for this finding have already been discussed (page 19).

Evidence that is apparently more positive is provided by Kiwimae et al (1969). They found that after administration of wheat treated either with mercuric nitrate, phenyl mercury hydroxide, or methoxyethyl hydroxide, to white leghorn hens, a proportion of the mercury compounds was transformed into methyl mercury in the hens. This would seem to prove that such a conversion can occur in a living organism above the bacterial level. However, although the percentages of methyl mercury in the blood and muscle are high after the administration of mercuric nitrate (80% and 85% respectively) the total amounts are only 0.2 ppm and 0.16 ppm. In the liver and the kidneys where there are high levels of mercury (2.65 ppm and 3.17 ppm) the percentages are only 12% and 9% respectively. The levels in the blood and muscle are hardly above background level

Since analyses by the National Institute of Public Health in Sweden since 1966 have shown that most of the mercury in hens' eggs is in the form of methyl mercury.

These findings are often quoted in the literature. Less often quoted is the fact that Westoo (1971) was not able to repeat these experiments. Also Stoewsand et al (1971) failed to corroborate their findings. Saha, Summer and Atton (1971) fed mercuric nitrate, mercuric

chloride andphenyl mercuric acetate to rainbow trout for two weeks and could find no evidence of a conversion to the methyl form.

Also often quoted is the finding that liver homogenate would convert inorganic mercury to organic mercury (Westoo, 1968). But this was found to occur when excess mercuric ions were added to an acidified aqueous suspension of liver and left overnight. It was found that the methyl mercury content of the liver increased after this treatment showing that inorganic mercury had been methylated.

Imura, Pan and Ukita (1972) made a comparative study of the activity of liver homogenates of seven species of fish and four mammalian species and their ability to form methyl mercury from mercuric chloride. They found that, compared with other samples tested, the livers of three types of tuna fish, the yellow fin tuna, Thunnus albacares, the bigeye tuna, Thunnus obesus, and the albacore, Thunnus alaunga, had remarkable abilities to methylate inorganic mercury. These results may be significant when it is considered that tuna fish meat has been shown to contain higher levels of methyl mercury than other sea foods (Aoki, 1970). They also found that mammalian livers had the ability to methylate inorganic mercury, but not to the same extent. Of these, the bovine liver had the most pronounced ability.

This latter example therefore seems to be the only real evidence that such a conversion can take place in animals.

Bertilsson and Neujahr (1971) produce further conflicting evidence. They found that thiols and cell proteins inhibit the methylation of mercuric chloride by methylcobalamin, the substance implicated in the transfer (Imura et al, 1971; Wood, Kennedy and Rosen, 1968). This inhibition is probably due to the thiols and cell proteins binding the mercury and making it unavailable for methylation. They suggest that a different mechanism may act in vivo than in vitro. Unless this is so, all the

evidence for such a conversion occurring in the environment looks much weaker.

There is thus no positive evidence for this conversion taking place but it is still a definite possibility. At the same time however the less discussed conversion from methyl mercury to inorganic mercury might be taking place.

Conversion from methyl mercury to inorganic mercury

A bacterial strain belonging to the Pseudomonas genus was shown to be capable of converting both inorganic and organic mercury into metallic mercury and the corresponding hydrocarbons (eg methyl mercury can be converted to methane and metallic mercury (Tonomura et al, 1968; Furukawa, Suzuki and Tonomura, 1969.)

Weiner, Levy and Mudge (1962) found that breakdown of organic mercurials proceeded more slowly in aqueous solution than in the presence of thiol containing compounds (cf Bertilsson and Neujahr, 1971; page 37).

Clarkson (1969) showed that biotransformation of seven types of organomercurials to inorganic mercury could occur but of those compounds investigated, methyl mercury was the most slowly metabolized. Norseth and Clarkson (1970) showed that after intravenous administration of radioactively-labelled methylmercuric chloride in the rat, inorganic mercury was found in all the tissues except the blood, where the mercury was almost completely bound in the red blood cells and not available for conversion.

Renfro et al (1974) also provides positive evidence of this breakdown of methyl mercury to inorganic mercury, as already mentioned. By following the fate of ^{14}C labelled methylmercuric chloride and comparing it with that of the ^{203}Hg labelled variety after absorption in to fish, they found that the mercury bond of the methyl mercury was rapidly split after uptake into the gills. The ^{203}Hg level remained high in the gills for a longer time than the ^{14}C label. This suggested that the methyl mercury was metabolized within the animal resulting in the production of inorganic mercury.

There are therefore two comparable processes, both known from experimental work, but with no definite evidence that one is more likely to occur than the other. Yet this dichotomy is seldom reported in the literature. There appears to be a bias towards reporting the conversion to the methyl form. For example, Jensen and Jernelov (1969) and Wood, Kennedy and Rosen (1968) are very often cited whereas Tonomura et al (1968) are cited less frequently. The work of Kiwimae et al (1969) is also often reported, but not the contra-evidence presented by Westoo (1971) and Stoewsand et al (1971). The bias extends apparently to failure to distinguish between the marine and freshwater environment. For example, Zitko et al (1970) report that fish contain 86% of total mercury as methyl mercury but the levels of mercury in the fish they sampled is vastly different. The average value for freshwater fish is 0.58 ppm and for marine fish it is only 0.05 ppm. Westoo (1966) reports high levels of methyl mercury in freshwater fish but only small amounts in marine fish, yet this is often reported as high levels in general (Lofroth 1970). Also it is rarely reported that Fujiki (1972) describes the mercury content of Minamata Bay as consisting chiefly of mercuric oxide and mercuric sulphide and that the highest concentration of

methyl mercury was 0.007 ppm. This was after effluent containing up to 140 ppm methyl mercury in an overall level of up to 366 ppm had been discharging into the Bay for a number of years. There is therefore no evidence that conversion to methyl mercury is occurring. Anaerobic bacteria that would be necessary for such a conversion were not found in the mud containing the methyl mercury. Since 1968 when the acetaldehyde plant was closed the methyl mercury content of the fish and shellfish in the Bay has decreased. Since the total mercury has not decreased significantly in the muds, methyl mercury cannot be being formed.

One reason for concern over this bias towards methyl mercury is that the analysis of material for methyl mercury is a difficult process. Saha (1972) presents a comparison between different analysis methods for total mercury and shows what limitations there are in analytical methods and human abilities, particularly with levels of less than 0.1 ppm. Gurba (1970) compares the results from two laboratories using neutron activitation analysis (NAA - this method of analysis will be described later). The results are extremely variable, but this variability is only found with low levels (less than 0.1 ppm). NAA is an extremely straightforward method whereas the methyl mercury determination is an extremely complex method (Westoo 1966, 1967, 1968). In the earliest method it involved a 30% correction factor for unfavourable partition coefficients of methyl mercury. This must lead to inaccuracies at low levels yet percentages of methyl mercury are quoted even when total mercury contents are extremely low. For example, Leatherland et al (1973) quote levels of over 90% methyl mercury in two Atlantic species while the total mercury contents were only 0.33 ppm and 0.16 ppm dry weight. It would seem that there is a possibility of miscalculation of the methyl

mercury proportion but this is not acknowledged. Uthe, Armstrong and Tam (1971) found indications of bias operating when they investigated the results for mercury analysis sent to twenty nine different laboratories. Results were widely disparate, but they indicated that many laboratories were conditioned to the 0.5 ppm level set as a guideline for judging fitness for human consumption. Such a finding is disturbing in view of the apparent bias towards methyl mercury in the literature.

These problems of analysis of methyl mercury are illustrated by Berglund et al (1971). They report how analyses of samples exchanged between Swedish and Japanese workers gave very disparate results. The Japanese gave consistently lower levels of methyl mercury as a percentage of the total mercury content. Westoo and Rydalv (1979) publish a table of the spread of the percentages of methyl mercury as part of the total mercury content and the most common value for methyl mercury content is 96 - 100% and the spread of percentages goes as high as 135%. This may suggest that Swedish levels are on the high side. Westoo suggests that the extraction methods used by the Japanese are not efficient, and that not all the methyl mercury present in a sample is measured (Westoo 1968; Kitamura et al 1966; Sumino 1968).

It can be seen that there are problems with the occurrence of mercury in the environment and the form that it takes. The purpose of this review is not to cast doubt on results from investigations of methyl mercury, but to suggest that there may be other explanations for the occurrence of methyl mercury in the environment than the commonly accepted view that it is formed by bacterial action; that possibly methyl mercury may not be such a great problem as generally believed, and that the results obtained from such enquiries be looked at without bias, and with a view to the different possibilities, and not just to confirm the

theory that methyl mercury is being formed in the environment.

In this study no analysis for methyl mercury has been carried out nor any experiments on the uptake of this compound. Since the techniques for studying methyl mercury are so complicated and in view of the conflicting evidence on this subject, analyses of this compound are best left to expert chemists. It may be that much of the controversy is due to inexpert analysis. Another reason for not studying methyl mercury is that it has not been shown to occur in the marine environment. to such a great extent as in certain freshwater ones. In the rush to study methyl mercury, inorganic mercury may be ignored and since it is the natural source ofmercury in the environment and because slight variations in the background levels due to other sources of mercury could affect the animals living in that environment, it is inorganic mercury that has been examined in this study.

From the examination of methods of studying mercury pollution, it can be seen that there are many possible approaches to be made. As already said, pollution as a subject traversing two scientific disciplines, calls for a varied approach to the problem so that all factors can be considered. Such an approach is exemplified by Fujiya (1965) who investigated numerous physiological parameters in his studies on the effects of Kraft pulp mill waste. In this study, however, the approach has been kept simple in order to make the findings as generally relevant as possible.

Methods of study used

The approach to the examination of the effects of mercury pollution in Homarus gammarus in this study is on various levels. It is first important to know what sort of accumulation of the metal is found

in the animal in nature. Some measurements have been made as already mentioned (page 7) but these are few in number and may in any case reflect regional variations. Since these background levels may be very low, an extremely sensitive analytical technique is required. This problem will be referred to later. The location of the metal in the tissues is already important as pointed out (page 14), since it may suggest whether the animal is able to control the level in the body, and suggest how it is doing this, or whether the metal accumulates in a particular tissue, which may be of relevance in an important food species.

These findings can then be amplified by studying the uptake from known concentrations of the metal for different periods of time, which should again give information on how the animal is controlling the levels. This may also call for sensitive analysis since the metal may remain at low concentration in some tissues whilst building up to high levels in others. Such a study may show that there are different control systems for different levels of exposure, and may also indicate the levels of exposure that the lobsters used for the background determinations have been subject to. Mount and Stephan (1967) showed that the past history of a fish's exposure to cadmium could be deduced from the distribution of the metal in its tissues.

Uptake via the food chain would also be an important factor to investigate particularly for a benthic omnivore such as the lobster. This was unfortunately not possible in this study because of restricted analysis facilities.

Uptake experiments carried out over a period of time will give

a static picture of concentrations in tissues resulting from controlled exposure but may not show the route of uptake. For example, uptake may occur through the stomach into the digestive gland while no actual accumulation occurs in the gland. Sparks (1972) suggests that cell damage due to heavy metals and other poisons will occur at sites of uptake and loss. Therefore if uptake is occurring in a region where accumulation does not also occur, then this area of likely cell damage might not be considered. It is therefore necessary to follow uptake over a short period of time to determine the sites of entry. This has been done in this study using radioactive tracers. Sites of exit are also important, since they too are susceptible to damage. It must first be determined if the animal is actually able to excrete the metal and at what rate, since then its potential for controlling its body levels can be understood. This has been investigated in the lobster by using radioactive isotopes and measuring the excretion of mercury in the urine.

An assessment of the long term effects of the sub-lethal levels is also essential since cellular changes induced by the metal, while not actually causing death, may cause damage that could affect the viability of the animal over a long period. Such effects might be seen with light microscopy, but more subtle changes might only be visible using the electron microscope. This damage is to be expected at sites of uptake and excretion and therefore following identification of these sites by the foregoing methods, they were examined by light microscopy and then additional studies were done using the electron microscope.

From a study of the literature the other necessity for the complete study of a pollutant is to look for possible weak links in the chain of the life cycle. Other workers have found larval stages considerably more susceptible to pollutants. The larval development in the lobster was therefore considered in this study. Other likely weak links in the Crustacea are animals about to moult. Wilson and Connor (1972) showed that shrimps were more susceptible to mercury and cadmium at this time. It is therefore a factor that needs studying, but usually in St Andrews only a few of the lobsters kept in the Aquarium ever moult, and only an exceptionally hot summer in 1975 made comments on the effect of mercury on pre-moulting lobsters possible.

Before giving a detailed plan of the course of study, there are two factors that have not been dealt with. They are both concerned with the difficulties of dealing with mercury as a pollutant.

As already shown the natural and polluted levels in the marine environment are both extremely low. Concentrations of mercury used in experiments to study mercury pollution therefore need to be realistically small. This fact immediately raises a problem: mercury is an extremely volatile substance having a vapour pressure that is relatively high ($8X10^{-3}$ Torr at 40^0C; $270X10^{-3}$ Torr at 100^0C). Mercury is readily lost from solutions with concentrations of less than 200 ppb (Newton and Ellis 1974). Coyne and Collins (1972) found that within three days, ninety per cent of the mercury (as mercuric chloride) was lost from an untreated sample of creek water. Corner and Rigler (1957) found that all the mercury (also as mercuric chloride) in a 100 ppb

solution was lost after four days. If the sea water was also enriched with nutrients that promoted bacterial growth, loss occurred at a greater rate.

When planning experiments with mercury a compromise has to be reached between two possible approaches: firstly, choosing realistically low levels of mercury for experimental solutions, which will require constant changing to maintain the initial concentrations, or the use of a constant flow mechanism, or secondly, working at slightly higher concentrations and taking into account the fact that the levels will be falling. In this study this latter approach was most often used.

The fact that mercury is volatile also makes for a difficulty in analysing material for the element. Loss can occur very easily with a subsequent loss in accuracy. The method of analysis chosen must be the one best suited to the particular analysis problem given. There are several methods of analysing for mercury but each one is not without its disadvantages. The three main nethods are:

a) The spectrophotometric method of determination, using the dithizone complex. This uses the characteristic of mercury to have a strong affinity for sulphur compounds

b) Atomic absorption, which utilises the volatile nature of mercury. Mercury is rendered into the elemental state by appropriate pre-treatment and then rapidly separated from other substances by a stream of air being bubbled through the medium. The amount of mercury vaporized in the stream of air is then measured by flameless atomic absorption.

c) The third method is that of neutron activation analysis (NAA). This uses the property of mercury that, when exposed to neutrons, the stable isotopes are converted to radioactive species, the emissions of

which can be measured and compared with those of a known amount of mercury.

In this study several factors had to be borne in mind. Since the aim was to determine both background levels of mercury in the lobster and those levels resulting from experimental exposure to mercury, it was necessary to use a method with a very high sensitivity. It also had to be remembered that the green glands, the chief excretory organs of the lobster, are extremely small; in some lobsters only 0.7 grams of this tissue was available for analysis. These are particularly important tissues in the study of mercury as they may be a major site for the control of mercury levels in the body. Their small size rules out the use of the dithizone method which requires large samples (10 g) to achieve accuracy with low mercury contents (Anon. 1971). Atomic absorption, while it does possess great sensitivity, is not very precise or accurate and its great advantage is that it can be used to give fairly speedy, cheap analyses of many samples.

In this study what was required was the accurate analysis of relatively few samples. For this purpose neutron activation analysis was the method of choice.

Neutron activation analysis

Neutron activation analysis (NAA) is a highly specific analysis technique. In it, a sample of mercury is bombarded with neutrons in a nuclear reactor and the naturally stable isotope ^{196}Hg is converted to the radioactive isotope ^{197}Hg with a half life of sixty five hours. This isotope emits a characteristic gamma radiation (77.6 KeV, 191.4 KeV, 269.1 KeV) together with gold X-rays (67.0 KeV, 68.8 KeV,

77.9 KeV and 80.1 KeV). These X-rays can be counted on a low energy photon detector (LEPD) with multi-channel analyser. By comparison with the emission from a known amount of mercury given the same treatment, the amount of mercury in the sample can be accurately determined. As it is the nucleus that is involved in the reaction, it is independent of the chemical state of the element. Therefore it is the total mercury that is determined.

The method of NAA used in this study was based on that of Johansen and Steinnes (1969). The sample for analysis is placed in a silica ampoule which is sealed prior to thermal neutron bombardment. Westmark and Sjostrand (1960) showed how easily loss of mercury could occur unless the mercury is in a sealed container as described here, or kept as mercuric sulphide below 80^0C. For greater sensitivity the samples are chemically digested after activation. This allows greater sensitivity as there will be no shielding by extraneous material, and also cuts out inaccuracies due to variations in tissue quantities and characteristics, and the geometry of the same (ie different tissues will be distributed differently in the ampoule and as counting efficiency is related to distance from the counter, results will be variable unless the samples are uniform).

One of the great advantages of NAA is to be found in this chemical separation process. Loss of mercury can occur very easily and in the other two methods described previously there can be no allowance made for any loss that may occur. This limits the accuracy of these techniques unless the analysis conditions are extremely stringent. NAA, on the other hand, does allow a correction for loss. This is done by the addition of non radio-active carrier mercury prior to chemical separation. An accurately known amount of mercury is added to the sample, such that the

small amount of mercury in the sample is negligible in comparison to the added amount. After the separation has been done the amount of carrier mercury remaining is determined by weighing and since the sample mercury will have been lost in the same proportion as the carrier mercury a correction can be made for any loss that has occurred.

NAA has been the method of choice in Sweden where possibly the most environmental mercury measurements have been made (Anon 1971). It has however not often been used for measurements of mercury accumulated in animals after controlled exposures and in view of its accuracy, Berlin (1972) pointed out that this lack should be rectified.

The inability to distinguish between different forms of mercury with this method is a pity in view of the significance of methyl mercury. However, neither of the other two methods enables this distinction to be made.

In addition to the great advantages of accuracy and precision, there are practical difficulties associated with NAA. One disadvantage is that as mercury must on no account escape in to the reactor as it can cause severe contamination, each ampoule has to be of silica, and after sealing each ampoule has to be pressure tested to ensure that it will not explode in the reactor. This is done by boiling the ampoules in water for half an hour, and ensuring that no weight change is detected in any ampoule, which would show that that ampoule was damaged. Each ampoule has to be individually blown and hand sealed, which makes for an additional expense. After the activation, each sample is individually chemically separated and this is also time-consuming. In this study, each lobster had to be taken individually to the Scottish Universities Reactor Centre, East Kilbride, to be

activated and analysed. Only one lobster could be done at a time because with the short half-life of the isotope being measured, samples had to be separated before the samples had decayed for too long. There were therefore restrictions on the planning of the experiments since the reactor, the glass blower and the laboratory facilities were not necessarily all available at the same time. (The numbers of experiments that could be done using this technique were also limited by personal expenses involved in working in East Kilbride.)

Thus, the overall plan of work for this study has been

a) to carry out accurate analysis of background levels of mercury found in the tissues of lobsters local to St Andrews, using neutron activation analysis

b) to carry out analysis of levels of mercury in different tissues resulting from exposure to known concentrations of mercury for certain periods of time, also using neutron activation analysis

c) to do short term uptake studies to determine the route of uptake into the body using radio-isotope tracers

d) to determine if the lobster is able to excrete mercury, and if so at what rate

e) to study the uptake into the lobster larvae using X-ray microanalysis and the effects of mercury on the development of the larvae

f) to examine histologically thetissues most likely to be affected by long term exposure to low levels of mercury and to amplify this work where necessary with electron microscopic examination.

EXPERIMENTAL INVESTIGATIONS

General method for the treatment of the adult lobsters during all experimental procedures

General care

Lobsters caught around the coast from St Andrews were kept in large circulation tanks prior to experimental treatments. In these tanks they were fed on fish every two or three days.

During all experimental treatments, the lobsters were kept either individually or in pairs, separated from each other by a perspex partition, in square, twenty litre, fibre glass tanks. The animals were not fed during experiments. Without running water to clear away debris after feeding, there are considerable problems in keeping the water sufficiently aerated, even with the use of air stones which were routinely kept in the tanks. The alternative would have been to have used different tanks for feeding but this was not possible. However, Stewart et al (1967) kept the american lobster, Homarus americanus, for 140-149 days without food and found no difference in mortality between fed and starved groups. No experiments in this study lasted for more than fifty days, and the common exposure times were either a week, or thirty days. More importantly, in uptake experiments it is necessary to know the source of the metal. Food can contain significant amounts of mercury (Kennedy and Smith, 1972, 1973). Uptake from food is an important variable but one that was not investigated in this study.

Exposure to mercury

Mercuric chloride, taken from a concentrated stock solution made up in distilled water, was used to give levels of mercury in the experimental tanks of 100 ppb and 10 ppb. The 100 ppb solutions were changed every alternate day and the 10 ppb solutions were changed every day. As mentioned before, loss would have occurred from these dilute solutions, and although this loss was minimised by the frequent changing of the solutions, these values for the concentrations must be regarded as maximum values only. The experimental animals could generally live healthily in these solutions for thirty days without outward signs of any illeffects due to the mercury. These levels are therefore not acutely toxic but there were found to be long term effects associated with this level of exposure. (Some lobsters did not survive for long at the 100 ppb level of exposure and this appeared to be associated with the inability to produce urine. This will be referred to later.)

A constant flow device was used in the experiment with lobster larvae. However the peristaltic pump available only allowed a differential rate of pumping of sixty four to one between the sea water and the mercury solution. This meant that the mercury solution had to be more dilute than the level from which loss would have been minimal. There was therefore no advantage in using this system for the adult lobsters.

a & b) Determination of background levels of inorganic mercury in local lobsters, and of the uptake after exposure to different levels of inorganic mercury for different periods of time using neutron activation analysis

Material and methods

Six intermoult lobsters were used in these experiments. The experiments performed were:

Experiment I	:	Lobster exposed to 100 ppb mercury for seven days
Experiment II	:	Lobster not exposed to mercury; to determine background levels
Experiment III	:	As Experiment II
Experiment IV	:	Lobster exposed to 100 ppb mercury for seven days
Experiment V	:	Lobster exposed to 100 ppb mercury for twenty four hours, and then kept in fresh sea water for six days
Experiment VI	:	Lobster exposed to 10 ppb for fourteen days

The lobsters were taken individually to the Scottish Universities Research and Reactor Centre, East Kilbride, wrapped in paper towels soaked in sea water and kept on ice in a polystyrene carrying box.

Two 2 ml samples of blood were taken from the tail of the lobster, with a hypodermic syringe and placed directly in silica ampoules made in the Centre. These ampoules had been previously washed in concentrated nitric acid and rinsed with distilled water and allowed to dry thoroughly before being accurately weighed. The lobster was then killed and two replicate samples of up to two grams in weight were taken of the digestive gland, tail muscle, and gills. (Gills were not analysed in Experiment I.) One sample was made up of the two green

glands. In Experiment II a sample of intestine was analysed. With female lobsters, (Experiments III and IV) samples of ovary were taken for analysis. Two mercury standards were made containing one microgram of mercury each. Two further ampoules contained samples of Fish Solubles provided by the International Atomic Agency, and samples of this were analysed each time (except in Experiment I) to provide comparisons between the different experiments. Samples of each tissue, except the green glands, were also taken to be dried in order to make calculations of dry weight concentrations of mercury. (The dry weight values for the concentration of mercury were determined for comparison, but only the wet weight values will be referred to in the text. There is debate about which system of expressing concentration should be used. Wet weight values may vary seasonally, particularly in some invertebrate species. However, since it is the wet weight value that is most relevant with regards to human consumption, and is also the more commonly used system, it is the one used in this study.) The ampoules were reweighed so that the weight of each tissue sample was accurately known. The ampoules were then sealed and pressure tested as described earlier. The samples were irradiated at a flux of 3.6×10^{12} neutrons/sq cm/sec for six hours. After irradiation they were left for three days to allow the short lived isotopes to decay and then the samples were chemically separated by the method of Johansen and Steinnes (1969).

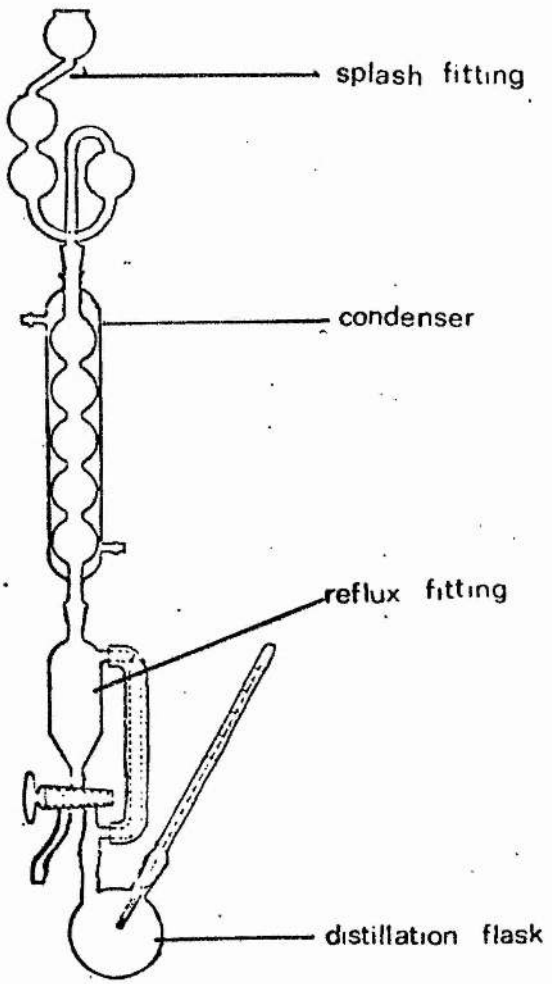
The ampoules were first washed in dilute nitric acid and distilled water to remove any surface contamination. All the ampoules were then kept in liquid nitrogen. (Samples were occasionally lost at this stage. In Experiment VI an accident prior to the samples being put in the reactor caused loss of most of the replicates.) The ampoule containing

the sample to be separated was then wrapped in a small square of 'Benchkote', (a plasticised non-absorbent paper). The ampoule was then broken inside this 'parcel' and the pieces were transferred as quickly as possible into a small distillation flask. The 'Benchkote' was rinsed with 15 ml 7N sulphuric acid into the flask so that no part of the sample was lost. Since the sample was in frozen pieces this complete transfer was easily ensured. 5 ml of concentrated nitric acid, 1 ml concentrated orthophosphoric acid, and 25 mg of carrier mercury were added to the flask.

The distillation flask was fitted in to the reflux apparatus (see diagram 1) and was heated on a Kjeldahl heater apparatus to 300^0C approximately, or until white fumes were forming. After cooling of the distillation flask, the distillate in the reflux condenser was returned to the flask. This was filtered into a 500 ml beaker and the apparatus was well rinsed into the beaker with distilled water. The pH of the solution was adjusted to 8 - 9 by the addition of ammonium hydroxide. Thioacetamide was then added and the solution gently warmed to precipitate the mercury as mercuric sulphide. [This is a form of mercury that is not volatile under 80^0C (Westermark and Sjostrand, 1960).] On cooling the precipitate was filtered on to a small planchette filter (Whatman's 2.1 cm glass fibre papers) using a vacuum pump. The precipitate was allowed to dry and then weighed. Correction could be made for any loss from the 25 mg of mercury carrier, allowing for the sulphide also present. The correction for loss of mercury during the chemical separation can only be done accurately if the precipitate obtained at the end of the procedure is pure mercuric sulphide. In

Diagram 1

Apparatus for chemical separation



order to test the purity of the samples obtained in these experiments, precipitates obtained in NAA experiments V and VI were analysed by the atomic absorption method.

The samples were dissolved in Aqua Regia and then made up to 100 ml in distilled water and filtered. Mercury standards for different levels around the 25 mg/100 ml level were made up and samples and standards were run through a Perkin Elmer Atomic Absorption Analyser. The results are given with the NAA results, Table I.

The precipitates were then counted on low energy photon detectors (LEPD), with multi-channel analysers. By a comparison of the peak areas for any of the gold X-rays for the sample with the same peaks for the mercury standards the amount of mercury in the samples could be determined. The calculation of peak areas was done by programmable calculator, or computer. (With the East Kilbride counter it was possible to use a programmable calculator. This counter was out of order for much of the work and the detector of the Geology department, University of St Andrews had to be used. With this the peak areas had to be calculated by computer and the rest of the calculations were done manually.)

The results from Experiment I were suspect because of inefficiency with the separation technique. (The results for this experiment have therefore been given as calculated for counting the undigested samples.) This process was therefore practised before the other five experiments were carried out so that human error as a source of inaccuracy could be cut down as much as possible.

TABLE I

Results from Neutron Activation Analysis

Experiment I

Lobster kept in 100 ppb mercury for seven days

Non-destructive analysis results

	Weight	Count corr for weight	ppm
Standard	1 g	32.66	1
Blood	2.2047	478.24	6.64 ± 0.27
Digestive gland	1.8017	749.95	12.73 ± 0.84
Muscle	2.2200	32.41	0.44 ± 0.23
Green gland	0.4894	1285.35	80.42 ± 3.87

Experiment II

Unpolluted lobster

Sample	Weight	Weight of Precipitate	Chemical Yield by weight	Chemical Yield by A.A.	Counts corrected for C.Y. and tissue weight	ppm	ppm dry weight
Mercury Std 1	1µg	0.0245	0.8284		2929.93	1	
Mercury Std 2	1µg	0.0178	0.6019		2676.20	1	
Blood	2.1860	0.0408	1.3795		27.86	0.009	0.08
Digestive gland 1	1.8989	0.0342	1.1564		97.06	0.035	0.08
Digestive gland 2	1.6217	0.0104	0.3516		1075.08	0.383	0.91
Muscle 1	1.1517	0.0227	0.7675		256.81	0.092	0.37
Muscle 2	1.2283	0.0241	0.8149		244.77	0.087	0.35
Green gland	0.3870	0.0247	0.8352		108.28	0.039	
Gill	1.0577	0.0310	1.0482		587.18	0.209	1.29
Intestine	0.8086	0.6055	3.57		866.93	0.309	
Fish standard	0.1947	0.0235	0.79		180.89	0.065	

Experiment III

Unpolluted lobster. Female. 405 grams

Sample	Weight	Weight of Precipitate	Chemical Yield by weight	Chemical Yield by A.A.	Counts corrected for C.Y. and tissue weight	ppm	ppm dry weight
Hg standard I	1µg	0.0336	1.14		3538 ± 114.7	1	
Hg standard II	1µg	0.0246	0.83		4482 ± 93.14	1	
Lobster larvae	0.5314	0.0217	0.73		86 ± 21.05	0.042 ± 0.010	
Blood	1.9787	0.064	2.16		41 ± 23.27	0.002 ± 0.001	0.027
Green gland	0.4485	0.0249	0.84		1791 ± 60.58	1.033 ± 0.043	
Digestive gland	1.2857	0.0233	0.79		1765 ± 96.90	0.38 ± 0.022	1.34
Digestive gland	1.3681	0.0244	0.83		1552 ± 92.7	0.301 ± 0.01	1.06
Ovary	0.5507	0.0298	1.01		3050 ± 87.40	1.203 ± 0.045	2.78
Gill	1.0677	0.0239	0.81		1392 ± 78.62	0.35 ± 0.021	2.95
Gill	0.9562	0.0160	0.54		2240 ± 93.70	0.941 ± 0.045	7.93
Muscle	1.2202	0.0240	0.81		6278 ± 126.11	1.386 ± 0.043	6.52
Fish standard	0.2064	0.0105	0.36		569.45±	0.391 ± 0.059	

Experiment IV

Female Lobster

Seven days in 100 ppb mercury

Sample	Weight	Weight of Precipitate	Chemical Yield by weight	Chemical Yield by Atomic Abs.	Counts corrected for C.Y. and tissue weight	ppm	ppm dry weight
Standard	1µg	0.0242	0.8104		661.39	1	
Standard	1µg	0.0245	0.8205		992.08	1	
Green gland	0.4195	0.0279	0.9343		27999.89	33.86 ± 7.59	
Ovary	0.5874	0.0257	0.8607		820.71	0.99 ± 0.22	1.30
Digestive gland	1.6822	0.0253	0.8472		18230.64	22.05 ± 5.13	68.22
Digestive gland	1.1799	0.0283	0.9477		22708.45	27.47 ± 6.03	84.99
Gill	1.1262	0.0297	0.9946		50935.99	61.61 ±16.03	449
Gill	0.7987	0.0303	1.01		52137.0	64.94 ±18.65	451.29
Blood	1.9467	0.0288	0.9645		3489.84	4.22 ± 0.95	57.34
Blood	2.1186	0.0349	1.1687		3505.0	4.31 ± 1.02	58.84
Muscle	1.3435	0.0242	0.8104		3547.87	4.29 ± 0.91	19.19
Muscle	1.3818	0.0295	0.9879		1527.0	1.90 ± 0.45	8.50
Fish A	0.4185	0.0279	0.93		68.28	0.08 ± 0.03	
Fish B	0.7831	0.0241	0.8071		93.70	0.11 ± 0.016	

Experiment V

Male lobster (356 grams) in 100 ppb for twenty four hours. Fresh sea water for six days

Sample	Weight	Weight of Precipitate	Chemical Yield by weight	Chemical Yield by Atomic Abs.	Counts corrected for C.Y. and tissue weight	ppm	ppm dry weight
Hg Standard I	1µg	0.0246	0.8484	0.84	1078.5	1	
Hg Standard II	1µg	0.0268	0.9242	0.92	933.78	1	
Green gland	0.6605	0.0256	0.8829	0.90	10383.17	10.32 ± 0.85	
Digestive gland	1.0724	0.0032	0.1098	0.12	1834.4	1.82 ± 0.18	7.6
Digestive gland	1.3010	0.0247	0.8519	0.92	1134.15	1.13 ± 0.12	4.73
Gill	0.5222	0.0235	0.8105	1.10	51795.29	51.48 ± 3.82	252.11
Gill	0.8003	$\frac{0.0423}{2}$	0.8932	1.32	43307.9	43.04 ± 2.86	210.77
Blood	2.0671	0.0250	0.8622	0.94	1496.42	1.49 ± 0.15	22.43
Blood	2.284	0.0223	0.7691	0.8	1387.89	1.38 ± 0.12	21.49
Muscle	0.827	0.0209	0.7208	0.8	1362.18	1.35 ± 0.13	6.61
Muscle	1.1579	0.0264	0.9105	0.90	692.42	0.69 ± 0.09	3.37
Fish Standard A	0.5456	0.0251	0.8657	0.82	167.26	0.16 ± 0.03	
Fish Standard B	0.6441	0.0267	0.9208	0.88	150.06	0.15 ± 0.06	

Experiment VI

Male lobster. 325 grams. 10 ppb for fourten days.

Sample	Weight	Weight of Precipitate	Chemical Yield by weight	Chemical Yield by Atomic Abs.	Counts corrected for weight and Chemical yield	ppm
Hg Standard	1µg	0.0294	0.99	0.88	1625.08	1
Hg Standard	1µg	0.0288	0.97	0.80	1547.06	1
Green gland	0.6207	0.0267	0.90	0.78	9030.27	5.69 ± 0.08
Gill	1.0064	0.0246	0.83	0.60	51614.0	32.54 ± 0.59
Muscle	0.7870	0.0235	0.79	0.64	1096.3	0.69 ± 0.01
Blood	2.0354	0.0268	0.91	0.96	128.1	0.08 ± 0.004
Blood	1.9682	0.0268	0.91	0.7	100.65	0.06 ± 0.003
Digestive gland	1.1726	0.0312	1.05	1.08	4394.44	2.77 ± 0.038
Fish standard	0.7721	0.03	1.01	0.86	178.49	0.11 ± 0.006

Figure 1

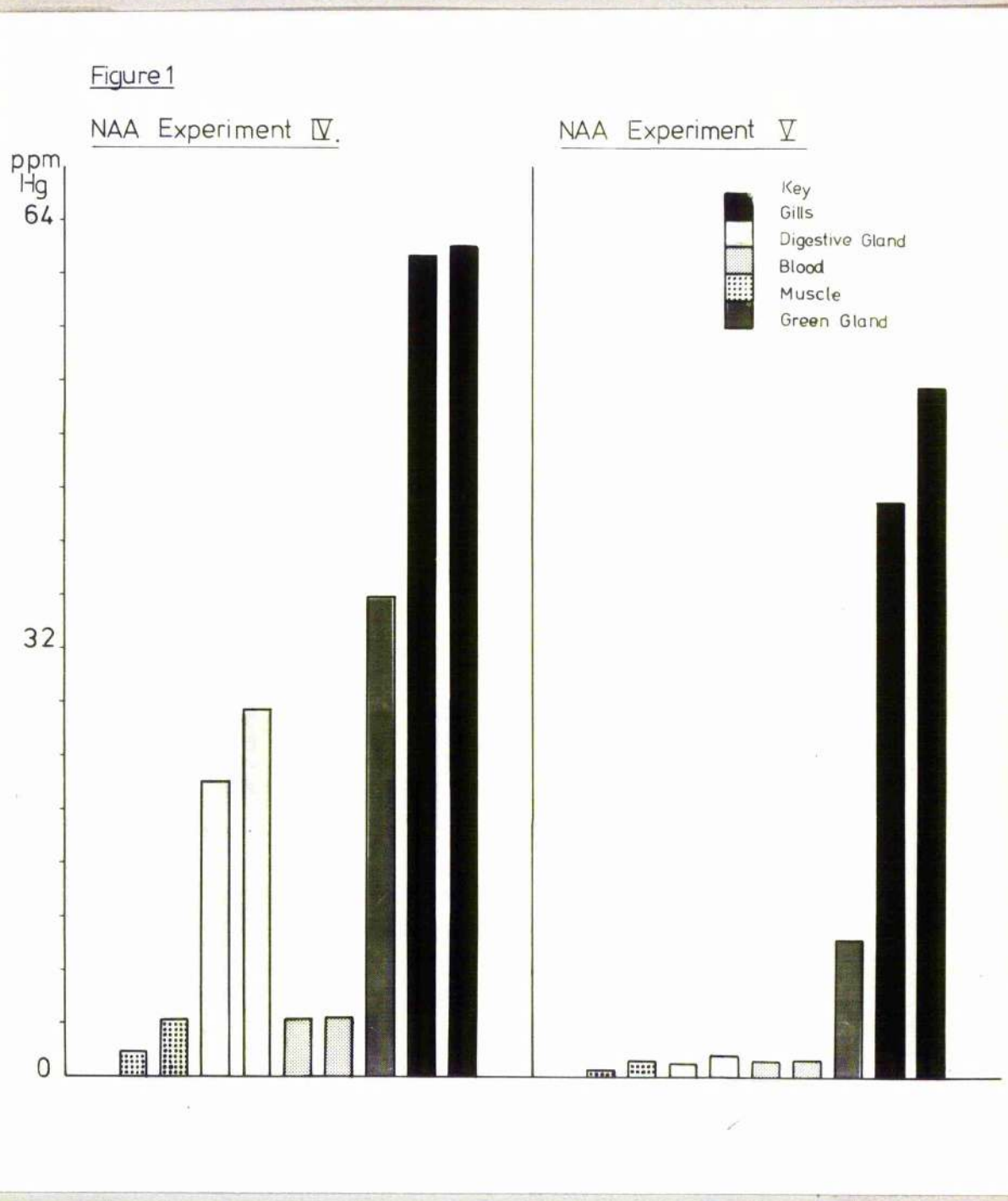
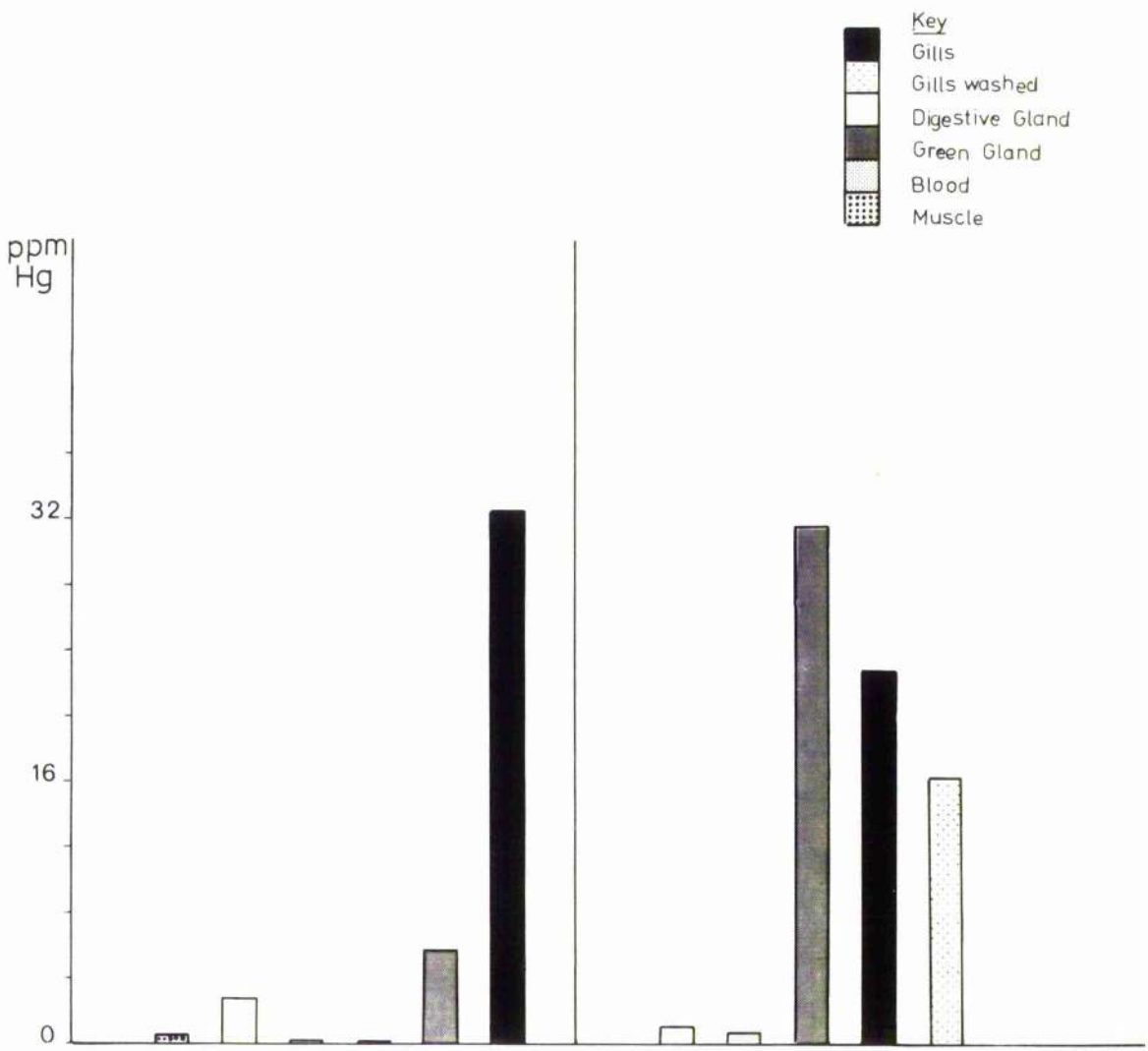

NAA Experiment IV.
NAA Experiment V
ppm Hg
64
32
0
Key
Gills
Digestive Gland
Blood
Muscle
Green Gland

Figure 2

NAA Experiment VI

Long term radio isotope tracer Experiment



Results

Since the investigations a, b, c and d, are all investigations of different aspects of the behaviour of mercury in lobsters, it has been convenient to discuss the results from each section separately, and then to discuss all the findings from each series of experiments together, in relation to other work, to assess the overall implications of these findings.

The results from the neutron activation analyses are presented in Table I and Figures 1 and 2.

It can be seen that while there is no particular pattern to mercury distribution, or constancy in background levels found in Experiments II and III, there is an overall pattern of distribution of mercury after exposure to the experimental solutions. The gills concentrate mercury to a considerable extent while the green glands concentrate it to a lesser extent but still considerably above the mercury levels in the experimental solutions. The digestive gland, blood and muscle appear to have a variable relationship to each other apparently dependent on the degree and length of exposure.

Discussion of results from neutron activation analysis

The results obtained using this method give an accurate picture of the background levels of mercury found in lobsters caught around St Andrews, and also, with the possible exception of Experiment I, of the concentrations of mercury resulting from various exposures to the metal. Unfortunately, due to the practical difficulties associated with

this method (ie distance from the Reactor Centre at East Kilbride and resulting expenses involved) only six lobsters were analysed. Since the overall patterns of accumulation were very similar, and because the resulting concentrations of mercury in the tissues varied with the concentration of mercury in the experimental solutions, and with time of exposure, it seems that the behaviour of mercury in lobsters follows a definite pattern.

There was however great variability found in the two unpolluted lobsters. In Experiment II the levels of mercury measured were extremely low, being less than 0.1 ppm in all tissues except the gill and digestive gland, where the levels were still less than 0.4 ppm. On the other hand, in Experiment III the levels were very much higher with the highest value in the muscle at 1.3 ppm. Ovary and green gland values were also high at 1.20 ppm and 1.0 ppm respectively. This is considerably higher than the 0.5 ppm limit to mercury content set for safe human consumption. Obviously these two lobsters are far too small a sample to deduce anything about natural background levels except that they can be variable. Since the previous history of the lobsters was not known, no explanation for these differences can be suggested.

Very little work has been done on the background levels of mercury in lobsters, but the findings from what other work there is suggest that these levels might not be so exceptional. Lobsters caught off the coast of Scotland had claw muscle levels within the range of 0.10 ppm to 0.55 ppm and tail muscle values of 0.28 ppm to 0.72 ppm (HMSO 1971, 1973). Holden and Topping (1972), also measuring mercury in Scottish lobsters, found tail muscle values of 0.12 ppm to

0.75 ppm. They also found that these values were far higher than those found for digestive gland or claw muscle. Freeman et al (1974), measuring mercury levels in the american lobster, found values ranging from 0.44 ppm to 1.53 ppm for tail muscle and 0.15 ppm to 1.13 ppm for claw muscle. Further evidence that it may not be exceptional for the greatest background concentration to be in the tail muscle is provided by Vereer (1972) who found that the crayfish, Orconectes virilis, contained three times as much mercury in the tail muscle as in the rest of the body.

The levels resulting from the exposure to different concentrations of mercury and different time periods can best be considered tissue by tissue, and mention will also be made of background levels for comparison.

Gills

The gills accumulated the highest concentration of mercury of any tissue when the animal was exposed to the experimental solution. The levels of mercury ranged from 0.2ppm in Experiment II to 64 ppm in Experiment IV (seven days at 100 ppb).

Since the gills are the major site of accumulation it is important to know what this accumulation represents. Since water is passed over the gills at a rate of 9.59 litres per hour for a 322 gm lobster (Thomas, 1954) the mercury could easily be adsorbed on to the gill surfaces. It may then be transported in to the gill tissue proper or remain bound to the cuticular covering of the gill. Alternatively, the gills are known to contain excretory cells known as nephrocytes and

phagocytes, and they are also the site of excretion of some nitrogenous products (Parry, 1960). The concentration of mercury in the gills may therefore represent an accumulation of mercury absorbed elsewhere (or by the gills) but awaiting excretion by the gills.

From these results, although it seems likely that the gills do absorb the mercury, it is not possible to say if the mercury is also controlled from the gills. The results from these experiments give a static picture and no definite evidence for uptake or loss, either active or passive, could be obtained. It may be possible however to deduce something of the behaviour of mercury in the gills from the results of Experiments IV and V. The levels resulting from seven days exposure to 100 ppb are not very much greater than those resulting from only twenty four hours in 100 ppb with six days in fresh water (61 - 64 ppm compared with 51 - 43 ppm). This suggests that uptake occurs very quickly, and therefore adsorption is likely to play an important part, and that loss from the gills does not occur at any great rate.

Green glands

The green glands are the second major site of mercury concentration. Levels vary from 0.03 ppm and 1.03 ppm in the unpolluted lobsters to 34 ppm in Experiment IV (seven days at 100 ppb). The mercury can only enter the green gland from the blood so it is obviously being concentrated in the green gland to a considerable extent since the blood concentrations are always very much less. Although the green gland is recognised as the main excretory organ in the lobster it must be remembered that it is only a relatively simple structure, being only a development of a single pair of nephrons. Therefore, while mercury can clearly be

concentrated in the glands from the blood, it is not clear that excretion of the metal is actually taking place. Evidence that it is, may be provided from Experiment V when the green gland level is far less than the value in Experiment IV (10.32 ppm compared with 33.86 ppm). Thus, while the level in the gills did not drop very much after six days in the fresh sea water, it does drop in the green gland suggesting that the green glands are able to excrete mercury. The level after fourteen days in 10 ppb mercury is also much lower at 5 ppm also suggesting a degree of control. This required further study and will be referred to later (section d).

Blood

Blood levels varied from less than 0.01 ppm in the unpolluted lobsters to 4.3 ppm in Experiment IV, and 6.64 ppm in Experiment I. The blood levels are always far less than levels for the green gland which could suggest that filtration by the green glands is very efficient. However the blood was taken from the tail where levels might be lower than immediately before passing through the green glands or after passage through the gills. This possibility was investigated later in the study.

Digestive gland

It is not really clear from the results for the digestive gland whether it has a role to play in the control of mercury. The levels range from 0.3 ppm in unpolluted lobsters to 22 ppm - 27 ppm in Experiment IV. The levels do appear to be related to the blood levels

since in Experiment IV while the digestive gland concentration was at 22 - 27 ppm the blood concentration was also high at 4 ppm. It may be that the digestive gland acts as a reservoir for levels higher than those with which the green gland can cope.

Muscle

Levels in the muscle are always low in comparison with the other tissues after exposure to mercury. This is in contrast to results from the unpolluted lobsters where in Experiment III the level was highest in the muscle at 1.39 ppm.

The muscle mercury values are more variable in replicate samples than the other tissues. In Experiments IV and V one sample gives values similar to those of the blood while the other replicate in each case was much lower. This could be due to differences in muscle properties, or difference in blood content of the muscle. Bryan (1967) found different concentrations of zinc in muscle and this was found to be related to the type of muscle sampled. It is possible that a similar selective concentration was occurring here but no evidence was found either for or against the hypothesis. On the other hand, Rucker and Amend (1969) studying the accumulation of mercury in rainbow trout , (Salmo gairdneri), suggested that mercury in muscle was entirely due to the blood content of the muscle. This is a more likely explanation for the variability found here, since the blood samples were drawn from the tail muscle and possibly the first sample of muscle would be taken from a blood deprived area. Since the samples were taken in reverse order to that shown in the tables, to prevent contamination of lower content samples by those of higher mercury content, it seems that this is the explanation for the different values.

Ovary

The level of mercury in the ovary is quite high in the unpolluted lobster in Experiment III at 1.203 ppm. However, after exposure to 100 ppb in Experiment IV the level is only 0.9 ppm and it is therefore unlikely that the ovary is a site of great accumulation, but levels may build up from long term exposures to low levels of mercury.

c) Determination of the route of uptake of mercury using radio-isotope tracers

Introduction

The results from NAA give accurate measurements of mercury concentrations in the different tissues. NAA is a time-consuming process and there were practical limitations on the numbers of results that could be obtained. Also the results from this method give a static picture, the concentrations were those that resulted from a fairly long exposure time. They therefore did not necessarily give any idea of what the route of uptake into the animal might be; whether the mercury was entering through the gills, through the stomach, or by a combination of these routes. In order to determine this pathway of uptake more analyses were needed after short sequential exposure times.

For this purpose Mercury-203 was used as a radio-active tracer. Mercury-203 is a very convenient isotope for tracer experiments having a half-life of forty seven days and an easily measurable radiation (βmax = 0.21 Me V, and γ = 0.28 Me V).

Method

The lobsters were held in a 100 ppb solution of mercury labelled with 2μCi of ^{203}Hg in twenty litres of sea water. The lobsters were exposed for different times and at the end of each time course, that individual lobster was removed from the tank and killed.

The exposure times were three hours, six hours, twelve hours, twenty hours and twenty four hours. One lobster that had been used in the experiments to determine the mercury levels in the urine (section d), that had been exposed to the radioactive mercury for seven days, and then kept for three days in clean sea water, was also analysed.

Blood samples were taken not from the tail as for NAA, but from the ventral aorta, for the reason discussed on page 61. Samples of digestive gland, green gland, and gills were also taken. The tissue samples were accurately weighed. They were then digested by the same method used for the chemical separation with NAA. A mercury standard was prepared using 100 μgm of the labelled mercury and this was then treated in the same way as the other samples.

An attempt was also made to determine whether uptake by the gills was adsorption on to the gill surfaces and could therefore possibly be removed by washing, or whether the mercury was actually entering into the cells of the gills. In Experiment 2 the gills were washed in potassium permanganate in sea water very briefly. This was possibly damaging to the cells and may have resulted in loss of mercury from within the cells as well as from the surface of the gills. In Experiment 3, therefore, the gills were washed by being shaken in three changes of sea water.

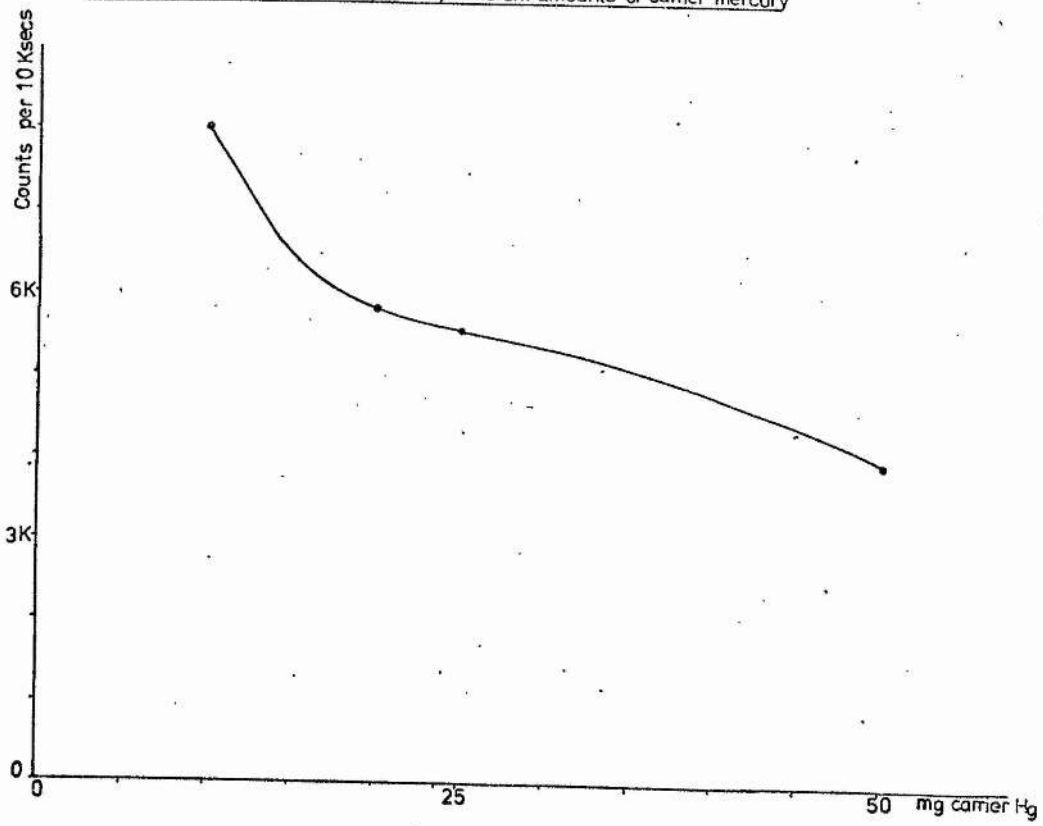
The precipitates of mercuric sulphide were weighed after drying and counted on a low background guarded geiger counter (Nuclear Enterprises Ltd). In this experiment it was the beta rays that were being counted. Corrections were therefore made for precipitate thickness by counting identical amounts of radioactivity in varying amounts of mercuric sulphide (see graph 1). Since the yields obtained were nearly all within the range of 20 - 25 mg of carrier mercury where differences in values due to selfabsorption are small, corrections were only made in a few cases. Corrections were also made for counter error where necessary (if counts were high) using the formula

$$\text{real time (cps)} = \frac{\text{observed counts (cps)}}{1 - \text{observed dead time}} \quad (0.0015 \text{ for this counter})$$

This method therefore involves the same separation steps as used for NAA but obviates the lengthy procedures prior to radiation. Accuracy is lost in this method by having to make the experimental solutions from the same active mercury solution, so that from decay and loss during storage, the mercury concentration may not be so accurately known after the initial experiments. The method cannot be as accurate as NAA but it is a far more accurate technique than normally used in radiotracer experiments. By digesting the tissue the problem of different characteristics of absorption of the tissues is removed; the addition of carrier mercury as in the NAA method allows a correction to be made for loss of mercury.

Graph 1

Calibration curve for self absorption by different amounts of carrier mercury



Results from radio-isotope tracer experiments

The results from these experiments are presented in Table II and in Figures 3 and 4.

Discussion of the results

The experiments using neutron activation analysis, whilst giving accurate results, could not be done quickly enough or often enough to give any information on the changing patterns of distribution with time. The radioisotope tracer experiments were therefore designed to show how the distribution of mercury varied with time in order to see if the actual pathway of the mercury into the different tissues could be followed.

The overall distribution pattern of mercury within the tissues however, stayed remarkably similar to that found after the longer time scale NAA experiment. The gills tended to accumulate the greatest concentration even after only three hours which suggests that uptake is due to their position with respect to the water, and is not related, at least at first, to the mercury content of the other tissues. One difference in pattern is that after three hours exposure, the blood and green gland concentrations are roughly similar, but then the level in the green gland increases rapidly with respect to the blood, and after twelve hours contains a far greater concentration than either the blood or the digestive gland. Also, in the NAA experiments the gills always contained the greatest concentration of mercury after the controlled exposures, while on three occasions in this series of experiments there were greater concentrations found in the green gland than the gills. (The reason for the comparatively low levels measured in lobster IV in

TABLE II

Results from Radioisotope Tracer Experiment

Experiment I

Lobster	Weight	Sex	Exposure times and dates	Tissue	Weight	Precipitate Weight	Yield	Corrected Counts	ppm
Lobster II	277gms	♀	10.00-13.00hrs 14.6.75 3 hours	Blood	0.8598	0.0354	1.22	34.31	0.11
				Digestive gland	1.6072	0.0415	1.4312	7.64	0.02
				Green gland	0.5627	0.0274	0.9622	14.14	0.14
				Gill	0.8475	0.0476	1.4692	1230.62	4.02
Lobster III	312gms	♂	10.00-16.00hrs 14.6.75 6 hours	Blood	0.8625	0.0334	1.1519	17.21	0.05
				Digestive gland	0.7448	0.0392	1.3519	27.70	0.09
				Green gland	0.5631	0.0289	0.9967	93.18	0.30
				Gill	0.7448	0.031	1.0691	1870.85	6.11
Lobster I	292gms	♀	21.20 13.6.75 to 9.20 14.6.75 12 hours	Blood	0.9486	0.0279	0.9622	170.47	0.55
				Digestive gland	1.6457	-	-	95.88	0.31
				Green gland	0.3223	0.0308	1.06224	2242.15	7.33
				Gill	1.1463	0.0386	1.3312	1119.03	3.65
Lobster IV	308gms	♂	13.00 14.6.75 to 13.00 15.6.75 24 hours	Blood	1.4459	0.0321	1.107	17.18	0.05
				Digestive gland	2.3576	0.0334	1.15	31.2	0.10
				Green gland	0.6997	0.0336	1.1588	128.02	0.41
				Gill	1.8019	0.0264	0.9104	807.64	2.64
Hg standard	100µg						0.8828	40022.65	305.88 is average for 1µg
	100µg						0.9139	21154.94	

Experiment 2

Lobster	Weight	Sex	Exposure times and dates	Tissue	Weight	Precipitate Weight	Yield	Corrected Counts	ppm
Lobster V	272gms	♂	21.30 16.6.75 to 9.30 17.6.75 12 hours	Green gland	0.5393	0.0301	1.038	921.77	3.01
				Gill	1.2225	0.0242	0.8346	8432.75	27.57
				Gill washed in KMnO4 in sea water	1.3626	0.0589*	2.03	1776.77	3.95
Lobster VI	357gms	♀	8.20 - 11.20 17.7.75 3 hours	Green gland	0.5472	0.0254	0.876	65.08	0.21
				Gill	1.5720	0.0426	1.46	919.56	3.00
				Gill washed in KMnO4/SW	1.3592	0.0472	1.62	238.52	0.77
Experiment 3									
Lobster VII	285gms	♀	30.6.75 3 hours	Blood	0.7650	0.0323	1.1139	93.70	0.30
				Digestive gland	1.3150	0.034	1.1726	31.42	0.10
				Green gland	0.5252	0.0284	0.9794	167.77	0.55
				Gill	1.0197	0.0325	1.1208	1371.8	4.48
				Gill washed in 3 1minute shake in SW	0.7726	0.0328	1.1312	1567.1	5.12
Lobster X	317gms (one claw)	♀	10.30 1.7.75 to 10.30 2.7.75 12 hours	Blood	0.7407	0.0276	0.9518	176.60	0.58
				Digestive gland	1.8828	0.0286	0.9863	98.92	0.32
				Green gland	0.6909	0.0272	0.9381	555.92	1.82
				Gill	2.0990	0.0308	1.0622	2249.25	7.35
				Gill washed	1.6059	0.0591*	2.0382	2024.92	6.62

*These samples have been corrected by a factor of 0.74 for thickness of precipitate (see correction graph - graph 1)

Lobster	Weight	Sex	Exposure times and dates	Tissue taken	Weight	Precipitate Weight	Yield	Corrected Counts	ppm
Lobster VIII	>400 gms	♂	30.6.75	Blood	None available - this lobster virtually dead due to air pump breakdown				
				Digestive gland	2.0305	0.0245	0.8449	370.26	1.21
				Green gland	0.5687	0.0287	0.9898	2447.33	8.00
				Gill	1.9043	0.0308	1.062	1860.35	6.08
			20 hours	Washed gill	1.8350	0.0402	1.3864	1157.25	3.78
Lobster IX	345gms	♂	30.6.75 - 1.7.75	Blood	1.2386	0.0305	1.05	191.92	0.63
				Digestive gland	1.3509	0.0289	0.9967	172.27	0.56
				Green gland	0.8267	0.0293	1.0105	1305.69	4.27
				Gill	1.6459	0.0323	1.1139	2268.53	7.42
			24 hours	Washed gill	1.8136	0.0309	1.065	2580.53	8.43
Lobster kept ten days in mercury; (from urine experiment)	304gms (no claws)	♂		Blood	0.5873	0.0321	1.1070	267.48	0.87
				Digestive gland	1.0292	0.0378	1.3036	390.67	1.28
				Green gland	1.0748	0.0333	1.1484	9692.94	31.69
				Gill	1.1276	0.0308	1.0622	8999.03	22.88
				Gill washed	1.2819	0.0308	1.22	4999.72	16.35

Figure 3

Radioisotope tracer Experiment 1

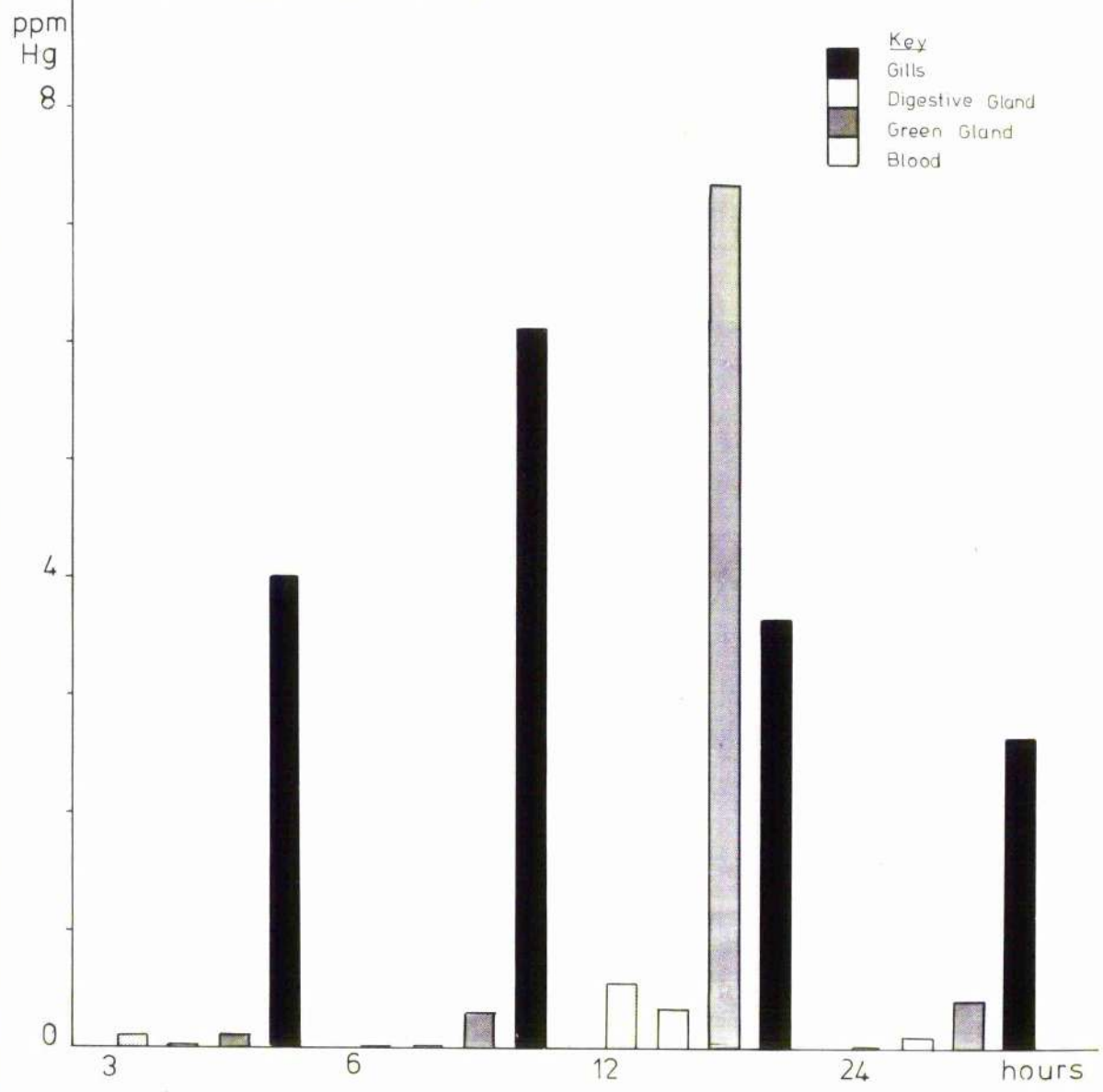
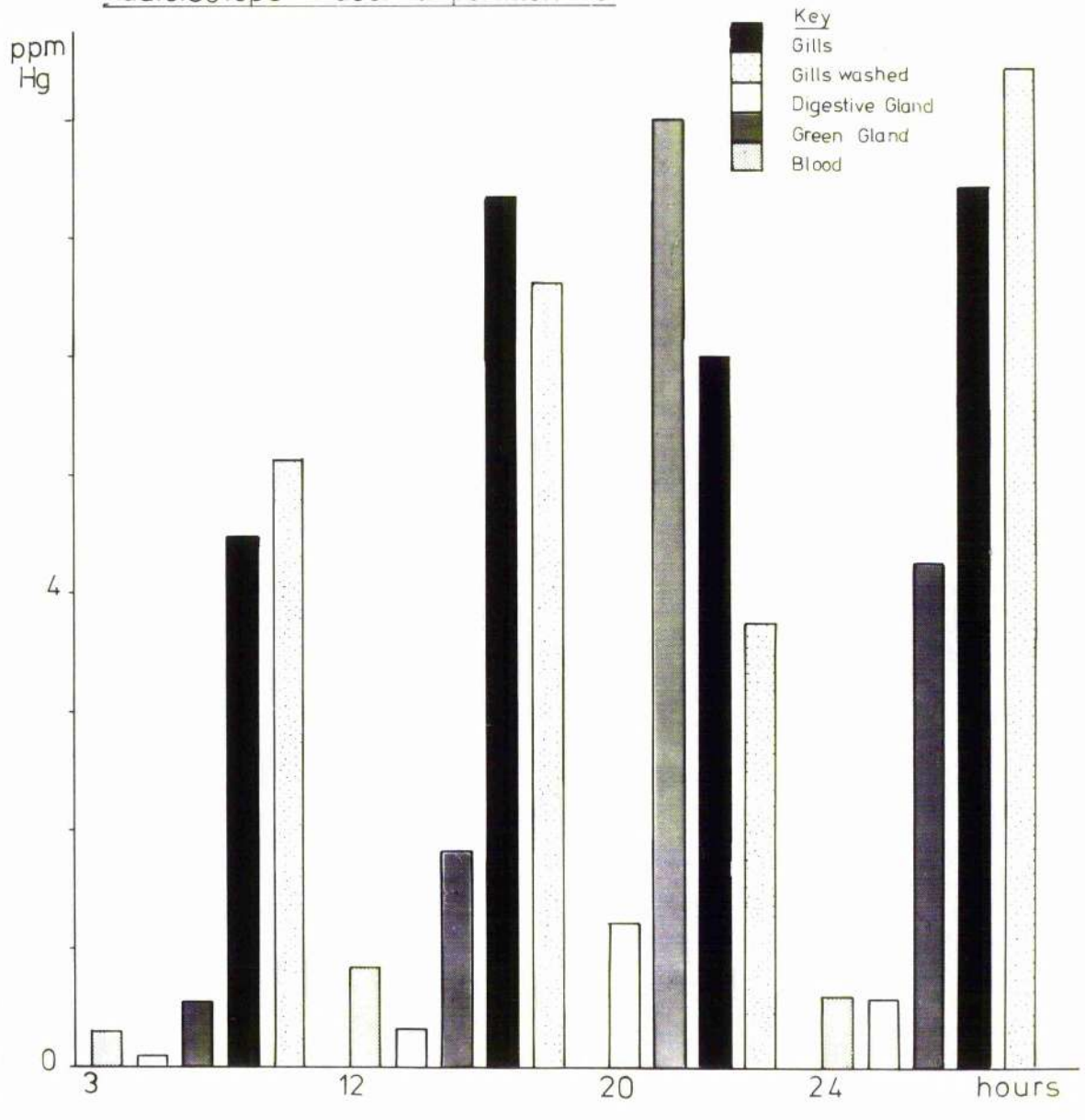


Figure 4

Radioisotope tracer Experiment 3



experiment 1 (24 hour exposure) is that the same tank of labelled mercury was used for all four lobsters in this experiment and lobster IV was the last lobster into the tank. In the later experiments solutions were made for each pair of lobsters exposed.)

The results from the washing of the gills were rather unclear. Washing with potassium permanganate in sea water did cause a considerable loss of mercury but this was as likely to be due to cell damage as to the washing off of mercury from the gill surfaces. With the three changes of sea water and vigorous shaking, the results were less clear still; two of the washed gills were found to contain more mercury than the unwashed. No definite conclusions as to the location of the mercury within the gills could be reached therefore.

It was hoped that this series of experiments would clarify the route by which the mercury entered the body. Since the level rises so quickly in the gills this must be a major route of entry but it cannot be determined conclusively from this series of experiments whether it is this mercury that then finds its way from the gills to the blood and thence to the green gland, or if mercury can also be taken up via the stomach and digestive gland. If this latter possibility occurred one would expect the digestive gland to be higher than the blood after short exposure times, but the digestive gland only has a higher value than the blood in the long term experiment.

The muscle concentrations were not determined in these experiments, since from the NAA results the muscle did not seem to be playing any great part in the control of mercury; the levels in muscle not appearing to differ much from that of the blood.

The results from the NAA experiments for the blood concentrations gave low values and this, it was thought might have been due to the site

where the blood was sampled. In these radiotracer experiments therefore, instead of taking the blood samples from the tail, they were taken from the ventral aorta. There was, however, little change in the levels measured.

d) Experiment to determine the role of the green gland in mercury control in the lobster

Introduction

It was known from the uptake experiments that the green glands were a major site of concentration of mercury. Although the green glands are the main excretory organs it was not known whether the glands could excrete mercury. Since death appears to occur in long term experiments of exposure to mercury approximately two days after urine production ceases (personal observation), the green glands are obviously important organs with respect to mercury poisoning. It was therefore considered important to determine to what extent the lobster is able to control the mercury content of the body by the green glands. In order to do this the lobsters were exposed to radioactive mercury and their urine was collected to determine if mercury was being excreted and at what concentrations.

Method

Lobsters were placed in the tanks containing the labelled mercury at 100 ppb as in the radio isotope tracer experiment. At this level (2 μCi per 20 litre tank) samples of the sea water were not above

background. Therefore in order to be able to measure mercury in the urine the lobster had to concentrate it above this level. Urine samples were taken at intervals by the method of Burger (1957). The lobsters are turned on their backs and their tails flexed forward towards the head. The lobster is squeezed at the base of its pereiopods and two jets of urine issue forth from the nephridiopores. It was however found to be more reliable not to block the nephridiopores between sampling as described by Burger. Other methods of blocking the nephridiopores were also tried but it was found that just allowing sufficient time to elapse between sampling to allow the bladder to fill gave a more reliable source of urine. (It seemed that blocking the nephridiopores actually inhibited urine production in many lobsters.) The urine was conveniently collected in needle guards from Gillette sterile hypodermic needles. These were used once only. The urine samples were transferred to scintillation tubes containing 10 ml Insta-gel scintillant (Packard Instrumental Company) using a hypodermic syringe. Samples were counted on a Nuclear Chicago Mark I liquid scintillation counter. A standard sample containing a known amount of mercury-203 was counted at the same time as the samples to give an approximate value to the quantity of mercury in the urine.

One lobster (A) used in these experiments was also analysed by the method used in the radio-isotope tracer experiment so that a comparison of urine mercury values and related tissue concentrations could be made.

Results

The results are shown in Table III. Graph 2 shows the pattern of excretion of mercury in the urine of Lobsters A, B and C.

TABLE III

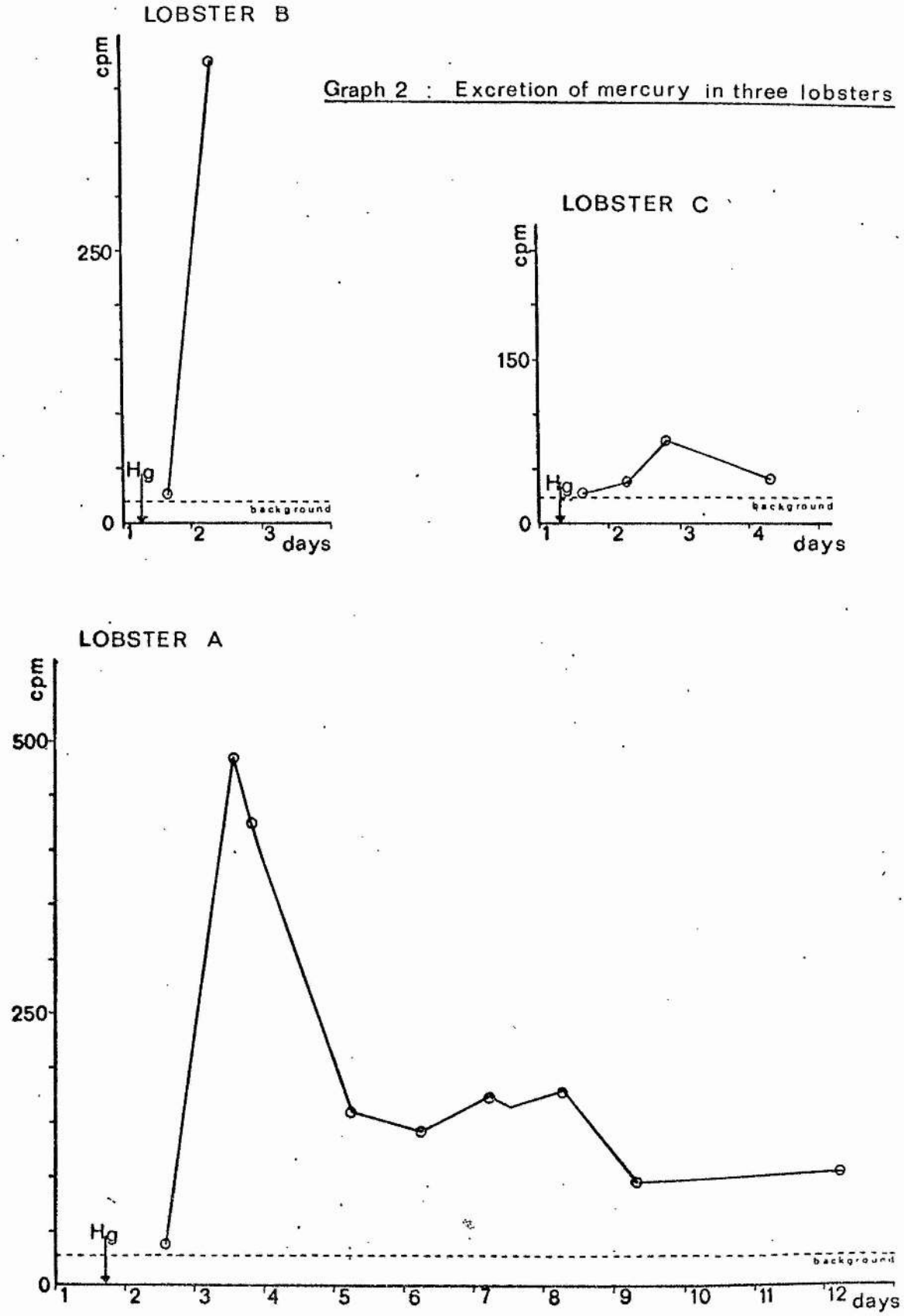
Results from Urine Experiment

Lobster	Volume of sample (ml)	Time taken	Day of experiment	Counts per minute
Lobster A	0.3	13.30	2	38.0
304 gms (no claws)	0.3	13.30	3	481.0
Male	0.3	19.00	3	436.0
Put in mercury 2µCi/2mgm20	0.3	9.15	5	160
litres sea water	0.3	9.30	6	142
21.20 hrs on 13.6.75 (Day 1)	0.3	9.00	7	178
Water temperature 16°C	0.3	13.00	7	163
	0.3	10.30	8	182
	0.3	10.30	9	94
Lobster then transferred to fresh sea water	0.3	11.00	12	101
Standard	0.1ml of Hg = 0.1µCi			93,466.00
Tank samples	0.3		5	40
" "	0.3		8	37

Lobster	Volume of Sample (ml)	Time Taken	Day of experiment	Counts per minute
Lobster B Male Put in mercury at 1100hrs on 1.9.75 Died 4.9.75	0.3 0.2	14.30 9.30	1 2	26 449
Lobster C Female 1.9.75 at 11.00hrs	0.3 0.25 0.3 0.3	14.30 9.30 14.00 9.30	1 2 2 4	28 37 73 34
Lobster D Male 322gms(1 claw) Put in mercury 15.00hrs 18.8.75	0.3	14.40	2	54
Lobster E Female 391gms 15.00hrs 18.8.75	0.3 0.15 0.15 0.2	14.40 9.40 12.20 17.00	2 3 3 3	45 44 46 44

Lobster	Volume of Sample (ml)	Time taken	Day of Experiment	Counts per minute
Lobster F Female Put in mercury 9.30 hrs 18.6.75	0.3	17.00	before putting in mercury	29
	0.3	9.30	1	29
	0.05	12.00	1	18
	0.3	17.00	1	31
	0.3	11.00	6	24
Lobster G 271gms (one claw) Male 15.6.75.	0.3	13.30	1	40
Lobster H 423gms 1300hrs 26.8.75	0.3	9.30	1	48
Lobster I 388 gms (as H)	0.3	9.30	1	28

(Both H and I died within 36 hours)



Graph 2 : Excretion of mercury in three lobsters

This experiment was mainly carried out in August 1975. This was after an exceptionally hot summer and many of the lobsters available were in the premoult stage. This seemed to make them far more susceptible to the low level of mercury and some became anuric and died after short exposure times. The other reason for difficulties in keeping the lobsters alive in concentrations of mercury previously found to be sub-lethal was that conditions for keeping the lobsters in the isotope laboratory available were not ideal. The temperature of the water in the tanks in the isotope laboratory were higher than in the aquarium from which the lobsters were taken (16° C compared with 11-12° C). There were also problems with keeping the water sufficiently well aerated. For these reasons the results from this experiment are extremely variable.

Of nine lobsters (four female) only three could be conclusively shown to produce urine containing mercury (having radioactive counts well above background). These were A, B and C (see Graph 2). These were all still hard shelled and not about to moult. Results from the other lobsters were much less clear. Lobsters H, D and E produced urine with radioactivity levels just above background but there were no urine samples taken from these lobsters before exposure to mercury available for comparison. Nor could sampling continue for very long because the lobsters became anuric and died shortly after.

Lobster F was kept for seven days in the mercury solution but none of the urine samples for this lobster contained levels of mercury higher than background. It was only possible to get samples from this lobster on Day 1 and Day 6 of the experiment. Lobsters G and I became anuric and also died shortly after being exposed to the mercury.

The results are therefore extremely variable. Nevertheless, excretion of mercury was shown to take place. Lobster A, kept for seven days in labelled mercury solution and for a further three days in fresh sea water gave the clearest results. It was possible to calculate the concentration of mercury in the urine of this lobster because the mercury standard was made at the same time as this experiment was done. The concentration of mercury measured at the start of the experiment reached approximately 16 ppm and this dropped rapidly to the steady state reached at the end of the experiment of 0.5 ppm. The urine samples of Lobster B had a similar activity to that of the initial rate of mercury excretion of Lobster A, while that of Lobster C had an activity more akin to that of the steady state of Lobster A. Due to radioactive decay in the mercury standard, and loss during storage, it was not possible to calculate the mercury content of these two lobsters' urine with any degree of confidence.

After the ten day experiment Lobster A was killed and its tissues were analysed by the method used in the radio isotope tracer experiment. The tissue mercury values corresponding to an excretion rate of 0.5 ppm are shown in Table II and Figure 2.

Discussion of the methods used

This use of two different methods of measuring accumulation has proved extremely informative. Neutron activation analysis is a tried and proven technique widely used in Sweden where they have had probably the most experience of measuring mercury levels in the environment (Anon 1971). However in this study it was difficult to use this technique as

much as would have been ideal because of restrictions imposed by finance, time, and organisational difficulties. These were peculiar to this situation and the technique itself, once perfected, is extremely reliable and accurate.

More measurements and more short term exposure results were needed, and so the radio tracer experiment was designed. The new venture of incorporating the chemical separation stages as used in the NAA experiments refine the technique markedly from such techniques used by Corner (1959) and Corner and Rigler (1958) when samples are not digested but mixed to a paste and dried by heating. A great deal of mercury must be lost in such procedures, (Westermark and Sjostrand, 1960). These methods must however, be more accurate than whole body counting as used by Kramer and Neidhart (1974) and Seymour (1971) where simple absorption and geometrical irregularities make accurate measurements of radiation extremely difficult.

The added advantage of this isotope tracer experiment described in this study is that by combining it with NAA analyses, the reliability of the results from the isotope tracer experiment could be assessed. Since the results corresponded well between the two experiments both results can probably be viewed with confidence.

This adaptation of the NAA chemical separation technique does not appear to have been used before and it provides a far more accurate technique for the measurement of mercury than the commonly used methods. The use of carrier mercury is a very particular advantage to attaining greater accuracy.

The ability of lobsters to excrete mercury in their urine has not

been measured before. The only comparable study is that of Corner on the spider crab, Maia squinado (1959). In this experiment the urine had to be evaporated to dryness after mixing with sodium sulphide. The development of scintillants such as Insta-gel, that enable such low levels of radioactive ions to be measured when present in complex mixtures such as urine, by liquid scintillation counting, provide a very great advantage over the methods that had to be used previously.

General discussion of uptake and excretion experiments

It has been shown in this combined study that the gills accumulate the greatest concentration of mercury even after short exposure times; that the next greatest concentration is found in the green glands, and that these organs can excrete mercury, and that the digestive gland, blood and muscle appear to concentrate mercury to varying extents depending upon the pre-treatment. Also, while the mercury levels will tend to be lowest in the muscle after exposure to controlled levels of mercury, this is not the case when background levels of mercury are measured in the body.

Little work has been done on metal uptake by lobsters, and certainly none with mercury. The uptake of mercury has been investigated in other species in which similar distribution patterns have been found, eg in the prawn, Leander serratus (Corner and Rigler 1958), and the spider crab, Maia squinado (Corner 1959), as well as many other species of crab, while uptake of other metals has been looked at in the lobster. Bryan (1964, 1965) has studied the uptake of zinc and manganese (with Ward) in the lobster. In the american lobster, Eisler, Zaroogian and Henneky (1972) studied the uptake of cadmium, and Fletcher (1971) has studied the uptake of yellow phosphorus. It is useful to make comparisons with these investigations, but the manner in which a particular species deals with different metals, and the way in which different species deal with the same metal can show important differences. These will be referred to in this discussion of the findings of this study.

Apart from determining the major concentration sites and patterns of accumulation it should be possible to answer several other questions from this study. The main questions are:

a) What is the route of uptake of mercury into the lobster?

b) In what manner, and to what extent, is the lobster able to

control the mercury level in the body?

a) The two possible routes of entry of mercury into the body are either through the gills, or through the mouth and stomach, and absorbed from there through the digestive gland.

Uptake through the gills

The first possibility is a likely one. A steady stream of water is passed in the respiratory current across the finely divided surfaces of the gills. For a 322 grams lobster, 9.59 litres per hour is pumped across the gills (Thomas 1954). Therefore, since in these experiments approximately half the volume of the holding tanks passed over the gills every hour, the gills were exposed to 1 mg/mercury per hour (in 100 ppb experiments). Since mercury is known to be attracted to surfaces (Coyne and Collins, 1972) it could easily be adsorbed on to the gill surface. However in the gill washing experiments described here, there appeared to be no clear evidence that the mercury was only attached to the surfaces and not actually in the gill tissue. Corner and Rigler (1958) investigated this problem in Leander serratus and found that immersing the gills in either Homarus ringer solution (Cole 1941) or in the same ringer solution but also containing reduced glutathione (thiol compounds such as this have marked affinities for heavy metals - Gurd 1954) did not reduce mercury concentration measured in the gills by any significant amount.

Bryan (1968) found that the gills were the main site of uptake and loss of zinc across the body surface in decapod crustaceans. He

suggests that absorption depends primarily on the adsorption of zinc on to the cuticle of the gills and then on the transfer of zinc into the gill cells attached to proteins. Zinc has a great tendency to bind to molecules such as proteins. Since mercury also shows the same tendency, it is possible that such a mechanism works for mercury. As pointed out before, mercury is an element alien to the body and whilst it might be expected that there would be mechanisms for the uptake of zinc there is no reason why there should be such a provision for mercury. Bryan does say however that the proteins carrying the zinc need not be specific carriers for zinc and that transfer could depend upon the normal random movements of the molecules. This does not account for an uptake against a concentration gradient (sea water zinc content = 0.3 - 9.8 ppb, gill content = 13 - 19 ppm), but Bryan found that most zinc was bound to protein in the blood and that the concentration for unbound zinc favoured uptake from the water. The blood levels of mercury measured in this study were low in comparison to normal blood zinc values, or mercury values measured in other uptake experiments where much higher levels of mercury were used (Corner and Rigler, 1958; Corner, 1959). Even so, the mercury must be bound to some extent for there to be a concentration gradient favouring the uptake of mercury from the external solution.

It is not known from this study if the mercury in the blood is bound to protein. This problem was investigated by Corner (1959) in uptake experiments with Maia squinado. By precipitating the protein fraction in the blood and identifying the location of the mercury between the precipitate and the non-protein fraction of the blood, he found that ninety five per cent of the mercury was associated with the protein. It is therefore likely that there is a similar uptake

mechanism for mercury to that suggested by Bryan (1968).

Evidence that adsorption is the primary mechanism is provided by Bryan (1971) again in his studies of zinc in the lobster. He showed that the rate of uptake is not proportional to the concentration of zinc in the sea water. He suggested that this is possibly evidence that adsorption has to occur before uptake into the gills proper. He showed this by comparing the uptake by isolated gills with that of the gills of whole lobsters, and how they varied with concentration of zinc in the external medium. A theoretical graph of uptake proportional to zinc concentration shows that the graphs for both gills are displaced from this line, the whole gills being the most displaced. This means that uptake is greatest at low concentration. It is likely that a similar mechanism does exist for mercury since at low mercury concentrations (Experiment VI) the gill levels are quite high while other tissue values are much lower.

There does, however, appear to be an important difference between the uptake of zinc and that of mercury. Gill levels measured in this study after exposure to 100 ppb mercury for seven days were 61 - 64 ppm while after exposure for just one day the levels were 43 - 51 ppm. The corresponding blood concentrations were 4.25 ppm and 1.4 ppm respectively. After exposure to the same level of zinc for much longer periods, gill concentrations were at a maximum of 37.5 ppm with blood levels of 10.0 ppm but with greatly raised levels in the digestive gland and the urine (Bryan, 1964). Therefore it seems likely that adsorption is as important a process for the initial uptake of both metals but thereafter, a far lesser proportion of mercury is transferred

into blood while the rest remains in the gills.

Uptake through mouth and stomach

The other possible route of uptake is through the mouth and stomach. Sea water would normally be taken in with the food, but the lobsters in these experiments were not fed. However, Burger (1957) describes how even non-feeding lobsters routinely swallow sea water. He said that the drinking was an intermittent affair and that water and monovalent ions were taken up from the stomach, although to a lesser extent than through the gills. Work by Corner and Rigler,(1958) and Corner (1959) give evidence for this being a possible route. They injected mercury into the prawn and spider crab and found a similar distribution of mercury within the body to that resulting from exposure to mercury in the external medium.

However, Burger (1957) gives the stomach capacity of a 500 gram lobster as ten millilitres. If such a lobster filled its stomach from an external solution of 100 ppb it would only take in one microgram. If the lobster filled its stomach three times a day it would still only take up three micrograms. The weight of the digestive gland of a 423 gm lobster is about sixteen grams (personal observation). The resulting concentration in the digestive gland following uptake of 3µg of mercury would therefore only be about 0.18 ppm. This cannot therefore be a major route of uptake since the concentrations found in the digestive gland were usually at this level, or higher after less than twenty four hours exposure.

Bryan (1964) found that zinc pipetted into the stomach would rapidly be absorbed. It was found that uptake from the stomach was

followed by a peak in the digestive gland content of zinc after seven hours, followed by a subsequent rise in blood concentrations. No such peak in the digestive gland preceding a rise in the blood levels was found in the radio tracer experiments, so this route can more or less be ruled out as of any importance for the uptake of mercury.

Therefore, uptake from solution is almost certainly by way of the gills. The primary mechanism is probably adsorption and a relatively small proportion of the adsorbed mercury then passes into the blood by a passive absorption process.

b) Control mechanism

The second question is to what extent is the lobster able to control the levels of mercury and by what means.

The green glands

The first place to look for control mechanisms is obviously the green glands since these are recognised as the main excretory organs. Here it was found that control was exerted by excretion of mercury in the urine. The initial mercury concentration of 16.66 ppm in the urine of Lobster A appears to be high. Bryan (1968) studied the excretion of zinc and copper in the Decapod Crustacea. The average concentration of these two metals in the lobster urine was found to be 2.2 ppm and 1.3 ppm respectively. When the lobster was put into sea water with a high zinc content (100 ppb) however, the urine content of zinc rose to up to 40 ppm (Bryan, 1964). As pointed out before, zinc and copper are both physiologically active and therefore it is to be expected that

there will be control mechanisms for these metals while the animal might be expected to have more difficulty in excreting a metal such as mercury.

Evidence that this is so is suggested by the fact that the urine to blood ration of mercury calculated from the results for Lobster A is 0.57. (This is calculated from the ratio of the blood concentration of mercury of 0.87 ppm to the final steady state urine concentration of 0.5 ppm.) Obviously the results from one lobster alone must be treated with caution but it is perhaps worth comparing this figure with values for other ions determined by Burger (1957). In his experiments the urine-plasma ratios for magnesium was 1.7, for calcium 0.81 and for sulphate it was 1.2. Bryan and Ward (1965) found urine to blood ratios for manganese of more than one and Bryan (1964) found the value for zinc to be up to four. These figures suggest that mercury is less easily excreted than these other elements.

Thus both rates of excretion of mercury found for Lobster A are within levels found for other elements, while the urine to blood ratio found at the end of the experiment suggests that mercury is less readily excreted than other elements. There may be an explanation for this change of urine concentration and low urine to blood ratio. Sloan, Thompson and Larkin (1974) when investigating the loss of mercury from the Dungeness crab, Cancer magister, also found a high initial rate of loss followed by a second phase of slower loss. They suggested that this might be due to degenerative pathological change. Cell destruction or blocking of enzyme pathways in excretory organs could lead to progressive reduced ability to eliminate free mercury which could then recycle into the organism. Evidence for this being a possible explanation for these different rates of excretion will be presented later in this study (section f, part 1).

Mercury excretion has not been measured in the lobster before, but Corner (1959) did look at the excretion of mercury in the spider crab, Maia squinado, and in this animal, after nineteen hours exposure to 10 ppm of inorganic mercury, the urine level of mercury was 1.64 ppm. This is after exposure to an extremely toxic level of mercury. Considering the high level of exposure it may be surprising that if the animal does have the ability to excrete mercury it does not do so at a higher rate, at least initially. This low rate of excretion may be due to the fact that lobsters and crabs were shown by Bryan (1966) to show different emphasis in the excretion of zinc; lobsters tend to lose excess zinc through the green glands, while crabs lose it mainly through the gills. In his study of decapod crustacea Bryan (1968) found that only the lobster, Homarus, and the crab, Atelecyclus, could produce urine with a concentration of zinc higher than 1 ppm.

Thus it has been shown in this study that mercury can be excreted by the lobster. How effective this excretion is as a means of control is less certain. By comparing the green gland concentration of mercury resulting from exposure to 100 ppb mercury, with blood concentrations it has been said that the green gland is able to concentrate mercury very efficiently from the blood. This may be misleading. This is because the mass of the green glands is very small compared with that of the blood. Burger and Smythe (1953) calculated a ratio of blood volume to weight for the american lobster, Homarus americanus, of 0.17. While this may differ from that for Homarus gammarus, it was used to calculate the blood volume for each of the lobsters used in the radio isotope tracer experiments, and two of the lobsters used in the NAA experiments. This enabled a rough estimate of the total mercury content

TABLE IV

Table to show comparison between total mercury levels in blood and green gland

Experiment	Weight of Lobster	Blood volume (ccs) (calculated from 0.17 ratio Burger & Smyth 1953)	Measured Hg concentration in green gland	Measured Hg concentration in blood	Actual green gland mercury	Actual Blood Hg content
Tracer expts Lobster II (3 hrs)	277 gms	47.09	0.144 in 0.5627gms	0.11	0.255µg	5.27µg
Lobster III (6 hrs)	312 gms	53.04	0.304 in 0.5631gms	0.05	0.5389µg	2.97µg
Lobster I (12 hrs)	292 gms	49.64	7.33 in 0.3223gms	0.55	22.74µg	27.66µg
Lobster IV (24 hrs)	308 gms	52.36	0.4185 in 0.6997gms	0.05	0.598µg	2.93µg
Lobster VII (3 hours)	285 gms	48.45	0.55 in 0.5252	0.30	1.04µg	14.83µg
Lobster X (12 hrs)	317 gms	60.69	1.82 in 0.6909	0.58	2.63µg	35.20µg
Lobster IX (24 hrs)	345 gms	58.65	4.27 in 0.8267	0.63	5.17µg	36.94µg
Longterm lobster 10 days	302 gms no claws (400)	68	41.69 in 1.0748	0.87	29.48µg	59.16µg
NAA V	356	60.52	10.32 in 0.6605	1.43	15.624µg	86.54µg
NAA VI	325	55.25	5.69 in 0.6207	0.07	9.16µg	3.87µg

of the green glands. These are shown in Table IV. These findings are very interesting in that it shows that the blood content of mercury is actually higher than that of the green gland, except in one experiment and that was the 10 ppb NAA experiment (Experiment VI). This may suggest that it is only at this level that the lobster is able to control the mercury level effectively.

It must be possible to deduce a level of mercury at which the animal is able to control the mercury level in the body, and therefore it might be expected that at that level there would be little deleterious effect caused by the mercury. The results from Experiment VI of the NAA experiments suggest that at 10 ppb mercury the lobster is able to exert quite effective control since the blood and the green gland levels are very low, and the total mercury content of the blood is lower than that of the green glands. Also, the muscle levels are within the range that has been found for background levels in Scottish lobsters. The gill levels are still high however, and if the mercury in the gills is excreted through the green gland there may be a long term pressure on the green glands. Evidence that this level of exposure is in fact harmful will be presented in section f.

The gills

The fact that Bryan (1966) showed that loss of zinc through the green glands is a more important control mechanism than loss through the gills in the lobster, and the fact that mercury has been shown to be excreted through the green glands in this study does not necessarily mean that the gills might not be important to the lobster as excretory organs. On the contrary, it is possible that the high concentrations of mercury in the gills do not merely represent the amount of mercury passively

taken up by the gills, but also sites of control of the mercury levels. This is suggested by experiments where mercury was injected into Leander serratus, (Corner and Rigler, 1958) and Maia squinado (Corner, 1959), when the mercury still accumulates to the greatest extent in the gills as after exposure to mercury in the external solution.

The gills are known to have an excretory function. They can transport substances such as urea and other nitrogenous waste across their surfaces (Burger, 1957). Corner (1959) found that if after Maia squinado had been exposed to mercury it was then placed in fresh sea water with the nephridiopores blocked, amounts of mercury were detected in the water. The crab is more likely to lose mercury via the gills (Bryan 1966) but at the same time it cannot be known how much the blocking of the nephridiopores affected the gill response. No investigation of whether the lobster actively lost mercury across its gill surfaces was carried out in this study. But as already pointed out, the fact that the level found after one day at 100 ppb and six days in fresh sea water were not very much less than the levels found after seven days at 100 ppb makes active excretion across the gill surfaces seem unlikely.

The gills also possess a different type of excretory system, that is they contain specialised cells called nephridiocytes and phagocytes which are known to be able to take up foreign substances such as dyes (Drach 1930). These cells are thought to be either retained throughout life, or the contents released during moulting (Drach 1930). Therefore, the accumulation of mercury in the gills could be due to the deposition of the metal within these cells. Localisation of this mercury was attempted using X-ray microanalysis and will be referred to later (section e).

However, the rapid build-up of mercury in the gills found in the radioisotope tracer experiment suggests that adsorption on the cuticle of the gills is more likely than uptake by excretory cells,

while the action of the excretory cells is possibly more likely to account for the high gill content following injection of mercury as found by Corner and Rigler (1958) and Corner (1959).

Another possible means of control is that the gills actually adsorb the mercury in such a way that it is not free to enter the blood except at a very slow rate. It is clear that mercury is entering through the gills but not to such an extent as zinc would for example (Bryan 1964). This may therefore represent a passive control, possibly simply due to properties of mercury itself.

Thus, apart from it appearing unlikely that mercury is actively excreted there can be little deduced positively from this study as to the exact location of the high levels of mercury accumulated by the gills.

Work on the fiddler crab has shown that mercury is actively transported from the gills to the digestive gland (Vernberg and O'Hara 1972). Vernberg and O'Hara also found that this transfer was decreased at low temperatures and this was thought to explain the greater mortality found in the crabs at low temperature. No evidence for such a transportation occurring in the lobster was found.

The digestive gland

The digestive gland is another possible site of control. Bryan (1964) found that it acted as a storage site for excess zinc, if the level in the blood became too high. The only evidence that this might occur in this study came from Experiment IV of the NAA experiments. In this the blood levels were quite high and the digestive gland levels were also correspondingly much higher than in other experiments. There may however be gradual accretion into the digestive gland since the

levels appear to rise gradually with longer exposures.

It seems possible that as long as blood levels remain low (less than 2 ppm) the green gland is able to cope, at least initially, and levels only rise markedly in the digestive glands when the green glands are no longer able to deal with the large amounts of mercury.

Loss of mercury could also occur through the faeces. This is not however likely in unfed lobsters, and Bryan (1964, 1966) showed that this route for loss of zinc is probably not important in the lobster in any case.

Thus the overall pattern of mercury uptake and control appears to be that the mercury is adsorbed on to the gill surfaces. A small proportion of this gradually transfers to the blood and is accumulated in the green gland from where it is excreted. At higher levels of exposure, blood levels will rise and then there will be accumulation of mercury in the digestive gland. The relevance of excretory cells or loss of mercury via the gills is not known but will be referred to with additional information later in the study.

e) Investigation of the uptake of mercury into lobster larvae and of the effect of mercury on their development

Introduction

It has been shown that larvae of marine animals tend to be less resistant to the action of pollutants such as mercury (Connor, 1972;

De Coursey and Vernberg, 1972).

It was therefore necessary to examine the effect of mercury in the larvae. It was not possible to carry out conventional toxicity tests since neither the necessary large numbers of larvae nor the experimental facilities for such procedures were available. In any case, this study has been more concerned with sub-lethal effects. It was therefore decided to approach the problem in the same way as to the study of the adults. That is, to determine the extent of accumulation of mercury in the larvae and its location; in this way to determine if the same pattern of accumulation was found or if it was possibly the lack of ability to control the location of mercury that led to increased mortality rather than only considerations of size, and after that, to determine possible differences in survival or development rate in the mercury-reared larvae.

Since it was not possible to dissect out, or analyse mercury in the different organs, as for the adults, it was thought that it might be possible to use X-ray microanalysis to locate the mercury and gain an understanding of at least the different ratios of mercury content in the different locations if not to get an actual measure of the concentrations.

X-ray analysis has been used successfully to locate various metals even within cells (eg calcium, zinc, copper, iron, barium, and cobalt) although it has not been used for mercury except in one study of the location of mercury in the gills of the rainbow trout (Olson and Fromm, 1973).

Method for rearing lobster larvae

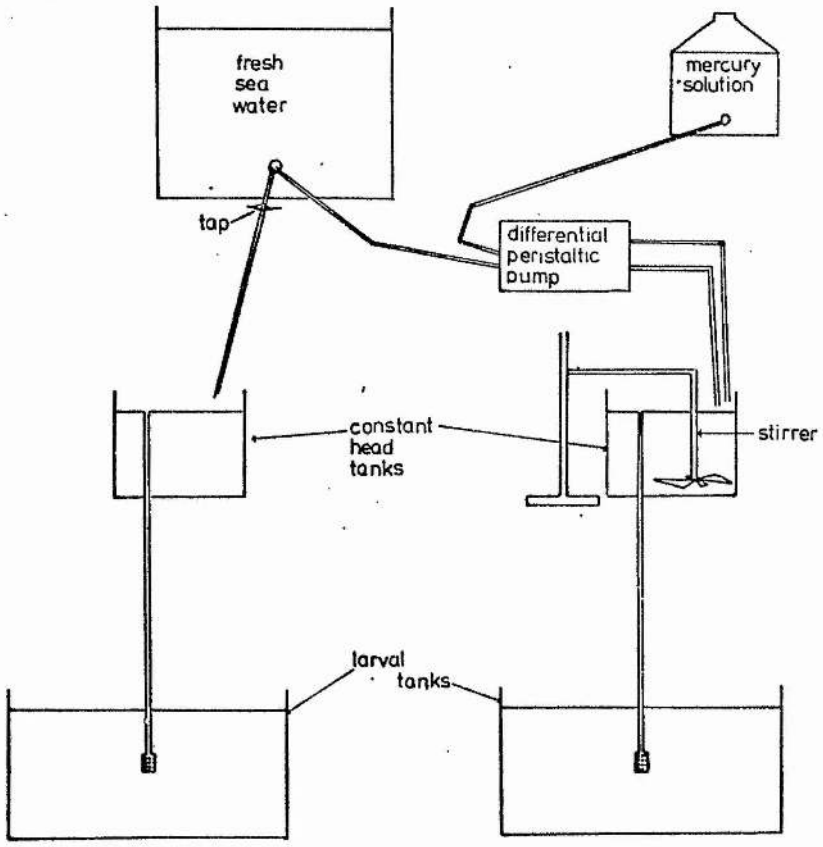
Lobster larvae were reared in rectangular tanks measuring 26 cms by 33 cms by 36 cms. A constant flow system was set up such that a slow stream of pure sea water flowed into one tank from a constant head device, while to the other, a slow steady stream of 100 ppb mercury in sea water solution was delivered. The 100 ppb mercury solution was made using a differential peristaltic pump delivering pure sea water and a stock solution of mercury at different rates into a mixing tank which was stirred constantly with a mechanical stirrer (see diagram 2). It is essential to rear lobster larvae in running sea water. The differential pump was used to cut down loss of mercury prior to adding to the rearing tank, although in practice, the differential provided by the pump only allowed for a difference in the rate of delivery of 64:1. Therefore the stock solution could not be concentrated enough to really minimise loss. It is likely therefore that the mercury level in the tank was less than 100 ppb even though the stock solution was changed every twenty four hours.

The overflow from the mercury tank was collected in a large tank (179 cms by 89 cms by 38 cms). After a week the solution was acidified and the mercury was precipitated as mercuric sulphide, using ammonium chloride as the precipitating agent. The precipitate could then be disposed of.

130 freshly hatched larvae were placed in each tank. They were fed on newly hatched Artemia salina, a diet found to give a higher percentage survival in the larvae of the american lobster under culture conditions than other diets (Hughes, Shleser, and Tchobanoglous, 1974).

Diagram 2

Diagram of apparatus used in larval rearing experiment



Part 1 - X-ray microanalysis study

Method

The main purpose of this experiment was to measure the uptake and location of mercury within the larvae. Larvae from both rearing tanks were sampled each day. They were placed in liquid nitrogen. Half of the daily sample was split longitudinally using a new razor blade and the rest were left whole. All samples were dehydrated through the alcohols to amyl acetate and then dried in carbon dioxide by the critical point method.

A few larvae from each tank were fixed in 2% glutaraldehyde and sucrose solution to give an osmolarity of 900 m osmols. They were then embedded in epon resin. These embedded samples were then sectioned longitudinally using the vertical head of a milling machine fitted with end mill cutters.

The specimens were fixed on graphite blocks and coated with spectrographically pure carbon (Johnson Matthey Metals Ltd). They were then examined in a Cambridge Mark 2A Scanning Electron Microscope fitted with an Edax energy dispersive X-ray microanalyser at the Marine Station, Menai Bridge.

Results

No trace of mercury was found in any sample after preparation by either critical point drying, or fixing in glutaraldehyde and embedding in epon.

This could be because the larvae had not accumulated any mercury at all. The more likely explanation is that this lack of positive results illustrates the difficulty of analysing for a volatile substance such as mercury. Any of the stages of preparation may have resulted in the loss of mercury by vaporisation. The stages that are particularly suspect are the drying by the critical point method, and the vacuum coating of carbon. The epon embedded samples should not have lost mercury during the preparation but they still have to withstand the vacuum in the Edax machine itself. The electron beam may also cause vaporisation.

After the failure to record mercury by this method, three larvae that had been used in these X-ray microanalysis studies were analysed for mercury using neutron activation analysis (Experiment III, Table I). The mercury content was found to be less than 0.1 ppm but this may show that the mercury had been lost in the preparation stages, (these larvae being ones that had been dried by the critical point method), and not necessarily that the mercury had not been there to measure initially, nor that the X-ray microanalysis method was not capable of measuring mercury content.

It was therefore considered worthwhile to use the technique to attempt to locate mercury within the gills after exposure of the adult lobster to mercury.

Measurement of mercury in the gills and green glands of adult lobsters following exposure to 100 ppb for seven days by X-ray microanalysis

Introduction

Following the analyses of mercury in the tissues of the adult lobsters, using NAA and radio isotope tracers, and the finding of high levels in the gills, there was an interesting problem as to whether the mercury was located in the cuticle of the gills, or was concentrated in the specialised excretory cells, or was just accumulated throughout the cells of the gills. It was thought that it might be possible to locate the mercury using X-ray microanalysis since the levels of mercury would be high (up to 64 ppm). It also provided an opportunity to see if the lack of results with the lobster larvae were due to lack of uptake of mercury by the larvae, loss of mercury during preparation, or the inability of the X-ray microanalyser to record the presence of mercury.

Method

Samples of gills and also green glands were taken from a lobster that had been exposed to 100 ppb for seven days (and therefore by comparison with the results from NAA the gills and green glands should have levels of mercury of 64 ppm and 33 ppm respectively). They were prepared by the same methods as those used for the lobster larvae.

These were examined in a Link systems energy dispersive X-ray microanalyser at the Biology Department, University of Dundee. A standard curve was first obtained using crystals of mercuric chloride

mounted on Silverdag (Acheson Colloids Ltd). Other samples of the same tissue was also examined in the same machine as used for the larval studies.

Results

Again, none of the samples, prepared by either preparation method, gave any results that corresponded at all to the standard curve.

Discussion of the results from X-ray microanalysis

It is possible that mercury is too volatile to be measured by X-ray micronalaysis. This technique has however been used to locate mercury in the gills of the rainbow trout, Salmo gairdneri, (Olson and Fromm, 1973). They found that methyl mercury was too volatile to be measured but they were able to measure high concentrations of inorganic mercury associated with the gill cartilage after the fish had been exposed to 250 ppb mercury (as mercuric chloride). The actual concentrations of mercury present in the gills are not given so it is not known if the levels they were measuring were higher than the levels present in the samples in this study.

Olson and Fromm (1973) prepared their tissue by freeze-drying which could also account for differences in the results. There may also have been too much interference from the lobster tissue since Olson and Fromm measured mercury in inverse proportion to other ions present. They also found that the peaks of mercury that they measured were mainly due to the mercury the trout had been exposed to the hour before sampling. This mercury was trapped in the inter-lamellar mucus. It may therefore only be possible to effectively measure surface mercury and in this study the surface mercury would probably have been removed by the preparation methods.

Thus it has been possible to locate mercury in other tissues with this technique but only to a limited extent. It certainly should be possible, in spite of the volatility of mercury, since mercury has been used as an ultra-histochemical stain for use in electron microscopy (see Kendall 1965; Mundkur 1964; Smith and Fishman 1969; Formanek and Formanek 1970). However, these techniques usually use mercury as part of a complex organic molecule. On the other hand Pepe and Feick (1961) also used a complex organo-mercurial compound as a stain for electron microscopy, but found that the mercury was reduced to its metallic form when the araldite-embedded specimen was exposed to the electron beam, and then either migrated in the section, or volatilised. They found that coating the tissue with carbon effectively prevented this loss. Carbon coating was used with the larvae, and the gills and the green glands were examined both with and without carbon coating. It is possible that levels were too low to measure in the larvae in any case, but that in the gills and green glands the mercury volatilised. However, it could be that since it is only the surface mercury in the inter-lamellar

spaces of the gills in the trout that was measured in the work of Olson and Fromm (1973) the mercury in the green gland and gill samples was bound too tightly within the tissue to be recorded.

With energy dispersive systems of X-ray microanalysis the limits to detection are said to be 10 ppm in terms of weight concentration or 10^{-15} - 10^{-11} gm in terms of absolute weight depending upon the element (Kimoto 1972). It should therefore have been possible to measure mercury at least in the green glands and gill samples. Kimoto (1972) does say that with large samples there may be swamping of the element that is being measured by X-rays from other elements present. This is however supposed to prevent accurate measurement but not to prevent recording of mercury at all. But this reason might account for mercury only being recorded in the interlamellar spaces of the trout gills (Olson and Fromm, 1973). It might also have been expected that if the mercury was adsorbed on to the external surface of the gills in the lobster, that it would have been able to record it by this method. This technique may therefore be indicating a site where mercury is not accumulated but no reliance can be placed on that.

There are therefore limitations to this method for measuring mercury and Olson and Fromm's work is the only work where mercury has been measured by this technique. Since it is such a convenient method to use for the location of other metals it would be useful if more work could be done to improve its potential for use in mercury studies.

Part II Assessment of the effect of inorganic mercury on the lobster larvae

Method

Subjective observations were made on the behaviour of the larvae in each of the two rearing tanks. Herrick (1895) describes how the normal healthy larvae of Homarus americanus float on the surface of the water and are a transparent bluish colour. Distressed animals turn red and tend to sink to the bottom of the holding tank. This appears to be the case for H. gammarus as well (personal observation) and these criteria of position in the tank, and colour were used in these rough observations (see Table V) to give an indication of the health or otherwise of the larvae. (Colour is not necessarily a guide to the vigour of the larvae and can be used as an indication only - Herrick 1895.) Mortality was also recorded but this can also only give an idea of possible differences between the two tanks since mortality will be high in any case with these methods of rearing. Without special water circulation devices it is impossible to prevent clumping of the larvae, and this leads to a high rate of cannibalism (Hughes, Shleser and Tchobanoglous, 1974). However, the mortality of the larvae could not be recorded in the initial stages of the experiment because any weak or dying larvae would have been eaten by the other larvae, and it was not possible to assess the mortality by counting the remaining larvae when the numbers were high.

Results from observations on lobster larvae reared in 100 ppb mercury

The results are presented in tabular form in Table V. This shows that there is possibly a greater degree of vigour in the unpolluted larvae but this initial advantage appears to be lost by the tenth day with greater numbers of larvae surviving in the polluted tank. However the proportion of second stage larvae is lower in this tank. These results are not significant since these rearing conditions are too far from ideal in any case. Lobster larvae are notoriously difficult to rear. Ideally the larvae would be reared in circular tanks with devices to prevent the clumping that leads to cannibalism (Hughes, Shleser and Tchobanoglous, 1974).

Since there does not appear to be a great difference in the mortality and effects on the larvae between the two tanks, contrary to expectations, a further short term experiment was devised to show how the larvae behave in a range of mercury exposure levels. It was likely that the levels of mercury in this experiment were lower than the calculated level. It was therefore relevant to obtain a guide to the behaviour of the larvae at different mercury levels so that the level actually used in this experiment might be indicated.

Investigation of the effects of a variety of mercury levels on the larvae of the lobster

Method

Ten newly hatched lobster larvae were placed in each of seven three litre jars containing 1 ppm, 500 ppb, 100 ppb, 50 ppb, 10 ppb and 5 ppb of mercury as mercuric chloride respectively with controls in

TABLE V

Observations on lobster larvae kept in pure sea water, and sea water containing 100 ppb mercury

Water temperature 13°C

130 larvae in each tank at the beginning of the experiment

	Pure sea water	Sea water with 100 ppb mercury
Day 1	None on bottom	40% on bottom but still all blue
Day 2 am	All at surface or near it. None on bottom for more than ten seconds at a time.	3 are orange. Some cannibalism apparent. Mostly on surface
pm	More on bottom. Majority still blue.	Much 'fighting' due to bunching in corner. About 50% red.
Day 3 am	5 still floating, rest on bottom	4 reddish larvae still floating. All the rest on bottom and many on their backs. A few are still blue
Day 4 am	2 floating; rest on bottom.	2 floating; rest bunched in corner.
	---------- Great deal of cannibalism. ----------	
pm	All on bottom except for 4.	Many appear dead but move spasmodically.
Day 5 am	----- None floating. Some in both tanks are blue --------	
pm	None floating	4 floating and appearing healthy
Day 6 am	1 floating. 14 dead removed	4 floating and appearing healthy. 4 dead
pm	6 dead removed	2 dead removed. Much 'fighting'
Day 8	3 dead removed	9 dead
Day 9	31 still alive, 24 moulted to second stage	29 alive, 17 moulted to second stage
Day 10	---------- None of surface ----------	
Day 11	9 still alive	15 still alive

pure sea water. The behaviour and mortality of the larvae were observed for forty eight hours and the observations were tabulated.

Results

TableVI summarises the observations. In the two highest concentrations all the larvae were dead within twenty four hours. In the other concentrations after thirty six hours, while the mercury was not acutely toxic, no larvae appeared healthy, even in pure sea water, and this illustrates the disadvantage of this rearing technique.

Discussion

It is only possible to say from this experiment that the two highest concentrations are acutely toxic. At lower concentrations there is no significant difference in mortality between exposed larvae and controls and far larger samples would be required to show such a difference.

However it may be possible that stage I larvae are genuinely more resistant to mercury while later stages which were not tested are more susceptible. De Coursey and Vernberg (1972) besides finding a great susceptibility to mercury in the larvae than adults of Uca pugilator also found that stage V larvae had less resistance to mercury than stage I larvae.

The findings of this study, although very limited, do not differ greatly from those of Connor (1972) with lobster larvae in mercury. He found that while mercury at levels of 100 ppb caused death of fifty per cent of the larvae within twenty three hours, the toxicity at lower concentrations dropped sharply so that at 10 ppb the time to kill more than fifty per cen (ET50) was more than 100 hours.

TABLE VI

Observations of the effects on lobster larvae of exposure to various levels of mercury pollution

Water temperature 14°C

	After six hours	After twenty four hours	After thirty six hours
1 ppm	Mostly lying on sides on bottom. Very few spontaneous movements.	All dead.	-
500 ppb	7 still bright blue but all on the bottom	All dead.	-
100 ppb	All swimming on surface	All on bottom but still moving.	All on bottom, virtually dead.
50 ppb	4 on bottom.	1 not on bottom	" " " "
10 ppb	2 on bottom	3 on surface	2 on surface
5 ppb	2 on bottom	3 on surface	All on bottom
sea water	2 on bottom	1 on surface	All on bottom

This does not however explain the lack of difference in the mortality rates between the larvae in the two tanks in the first larval experiment. It must therefore mean that the concentration of mercury resulting from the use of the constant flow system was much lower than the calculated level due to mercury loss.

This type of experiment really needs to be done with very large samples of larvae as mortality will tend to be high in any case unless ideal rearing conditions are available. For example, Hughes, Shleser and Tchobanoglous (1974) had a maximum survival rate of 75 - 80% with special rearing tanks and a diet of Artemia salina, but with less well suited food but still in special rearing tanks the survival rate dropped to 10 - 12%.

But there is an indication that lobster larvae are not as susceptible to mercury as other larval forms, eg oysters and shrimps (Connor 1972), and possibly they are not therefore as relevant to such studies as other larvae. This is based on results from toxicity studies, but De Coursey and Venberg (1972) were able to demonstrate effects on the behaviour and long term survival of larvae with levels as low as 1.8 ppb and even a reduced ability to survive at 0.018 ppb. It might be interesting therefore to use more sophisticated techniques to determine if lobster larvae were similarly affected.

The survival rate of such larval forms is, however, so small in nature, that such changes may be of little relevance. This question will be referred to later in the General Discussion.

f) Part I Histological study of tissues most likely to be affected by long term exposure to low levels of mercury

Introduction

Chemical agents can be expected to cause damage at their sites of entrance or exit from the body, or at the sites of greatest accumulation (Sparks, 1972). The green glands and the gills of the lobster have been identified in this study, using the NAA and radioisotope tracer techniques, as the tissues where the highest concentrations of inorganic mercury occur. It is not known for certain from this study whether the gills actively excrete the metal as well as concentrating it, but the green glands have actually been shown to excrete the metal. The green glands are then probably even more likely to show histological damage following exposure to mercury, and therefore a brief description of their structure and mode of functioning will be given here. It may be possible to predict from what is known of the excretion mechanism what damage mercury is likely to cause.

The structure and function of the normal green gland of Homarus gammarus

The green glands are recognised as the main excretory organs of the lobster. They are a pair of structures, one situated at the base of each antenna. Each one consists of three main parts: (i) an internal end sac or coelomosac, separated by a sphincter from (ii) the tubular section, the labyrinth, and (iii) a bladder. A short ureter leads from the bladder to the opening, or nephridiopore, situated at the base of the antenna on the ventral surface. The coelomosac and labyrinth are easily distinguishable histologically. The labyrinth

cells are clearly arranged in very convoluted tubules, while the coelomosac cells are smaller and less regular in shape and of less definite arrangement (Plates 1 - 4). Both areas have a rich blood supply from the antennal artery (Peters, 1935).

There has been a great deal of debate about the mechanism of urine production by the green gland. The two main possibilities are that the primary urine can be produced by secretion, or by ultrafiltration. (Ultrafiltration is the bulk movement of a solution through a porous membrane by the application of a head of pressure.) At various times the argument has been resolved in favour of secretion being the principle process (eg Maluf, 1941a), or in favour of filtration being the main process (Riegel and Kirschner, 1960). However, Peters had suggested as early as 1935 that a filtrate of the blood is formed in the coelomosac, and that this primary urine is added to from the secretion through the wall of the labyrinth; secretion droplets being clearly visible extruding through the labyrinth cells in histological sections of this region (Peters, 1935) (see plate 4 (s)).

Further evidence for this dual mechanism is suggested by the finding of Kirshner and Wagner (1965) that the green gland of the crayfish is only freely permeable to dextrans of molecular weights of up to 20,000 to 40,000. An alternative method must therefore be available for the excretion of larger molecules. Riegel (1966 a and b) describes formed-bodies obtained from both the coelomosac and the labyrinth. He suggests that there are involved in the secretory process. The coelomosac cells are also seen to take up materials in a similar way to the labyrinth cells, eg calcium (Maluf, 1941b). Size may not

therefore be the sole method of determining how a substance is excreted.

Riegel (1966b) suggests as a possibility, that relatively neutral molecules (such as lipids, sugars, and conjugated proteins) are eliminated by the coelomosac, whilst charged particles (dyes, unconjugated proteins, etc) are eliminated by the labyrinth.

Thus it seems that secretion occurs in both the labyrinth and the coelomosac, and that filtration probably only occurs in the coelomosac. However, it is not known definitely what materials would be excreted in a particular manner. The way in which mercury might affect the green glands cannot therefore be deduced from what is known of the green glands.

Both the green glands and the gills were examined histologically for signs of cellular damage. The digestive gland was also examined. This tissue was not shown to accumulate mercury to anything like the same extent as the other two tissues, but there was some evidence that it might act as a storage system for mercury at levels higher than those with which the green glands can cope. Also, other workers have found cell degeneration in this tissue in the american lobster, Homarus americanus, following exposure to yellow phosphorus (Aiken and Byard, 1972).

Material and methods

Treatment of lobsters

Intermoult lobsters were kept in tanks containing either 100 ppb or 10 ppb mercury, or pure sea water, as controls, as described in the general method (page 51). The length of the exposure was usually thirty days, but some lobsters were kept for longer periods, up to a maximum exposure time of forty nine days.

The experiments were carried out in two main series: one in the period from April to June, when the temperature ranged from 7° C to 9° C, and the other series in October and November when the temperature range was 12° C to 13° C.

The lobsters were tested each day to see if urine was being produced since it was noticed that if the lobsters became anuric, death would occur within a short period. (This testing was done in the manner described for the collection of urine in section d.). If urine was not produced for two days the lobster was killed while still showing muscle tone in the extensor muscles. (Once a lobster has lost the muscle tone in the abdomen, death is inevitable.)

Organ preparation for histology

After the exposure period, or after cessation of urine production, the lobsters were killed, and the green glands, samples of gills, and digestive gland were fixed in sea water Bouin's solution for a minimum

of thirty six hours.

The samples were dehydrated through an alcoholic series to tertiary butyl alcohol and embedded in paraffin wax. They were then sectioned with a rotary microtome and for routine examination they were stained with Ehrlich's acid haemotoxylin and counter stained with alcoholic eosin.

Histochemical methods used to identify material forming the 'brick-red' lesion

Other stains were used in the course of the investigaiton, for histochemical studies. These were:

a) Masson's trichrome stain

b) Long Ziehl-Neelson

c) Melanin bleach

d) Masson's fontana method

e) Bethe's test for chitin

f) Iodine in potassium iodide with zinc chloride method for chitin, (Schulze's method)

g) von Kóssa's method for calcium

h) X-ray micronalaysis was also used to determine the nature of the lesion

Photography

Photographs were taken with a Hasselblad 500 fitted on a Leitz Laborlux microscope.

Results

Gills and digestive gland

Histological examination of the gills and the digestive gland showed no apparent cellular changes due to the mercury, when compared with the control tissue. No further studies were made of these organs.

Green glands

In the first experimental series, two lobsters that had been kept in 100 ppb mercury for 37 - 38 days showed obvious lesions in the green glands. One of the glands in each of the lobsters, instead of appearing as green-yellow soft tissue as normal, had a sheet-like deposit of hard red-brown material over the gland. A similarly coloured hard material was also found within the coelomosac on sectioning one of the glands of a lobster exposed to 10 ppb mercury. In this first series, the lesion, which for convenience will be referred to as the 'brick-red lesion', was found in three out of four mercury exposed lobsters. No similar structure or colouration was seen in any control tissue, or in any lobster that had been taken from the holding tanks for other experimental purposes (at least twenty five lobsters). This lesion was not found in any of the lobsters exposed to mercury in the second series. Nevertheless, there is a clear sequence of histological changes found in the green glands following exposure to inorganic mercury.

a) Controls

The control tissues can be seen in plates 1 - 4. The normal labyrinth cells are shown to be clearly arranged in tubules (plates 1 and 2). The cells are regular in shape and have rounded nuclei with normal distribution of chromatin. The luminal surfaces of the cells have clearly visible brush borders (bb). Blood spaces (b) with blood cells (wandering cells (w), Peters 1935) can be seen between the tubules.

The coelomosac lies outside the labyrinth. The cells of the coelomosac are small and irregular in shape and the lumen (l) of the coelomosac is large (plate 3). The epithelium of the coelomosac appears to be arranged in folds, thus compartmentalising the lumen.

The relationship between the coelomosac and the labyrinth is illustrated in plate 4. Blood vessels (bv) can be seen to be distributed around the margin of the coelomosac. Areas where active secretion is occurring can be seen in the labyrinth (s). (These are the 'secretory bubbles' [sekretenblasen] of Peters 1935.)

b) Changes in the labyrinth following exposure to mercury

The first signs of cellular damage following exposure to mercury are found in the labyrinth. The cells become vacuolized in the apical region giving the brush border the appearance of bulging into the lumen. In places the brush border appears to be lost (plate 5). Nuclear damage is also seen in restricted areas. The initial stages of this are marked by clumping of the chromatin around the nuclear

membrane. The nuclei can also be seen splitting apart (karryorhexis) (kn). This is seen after forty nine days at 10 ppb (plate 5).

Greater damage follows, and after thirty days at 100 ppb large scale cellular disintegration has occurred and the brush border has been lost in many places (plate 6). In many parts there is complete cytoplasmic and nuclear breakdown, karryolytic nuclei can be seen (kn) and also, cytoplasmic remnants with pyknotic (shrunken) nuclei in the lumen (cr).

After forty seven days at 100 ppb the labyrinth cells have mostly become completely shrunken and there is much cytoplasmic debris in the lumen, obvious karryorhexis, and a general lack of cellular organisation (plates 7 and 8).

However, in areas of the labyrinth, fairly normal organisation can still be found. Plate 9 shows an isolated area of the same labyrinth illustrated in plates 7 and 8. This area still shows a fairly intact brush border although the cells are somewhat vacuolized and swollen in appearance. Cell damage appears to progress from the exterior of the labyrinth inwards.

c) Changes in the coelomosac following exposure to mercury

The swelling of the cells of the labyrinth is followed by disorientation of the coelomosac tissue. After thirty days exposure to 100 ppb mercury (second series of exposures) the coelomosac is somewhat disorganised and showing signs of nuclear breakdown (kn) (plate 10).

After fifty days at the lesser concentration (also second series)

the disorganisation is more noticeable and cytoplasmic disintegration and nuclear breakdown is more apparent (plate 11).

In the first series of experiments, the changes in the coelomosac are far more extensive. After exposure to 100 ppb mercury for thirty seven days, the disorientation of the coelomosac becomes complete and there are gross changes to the cellular arrangement. In some areas there are dense masses of cells (top half of plate 12), while there is oedema in other areas (bottom half of plate 12). There appears to be an influx of different cell types; most prominent of these are cells with long processes and elongated nuclei (f). These resemble the usual fibroblast cells of vertebrate tissue (Bloom and Fawcett 1975). Other cell types involved are larger cells (h), possibly hemocytes (plates 13 and 14).

In some regions of the coelomosac 'granulomas' develop (g) (plates 15 - 18). These will be referred to later.

The general progression is such as to give the impression of a chronic inflammatory condition (plate 19). Typical features of such a reaction are the generalised necrosis of cells with oedema and cellular disorientation, formation of granulomatous tissue and at the same time, the inlux of fibroblast type cells signifying tissue repair.

The 'brick-red lesion', that has already been mentioned as the most obvious change occurring in the first series of exposures, can be seen in plate 20. Whilst after exposure to 100 ppb mercury, the sheet of hard material was found lying over one of each pair of green glands, after exposure to 10 ppb mercury, the lesion was only found on sectioning the gland as the lesion was embedded deeply in the coelomosac (plate 20). In plate 20 the lesion can be seen to be encased in necrotic, eosinophilic tissue. A clear gradation can be seen from

fairly normal cells (i), progressing to necrotic eosinophilic areas (ii), to the 'brick-red lesion' itself (iii). The lesion is bounded by cells with elongated nuclei (bc). These form a boundary to the lesion.

The invasion of the fibroblast type cells (plates 13 and 14) suggest that tissue repair is in progress, which is characteristic of chronic inflammatory conditions as already said. However, this repair process may in fact have some success since the lesion does appear to have become completely walled off from what appears to be fairly normal coelomosac tissue in one of the 100 ppb lobsters (plate 21).

Study of the formation and nature of the 'brick-red lesion', and the granulomatous changes in the coelomosac

The changes in the green glands following exposure to inorganic mercury are very interesting as a whole, but the most striking finding was that of the 'brick-red lesion' in three out of four lobsters in the initial series of exposures, that is in the April-May series. Two of these lobsters had been in 100 ppb for thirty eight days, and urine production had continued until two days before the lobsters had been killed. In these the lesion was seen as a sheet of hard chitinous material over one of the green glands. In the 10 ppb lobster, exposure had only lasted thirty days. In this lobster, the lesion was only found upon sectioning the coelomosac. This was in a green gland that had not produced urine for fifteen days before the animal had been killed.

Since there have been no descriptions of similar developments occurring after exposure to heavy metals, or indeed of any similar reaction that is obviously akin to this development, it was interesting to determine the nature and derivation of this lesion.

The most likely explanation is suggested by the finding of the 'granuloma' formations, as seen in plates 15-18.

The response of any tissue to injury or cell destruction is inflammation and this process dilutes, destroys, or walls off the injurious agent and the dead or damaged tissue. Scar tissue is typically laid down by the influx of fibroblasts and the deposition of collagen, as the tissue tries to repair itself (Sparks 1972).

This is what can be seen in the green gland tissue of the lobsters that have been exposed to 100 ppb mercury for thirty eight days. Typical oedema, cell breakdown, and necrosis can be seen, with at the same time, an influx of the sigmoid shaped cells with elongated nuclei similar to the fibroblast tissue of the vertebrates (Bloom and Fawcett 1975).

i) This similarity to the fibroblast cells of the vertebrates was further illustrated by demonstrating the production of collagen from these cells. This was done by the use of Masson's trichrome stain. This showed green staining areas, indicative of collagen, around the fibroblast type cells.

In some areas the actual 'walling off' process can be seen. Eosinophilic granules can be seen surrounded by masses of cells (plates 15 - 18). These are typical 'granuloma' formations. The term granuloma originally referred to the reaction observed in human tissue, to the

presence of the tuberculous bacilli, Mycobacterium tuberculosis, but has since been redefined to include all lesions showing similar features (see Timur 1975). Granulomatous chronic inflammation is characterised in vertebrate tissue by the local accumulation of a variety of cell types, namely macrophages, lymphocytes, plasma cells, fibroblasts, and giant cells (Timur 1975). Such reactions are seen after the introduction into the body of foreign material such as bacteria, and inorganic matter such as talc, silica, and beryllium dust (Ogilvie 1967).

All these cell types may not be present in invertebrate tissue, but the appearance of the reaction is very similar. Such reactions have also been described in response to talc introduced into the cockroach, Periplaneta americana, and in oysters, (Schlumberger 1952; Pauley and Sparks 1967).

The process seen in the lobster tissue in this study is different in that it is not caused by any particulate material. It is probably formed in response to cell destruction. Necrotic tissue is often seen at the centre of these formations in vertebrate tissue and appears as strongly acidophilic granules (Ogilvie 1967). This appearance is seen in the granulomas in the green glands. It is likely therefore that the formation of the granulomas is in response to cell breakdown leading to an accumulation of lipofuscin which as an irritant substance, provokes this 'walling off' reaction. (Cell breakdown first leads to the production of lipoprotein. The animal can resorb the protein but is unable to utilize the remaining lipofuscin.)

It is highly probable that the brick-red lesion has been formed in this way, either due to massive cell death leading to the walling off process occurring on a large scale, or by the gradual accumulation of such granuloma cores.

In order to test this hypothesis, a number of histochemical tests were carried out.

ii) Tests for lipofuscin and other melanin type cell breakdown pigments

All cell breakdown pigments are now known to belong to the melanin class of pigment, even though they may be derived from different sources to the melanin (Edelstein 1971). Selective stains can be used to identify these pigments and were used for this purpose in this study.

a) Long Ziehl-Neelson method

This stains acid fast lipofuscins bright red and lipoproteins pink. This test was positive for the brick-red lesion, suggesting that it was lipofuscin.

b) Melanin bleach

Treatment with hydrogen peroxide for twenty four hours decolourised the pigment. The facility with which the melanins bleach in strong oxidising agents is one of their special characteristics.

c) Masson's Fontana method

This stains melanin granules black. This test gave a negative result but it is known that silver stains are very specific for different tissues, and usually have to be adapted for use with each new tissue. This was not possible in this study.

Therefore, two out of three tests were positive for the lesion being formed of a melanin type substance.

Disproof of other possible explanations for the origin of the 'brick-red lesion'

Since no studies on such inflammatory reactions have been made in the Crustacea, the possibility of the lesion being due to another cause, known in the literature, had to be explored.

One possibility was that the lesion was an anomalous growth of chitin, which it very much resembled. Such a growth was described by Hanstrom (1926) who found a tumour-like growth in the primitive arthropod, Limulus polyphemus. It appeared as a chitinous foreign body near the anterior of the brain, and was found to be formed by the recession of a sac-like portion of the ectoderm into the body, where it continued to secrete successive chitin layers. Schlumberger (1952) also found growths of chitin following injections of talc and methylcholanthrene into the body of the cockroach, Periplaneta americana. (This however appeared to be due to disruption of the tracheal cells which Beard (1942) showed led to increased secretion of chitin.) Goodrich (1928) describes the growth of chitinous nodules in Gammarus pulex L following infection with yeasts.

iii) Histochemical tests for chitin

Two histochemical tests for chitin were therefore carried out (Bolles Lee 1921):

a) Bethe's stain for chitin. This should impart a green colouration at first, becoming blue on addition of tap water. This test was negative.

b) Schulze's test for chitin. This should give a violet reaction. This was also negative.

Another strong possibility was that the lesion was connected with normal premoult changes. This was particularly likely, since the lesion was only found in the first series of exposures.

Riegel (1966) describes a deep orange staining of the coelomosac of the premoult crayfish (Stage D). This is due to the presence of a pigment, the so called 'lipochrome', which is derived from the blood, and is first accumulated from the blood into the coelomosac cells from where it is eventually eliminated. However, the lesion described in this study can be seen to be derived from broken down cells rather than from pigment within healthy cells. It is still possible that it is excessive demands on the excretory powers of the green glands that cause the mercury to have a more destructive effect on the gland than is found later in the year. The lobsters in this study were not in premoult condition, but it is possible that changes do occur in the excretory patterns before the premoult condition is reached.

Maluf (1941a) also describes the presence of anomalous structures in the green gland of a crayfish. These were hard yellowish brown concretions, the size of which showed that they could not have been derived intra-cellularly. The material was found to be insoluble in hot or cold water, and in absolute ethyl alcohol. The alcohol decomposed the surrounding yellowish brown organic material to expose white crystals inside. These dissolved in dilute hydrochloric acid with the energetic release of gas, and were slowly soluble in 10% ammonium chloride, and were therefore taken to be calcium carbonate crystals.

Deposits of calcium may be fairly common in other kidney tissue. For example, it was commonly found in kidney tissue of the wild turbot, Scopthalmus maximus (Anderson et al 1976) and is also a common adjunct of necrosis in vertebrate tissue (Ogilvie 1967).

iv) Test for calcium

Therefore the von Kőssa stain for calcium was carried out. This proved negative. Further evidence for the lesion not being formed of calcium will be provided later.

v) X-ray microanalysis investigation

A further possibility that was investigated was that the brick-red lesion could be formed by the presence of mercury in the green glands interfering with the normal excretion of other ions. Particularly it was thought that it might be cobalt that was being deposited in this form. Cobalt compounds often have a brick red colour, and the element is an essential part of vitamin B_{12} . It is this vitamin, and its derivatives, eg methylcobalamine, that are implicated in the methylation of mercury (see general introduction, page 35).

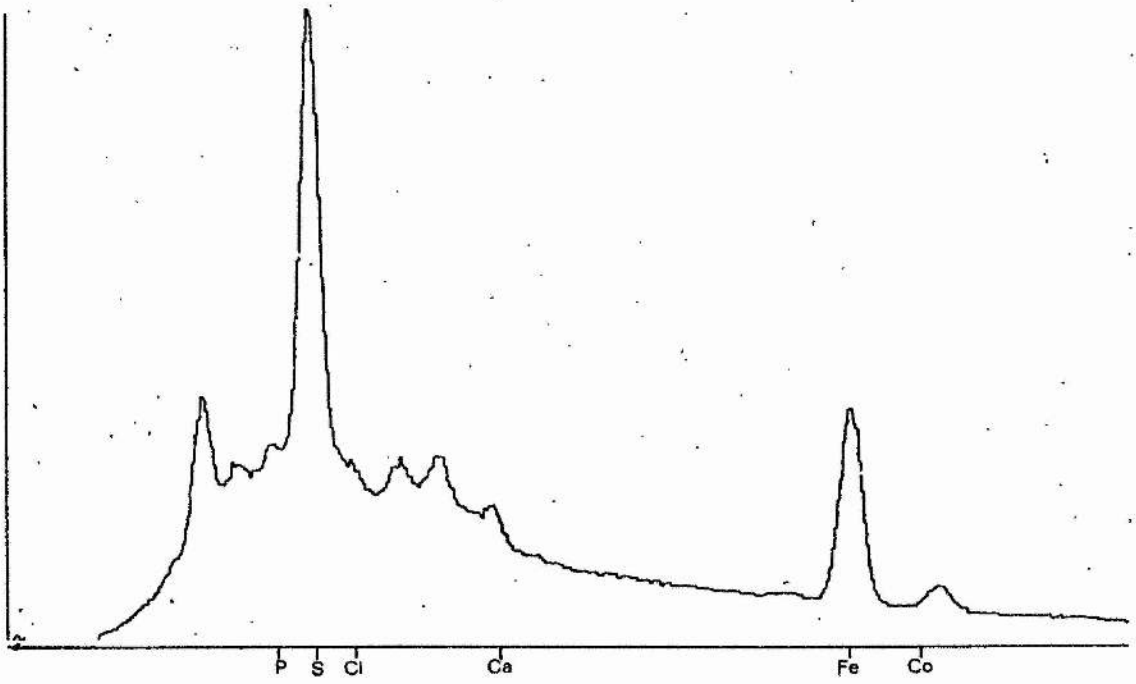
Therefore, a sample of the green gland with the brick-red lesion forming a coating over its outer surface, was dried by the critical point method and then examined in a Link systems energy dispersive X-ray microanalyser, (Biology Department, University of Dundee). The traces are shown in graphs 3 and 4.

While there is a very faint peak near cobalt, there is a very clearly marked peak for iron. The cobalt peak is probably too displaced to be taken of proof of the presence of that metal, but the iron peak is clear and unmistakable.

Copper is the main constituent of lobster respiratory pigment, haemocyanin, and it might be expected that with such cell breakdown, copper might be deposited in the tissue. (Iron pigments, haemosiderin, are commonly found in vertebrate necrotic tissue - Cappell and Anderson, 1974). However, there is no peak for copper.

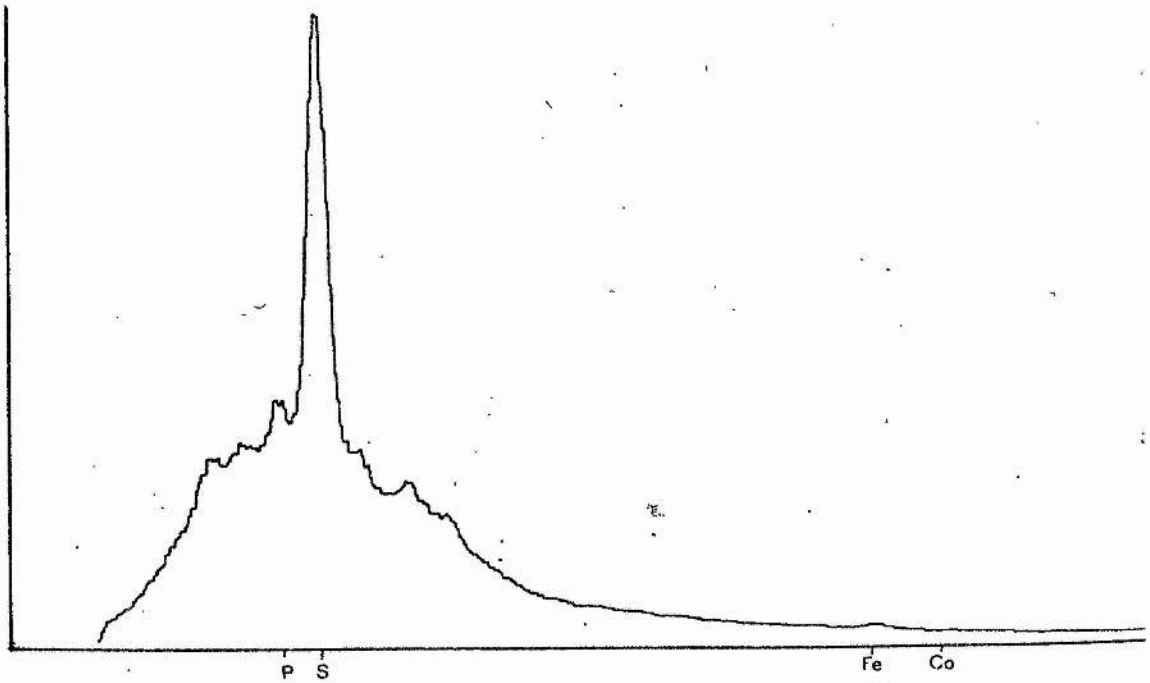
Graph 3

X ray microanalysis of 'brick-red lesion' of green gland



Graph 4

X ray microanalysis of normal green gland.



It is therefore most likely that the brick-red lesion does consist of a melanin-type pigment resulting from gross cell breakdown. (The presence of iron may be explained with reference to studies on the histochemical properties of the melanins. Lillie (1957) found that melanin had an affinity for ferrous ions. X-ray microanalysis does not allow the identification of the valency state of an element, but this property of melanin could be the reason forthe presence of iron.)

In the second series of exposures, there was no brick-red lesion found, out of eight mercury treated lobsters. The coelomosac only showed mild disarray in the 100 ppb lobsters and there may have been fibroblasts present but only to a very limited extent. Cellular damage in the labyrinth was equally severe in both series (see figures 5, 6, 7 and 8). This is difficult to explain. The exposure periods of the second series were all of thirty days except for one lobster that was kept at 10 ppb for forty nine days. However, the second series was carried out at a higher temperature, and so that it would be expected that any pathological change that was going to occur would do so more quickly than in the first series of exposures.

In the second series of exposures, the lobsters were all producing urine when they were killed. In the first series, none of the glands in which the lesion was found had been producing urine within at least two days of the animals being killed. In the case of the 10 ppb lobster with the lesion, no urine had been seen to be produced from the affected gland for fifteen days before killing the lobster. However, there is no way of knowing whether the presence of the lesion was the cause of the cessation of urine production or vica versa. It is also difficult to suggest why the lesion should only occur in one of the pair of glands

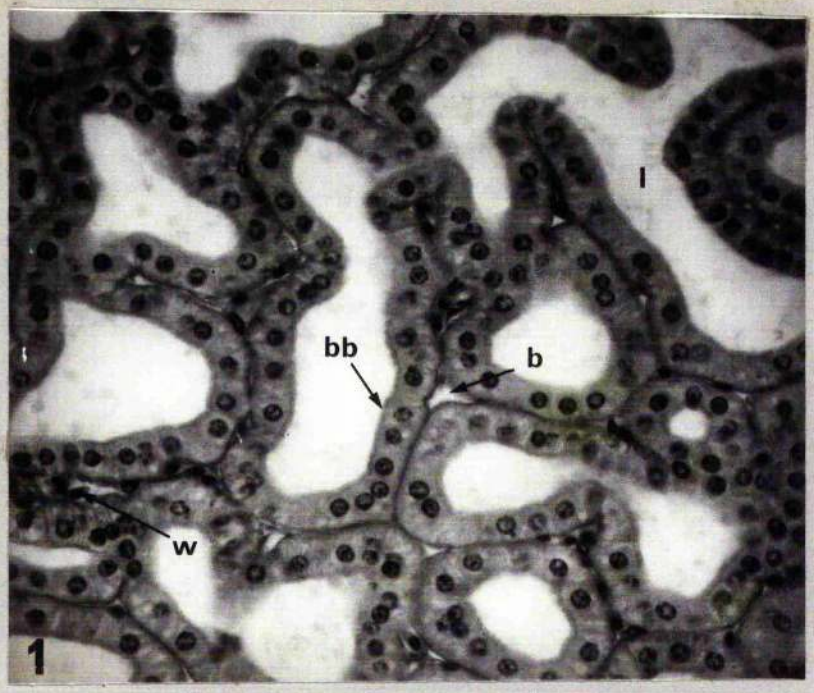
on each occasion it was found. This is the first study to be made of this type of reaction in crustacea following exposure to heavy metals, and much more would need to be done to clarify the matter.

Plate 1 (X 500) Control: shows normal labyrinth.

blood space (b) brush border (bb)

lumen (l) blood (wander) cells (w)

Plate 2 (X 800) Control: as plate 1.


l
bb
b
w
1

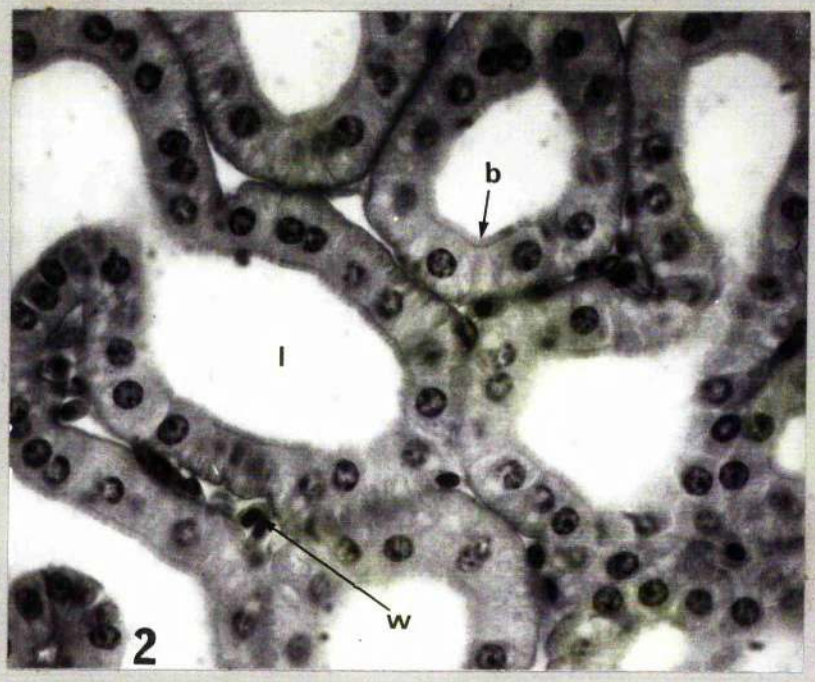
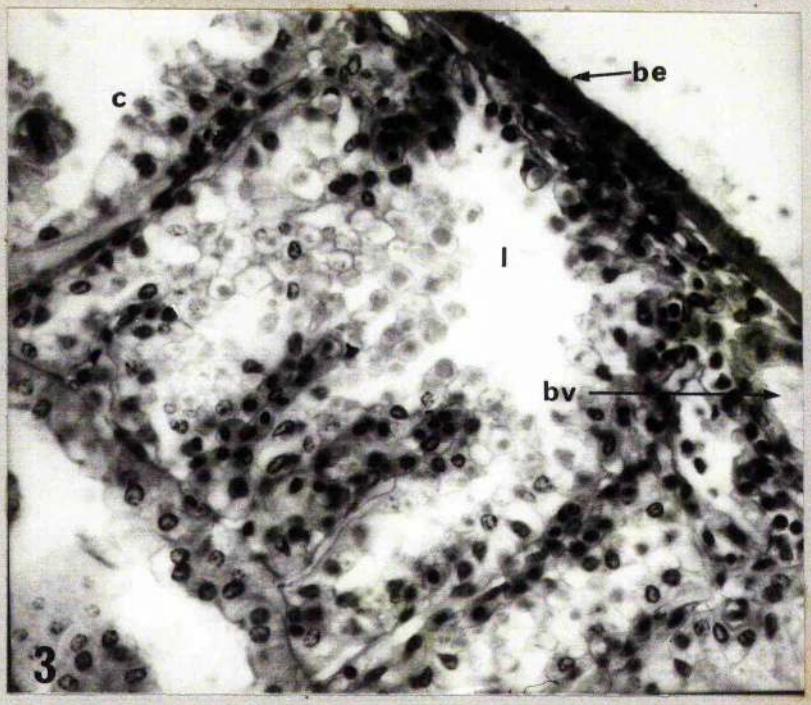

b
l
w
2

Plate 3 (X 500) Control: coelomosac. lumen (l) blood vessel (bv) boundary epithelium (be) labyrinth (la).

Plate 4 (X 200) Control: coelomosac (c) and labyrinth (la) and with secretion droplets (s) in lumen of the labyrinth.


c
be
l
bv
3

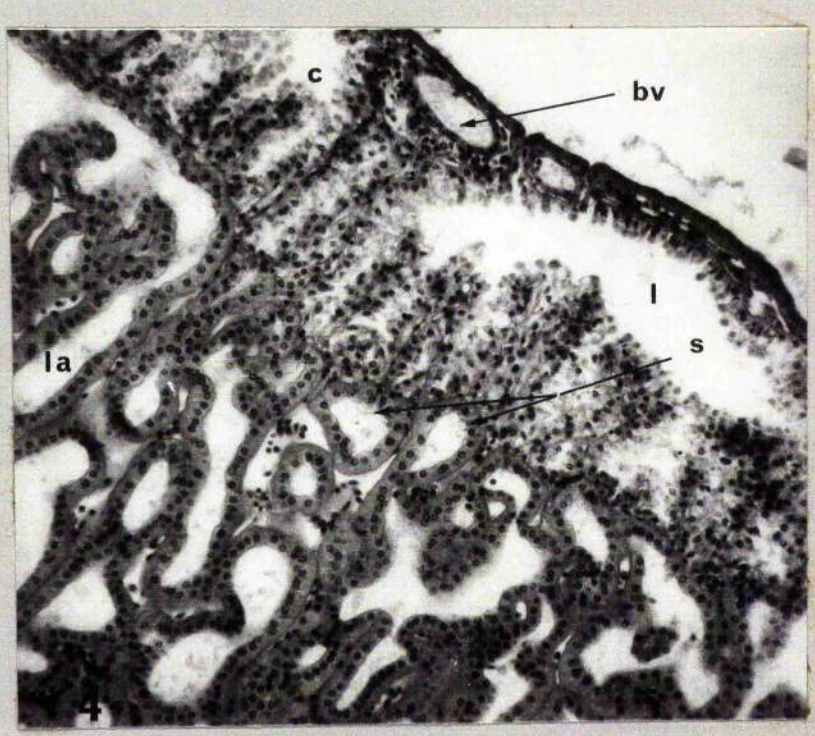
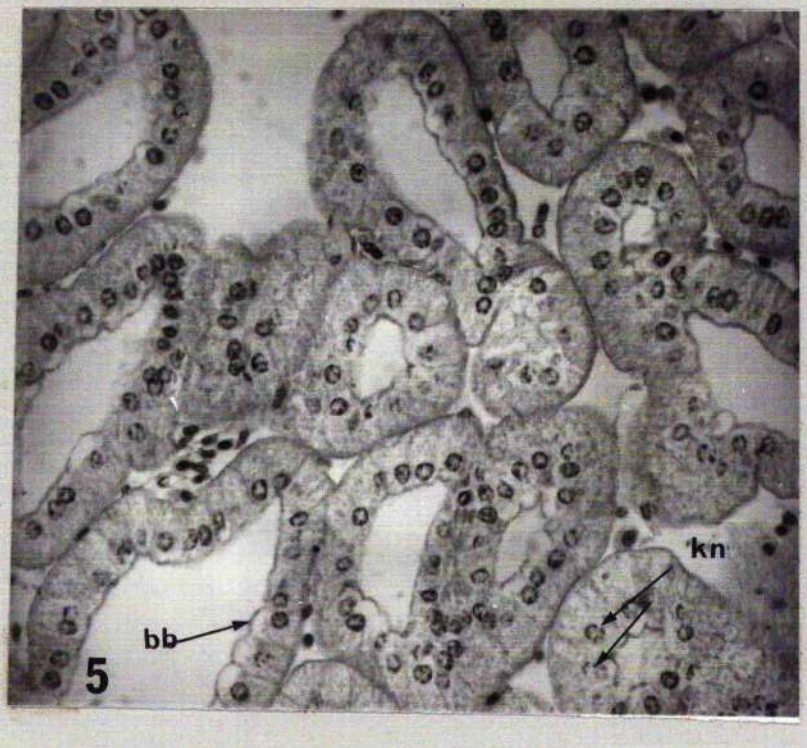

c
bv
l
la
s
4

Plate 5 (X 500)

49 days at 10 ppb. Series 2:
labyrinth cells showing vacuolisation brush borders (bb) displaced into the lumen and initial nuclear breakdown (kn) can be seen.

Plate 6 (X 500)

30 days at 100 ppb. Series 2 :
greater cellular damage, cytoplasmic disintegration, loss of brush borders; karyolytic nuclei (kn) and cytoplasmic remnants with pyknotic nuclei (cr) can be seen in the lumen.


kn
bb
5

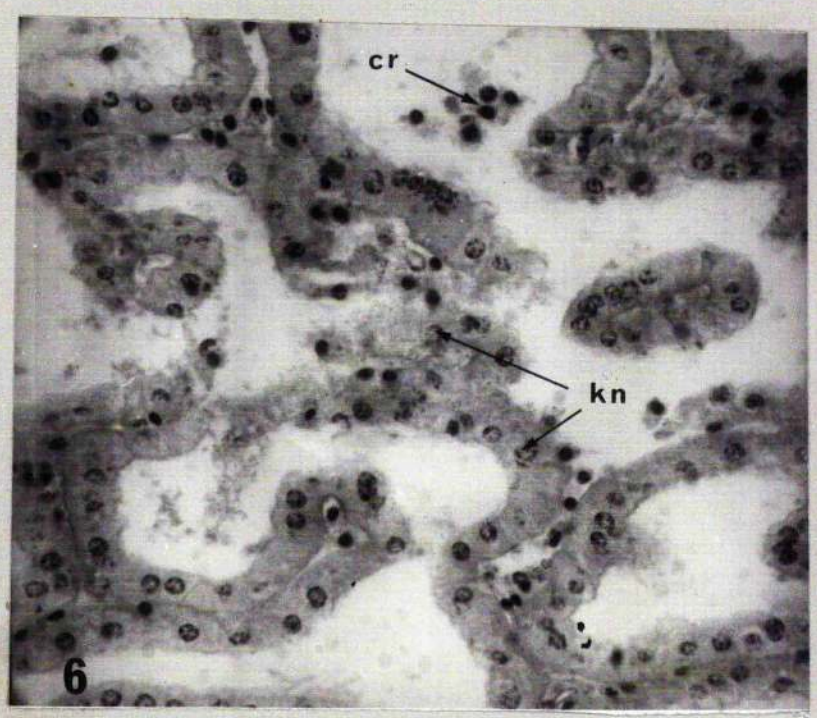
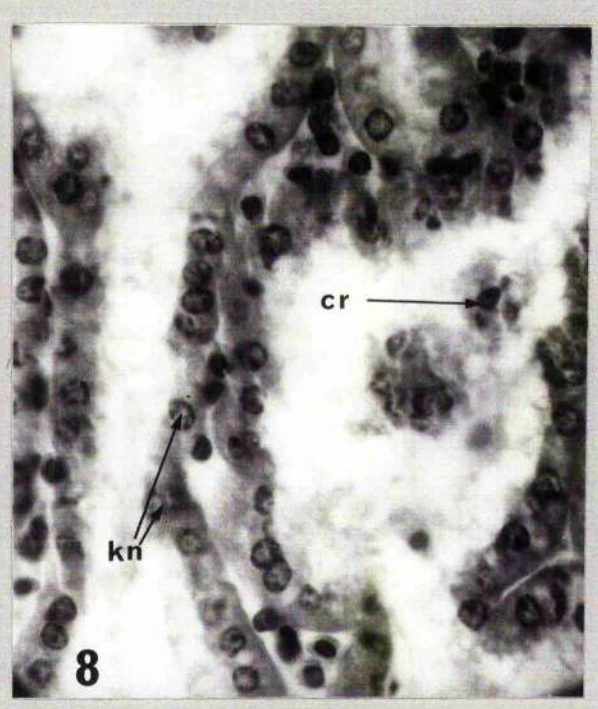

cr
kn
6

Plate 7 (X 500) — 47 days at 100 ppb. Series 1: complete shrinking of labyrinth cells, much cytoplasmic debris in lumen, and extensive nuclear breakdown.

Plate 8 (X 800) — As plate 7: karyolytic nuclei (kn) and cytoplasmic remnants with pyknotic nuclei (cr).

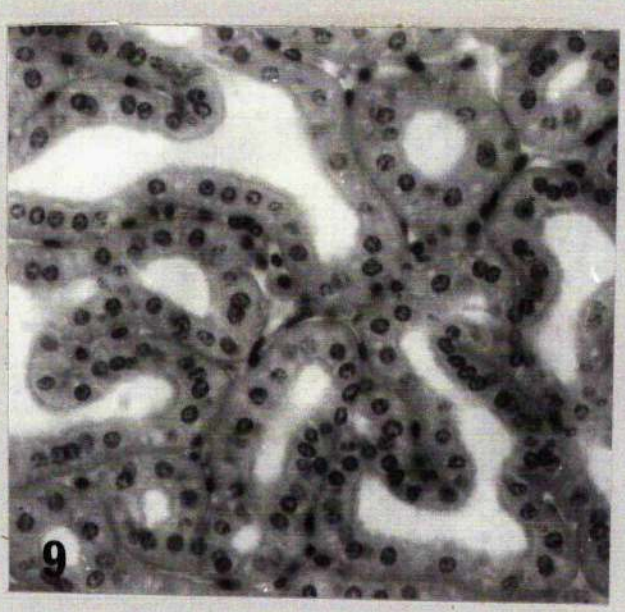
7



8

Plate 9 (X 500) Same green gland as shown in plates 7 and 8: showing the localised presence of healthy cells, although there is some cytoplasmic swelling.

Plate 10 (X 800) 30 days at 100 ppb. Series 2: coelomosac tissue showing some disorganisation and presence of karryolytic nuclei (kn).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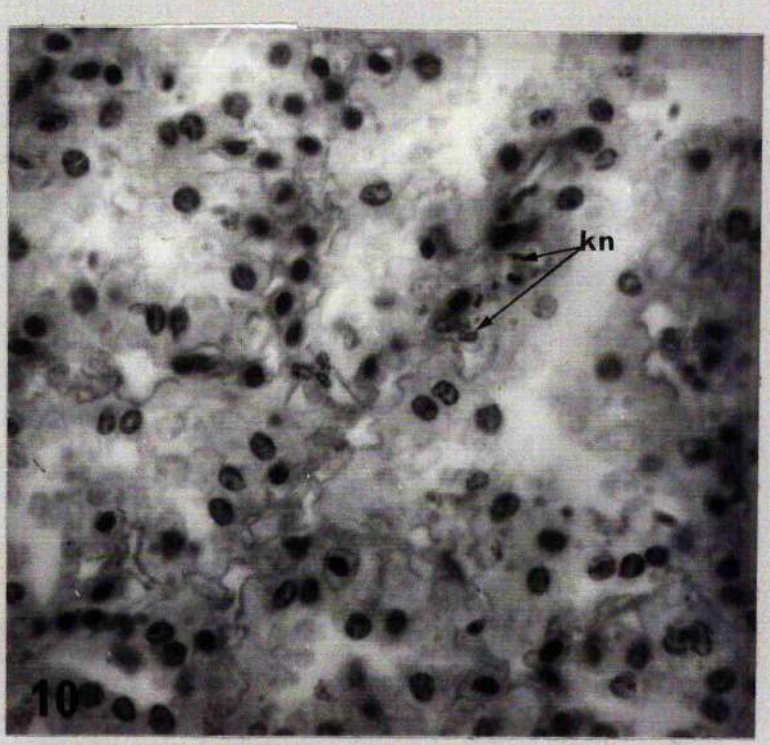
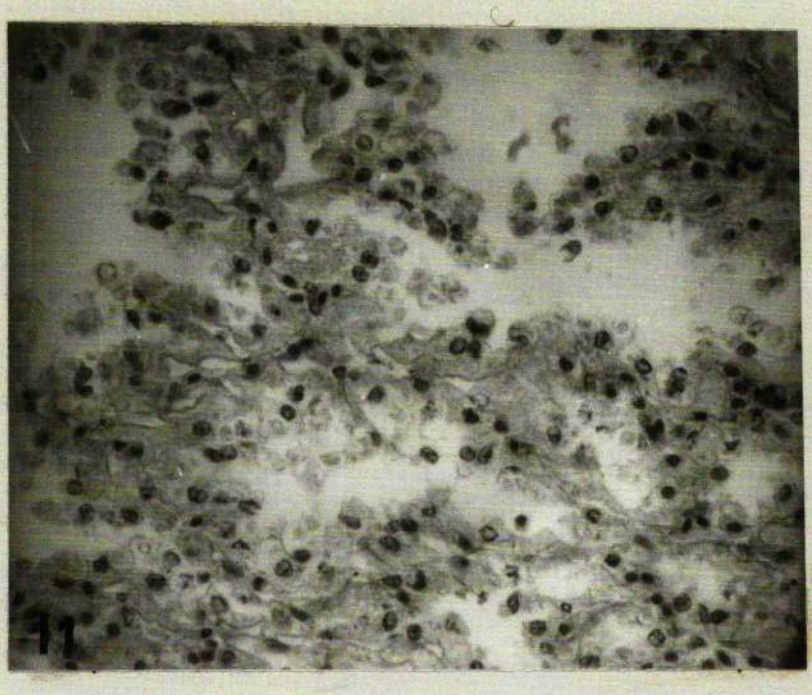

kn
10

Plate 11 (X 500) — 50 days at 10 ppb. Series 2: showing greater cellular disorganisation with cytoplasmic debris in lumen, and pyknotic and karryolytic nuclei can be seen.

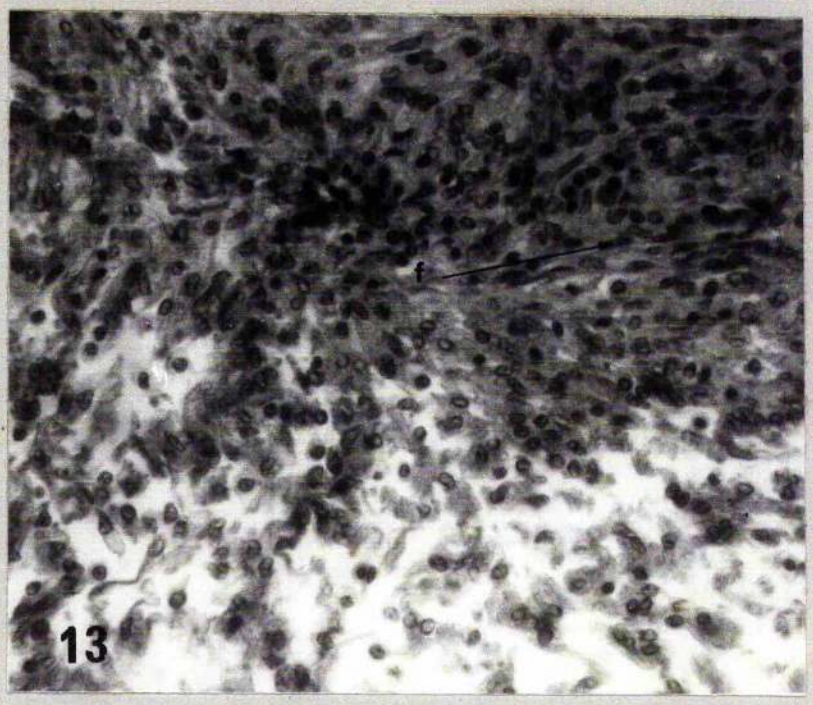
Plate 12 (X 200) — 37 days at 100 ppb. Series 1: showing dense mass of cells in the top half of the plate, whilst clearly oedematous tissue can be seen in the bottom half.


11


12.

Plate 13 (X 500) — As plate 12: showing the influx of different cell types, particularly fibroblast-type cells (f).

Plate 14 (X 800) — As plate 12 and 13: at higher power the elongated fibroblast-type cells (f) are obvious. Other cell types, probably hemocytes (h) can be seen.


f
13

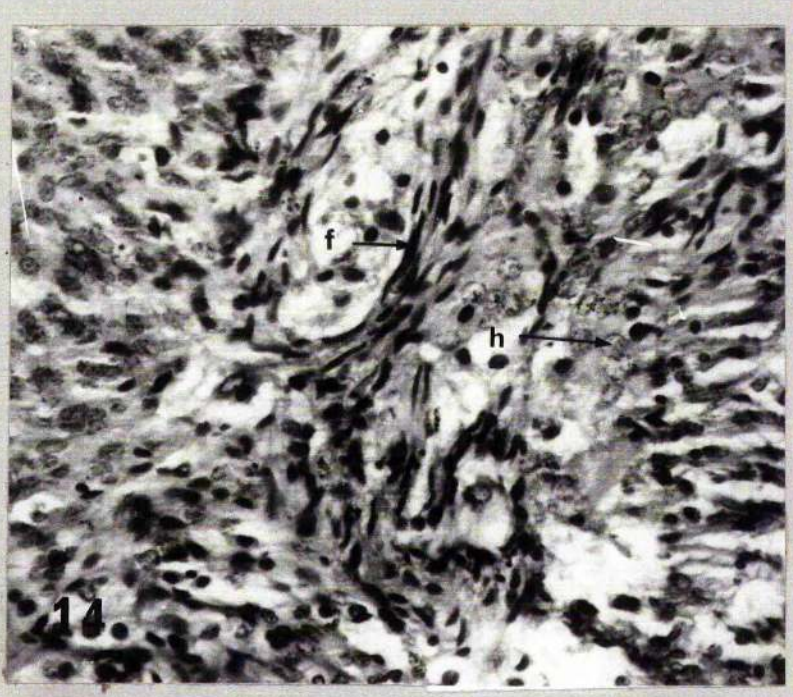

f
h
14.

Plate 15 (X 500) — 38 days at 100 ppb. Series 1: coelomosac showing the development of granulomas (g)

Plate 16 (X 500) — as plate 15: besides showing a granuloma developed by the labyrinth, illustrates the complete loss of coelomosac cellular arrangement and the influx of the fibroblast type cells.


g
15

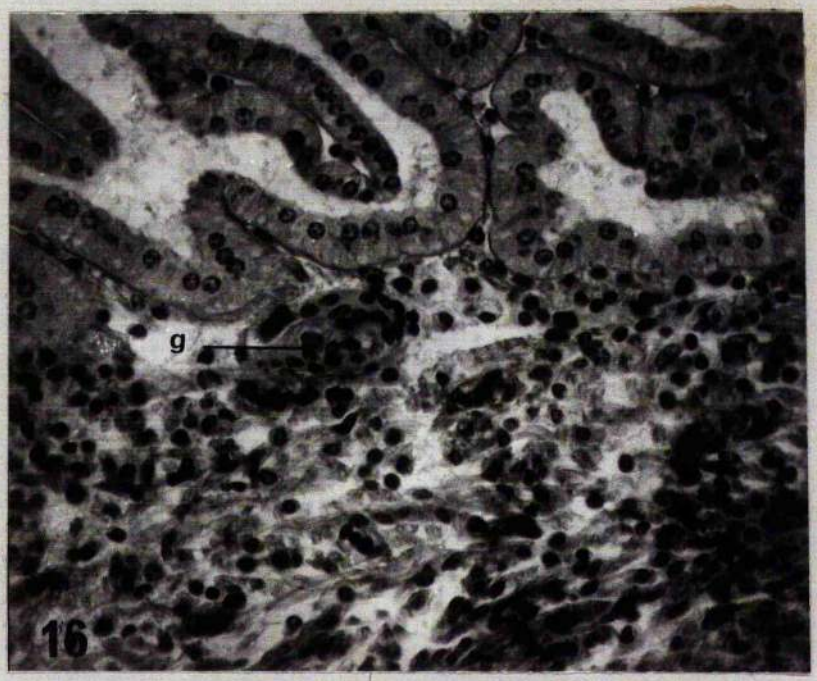
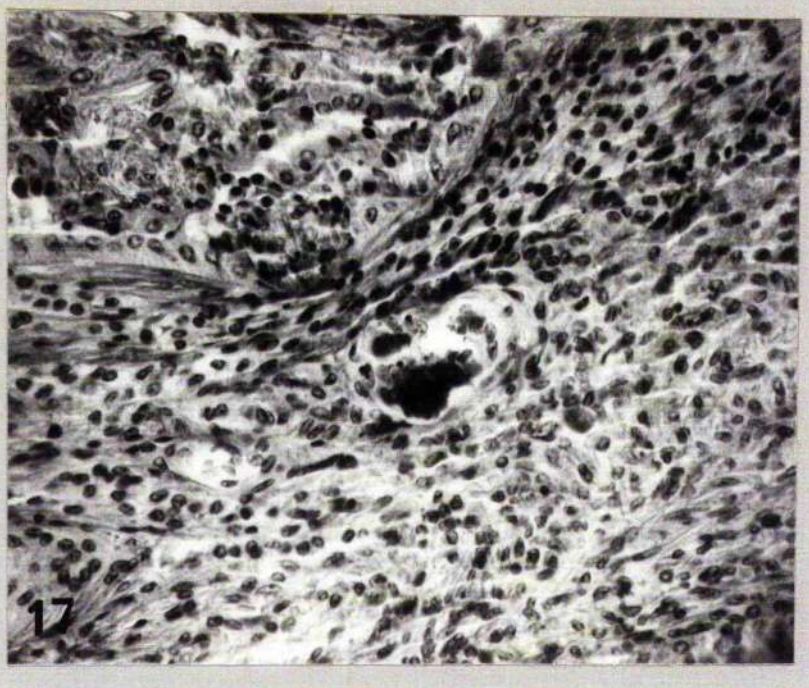

g
16

Plate 17 (X 500) As plates 15 and 16

Plate 18 (X 800) As plate 16 : showing necrotic eosinophilic tissue at core of the granuloma (et) and surrounded by boundary of cells (bc).


17

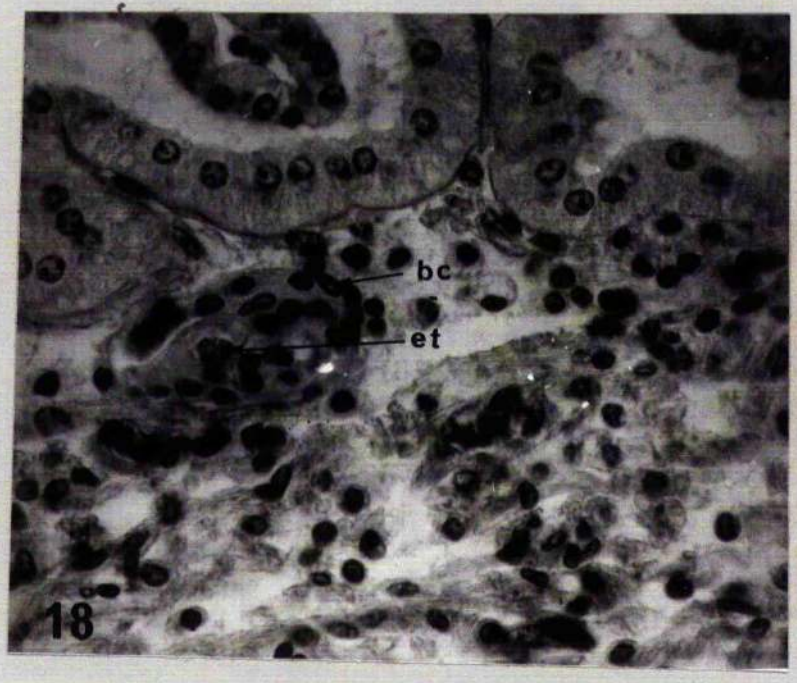
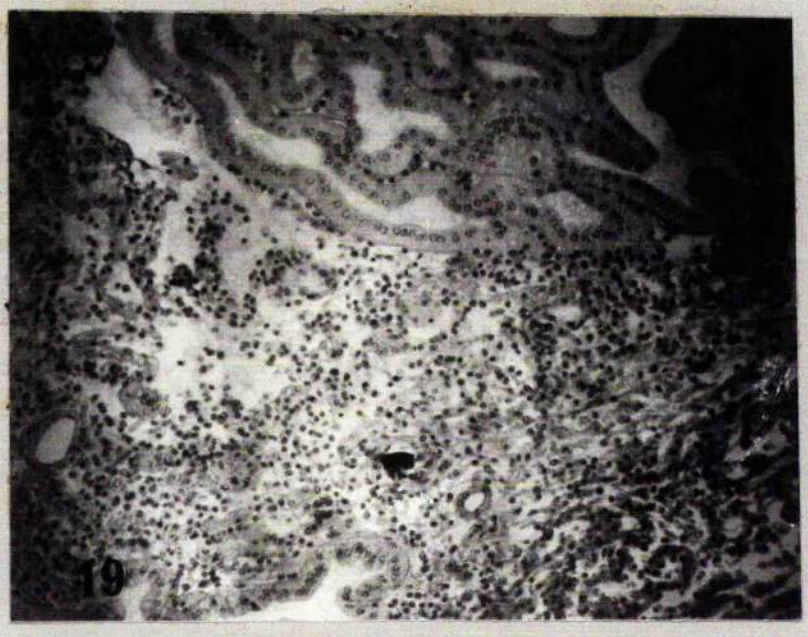

bc
et
18

Plate 19 (X 200)

38 days at 100 ppb. Series 1: illustrating the features of chronic inflammation. Generalised loss of cellular organisation, with oedema, development of granulomas, with at the same time, the influx of the fibroblast type cells.

Plate 20 (X 100)

As plate 19: showing the 'brick-red lesion' embedded deeply amongst coelomosac cells. A clear progression from fairly healthy cells (i) to necrotic, eosinophilic, material (ii) to the 'brick-red lesion' itself (iii) can be seen. A boundary of elongated cells can be seen in places around the lesion (bc).


19


ii
bc
iii

Plate 21 (X 200)

This plate possibly illustrates the healing process, as the lesion is walled off by the masses of fibroblast cells from apparently healthy coelomosac tissue.


21

Discussion

There has been very little work done on the effects of heavy metals on invertebrate tissue. This is surprising since the techniques are comparatively simple, and generally available, and as can be seen from this study, the results can show very clear cellular changes from even low exposure levels.

The work that has been done on the effects of metals in crustacean tissue; that is, the investigations of the histological effects of copper in the crayfish, Orconectes rusticus (Hubschman 1967), and the effects of yellow phosphorus on the american lobster, Homarus americanus, (Aiken and Byard 1972), while showing that some of the tissues were damaged by the metals, do not give much detail on the cellular changes found. This has been a disadvantage when trying to compare the effects of the three metals. It is, perhaps, only necessary for a pollution scientist to show that certain levels, or periods of exposure have a harmful effect, but it would be most useful to be able to assess cell damage due to different metals, and to be able to make comparisons between their modes of action. It might then be possible to predict how a particular metal would affect a particular organ, to assess chances of recovery, etc. This will not be possible unless the histological changes are understood.

A further difficulty in this aim is that very little is known of the effects of injury in crustacean tissue. The general reaction of all tissue to injury or destruction in inflammation. This reaction has been mainly studied in vertebrate tissue but more is becoming known about inflammatory processes in some invertebrate tissues, eg oysters (Pauley and Sparks 1966, 1967). Despite some of the first work on

inflammation being done on the crustacean, Daphnia, (Metchnikoff 1895) very little further work has been done on this process in the Crustacea since (Sparks 1972). Therefore the cellular changes in this study have been described in detail and will be discussed at length, so that at least a start can be made at understanding the effects of mercury on crustacean tissue, as well as giving general information on crustacean response to histological injury.

This may not be of direct relevance to pollution studies but it would be useful to know if all metals cause the same or different pathological effects. These cannot be compared unless the types of response to injury are known in detail.

In this study, exposure to both 10 ppb and 100 ppb inorganic mercury have been found to cause varying degrees of cellular damage in both the labyrinth and the coelomosac of the green glands, whilst histological changes due to the mercury were not apparent in either the digestive glands or the gills.

Digestive gland

In the case of the digestive gland this lack of histological change is not surprising since it has been found in this study that little accumulation of mercury occurs in this gland, compared with that

in the green glands and the gills.

Yelbw phosphorus was found to cause considerable degenerative changes in the digestive glands of the american lobster (Aiken and Byard 1972), but then the digestive gland is the main site of accumulation of this metal. Fletcher (1971) calculated that after exposure to 15 ppb phosphorus for forty eight hours, the digestive gland contained 92% of the total body content of phosphorus.

The gills

It is more surprising that no histological changes were found in the gills. These are the organs that show the greatest tendency to accumulate mercury and therefore cell damage might be expected. This lack of effect may be due to the manner in which this accumulation occurs.

If nephridiocytes did accumulate mercury, there might be an increase in the numbers of large phagocytic cells in the gill tissue. This does not appear to happen, but it may be that the cells that are already present accumulate the mercury.

The alternative explanation for there being no cellular changes in the gills, is that the mercury is tightly bound to the cell walls of the gill epithelium and that no active movement of the mercury is occurring. In the green gland on the other hand, where excretion has been shown to occur, the damage is considerable.

It would be interesting to attempt to locate mercury in the gills by histochemical methods (eg silver sulphide method of Timm (Pearse 1972)) following the failure to get results with X-ray micronanalysis

but this was not done.

Supporting evidence for the hypothesis that the mercury is bound to the cell walls and therefore does not cause damage to the cells, is provided by the findings of Vogel (1959). He showed that copper was accumulated in the epithelial cells covering the gills of the goldfish (after exposure to 200 ppb copper for up to thirty five weeks) while there were much lesser amounts present in the underlying cartilagenous stroma. Cytological changes were not found in the gill tissue at all, nor were they found in the liver of the fish, and this is a tissue somewhat akin to the digestive gland of the lobster (Vonk 1960). On the other hand, the kidney showed major cytological changes; coagulative necrosis was found and was characterised by either lysis, or by hypereosinophilia of the cytoplasm and pyknosis or karryorhexis of the nuclei. This is similar to the damage found in the green gland (although in addition there was calcification of the tissue). This lends support to a further hypothesis; that histological and cytological damage only occur where there is active control as demonstrated in the green gland in this study.

Gill damage was not found in the crayfish nor in the american lobster following exposure to copper and yellow phosphorus respectively (Hubschman 1967; Aiken and Byard 1972) but then Fletcher (1971) showed that very little phosphorus accumulated in the gills in comparison with the levels found in the digestive gland and ovaries, while it is not known to what extent copper accumulates in the various tissues of the crayfish.

The findings of this study, together with those of Vogel (1959) and possibly Hubschman (1967) may suggest a modification to the statement of Sparks (1972) that heavy metals cause damage at their site of greatest

concentration or at sites of entrance or exit. That is, that metals cause damage only in the organs in which active control occurs.

This may be a simplified view since Baker (1969) did find changes in the gills after exposing the winter flounder, Pseudopleuronectes americanus to levels of copper from 3.2 ppm to 0.18 ppm (180 ppb). At the lowest level the effects are only very slight with vacuolation and shrinking of the epithelial cells but at higher levels changes were more gross, until complete cell breakdown in the gill lamellae was seen after exposure to 3.2 ppm. (Vogel, 1959, found no effects on the gills of the goldfish after exposure to 200 ppb but this difference may be due to difference in susceptibility between sea water and fresh water fish, apart from any specific difference.) This finding of Baker may not in fact invalidate the hypothesis that mercury is bound to the cell walls because if it is binding of the metal to the cell walls that prevents damage to the cells, there will be a saturation level at which no more binding will be possible, and then damage may occur. However, since the organs showing effects at the lowest level of exposure must be the limiting factor to an animal's survival in a pollutant, effects found on other organs at higher levels are largely irrelevant to pollution studies.

A further possible contra-indication to the suggestion that cell damage only occurs at sites of active control is given by the findings of Crandall and Goodnight (1963) who found generalised cell changes in the guppy, Lebistes reticulatus, following exposure to lead, zinc and sodium pentachlorophenate. They suggest that most histological abnormalities were secondary, and were more or less non-specific results of chronic toxicity. (Levels of exposure used were 1.15 ppm of zinc,

2.48 ppm lead, and 0.5 ppm of sodium pentachlorophenate.)

However, one particularly important factor is the levels of pollutant the animal is exposed to. There may well be generalized histological effects following exposure to levels above which the animal is unable to make any attempt at control. For example, the finding of cellular disintegration in the digestive gland of the american lobster following exposure to yellow phosphorus (Aiken and Byard 1972) may only be found at levels where the gland is being used as a 'sponge' or storage site as suggested by Bryan (1964) when the lobster is exposed to high levels of zinc, but the levels of phosphorus used in these studies are not given. It is important to know how the animal deals with different levels so that the minimal level for finding damaging effects can be found.

At this minimal level of exposure it is probably very likely that it will only be those organs that are actually controlling the pollutant that will show any cellular effects.

The green glands

The cellular changes found in the green glands are particularly interesting in that they suggest that there may be a difference in the action of mercury on the tissue at different times of the year. That is, that while similar cellular changes were found in both series of experiments, the effects found on the coelomosac differed considerably.

In the labyrinth, at the lower concentrations of mercury, cytoplasmic swelling was the most obvious feature, which suggested that the tissue was under osmotic stress. At the same time, nuclear damage could

be seen and this would indicate irreversible cell damage, and that even at this low level, longer term exposure could cause serious histological damage. At the higher level of exposure, cytoplasmic destruction was clearly apparent and was greater after longer exposure periods. This damage did tend to be localised at first and this makes it likely that if exposure was stopped the tissue as a whole could recover.

In the coelomosac, however, the results varied. In the second autumnal series of experiments, there was some evidence of cellular disorganisation and nuclear breakdown, but in the first series of experiments, the effects were far greater. There was a clear chronic inflammatory condition, with massive cell death, oedema, loss of histological organisation, and at the same time, the influx of fibroblast type cells typical of this type of reaction, suggested that repair was being attempted.

These differences between the results may suggest that while mercury excretion imposes an unchanging stress on the secretory processes of the labyrinth, there is a seasonal change in excretory pattern that causes great stress on the coelomosac only at a certain time in the moult cycle of the lobster.

As already explained, there is not enough known about the actual mechanisms of excretion for there to be a clear explanation for this variation. It is possible that there could be a change in the amount of mercury bound in the blood depending on the stage in the moult cycle. Unbound mercury is more likely to be excreted by filtration and this would therefore affect the coelomosac. Also, being unbound it is likely to cause more cellular damage.

This must be pure speculation, but this is not the first evidence in this study for the possibility of there being different susceptibilities

in lobsters at different times in their moult cycle. While none of the lobsters in either of the two series of experiments were in the pre-moult condition, it is possible that there are changes in the excretory patterns through the whole cycle.

With histological techniques it would be very simple to determine whether there is in fact different cellular responses to mercury at different stages of the moult cycle.

The actual cellular changes observed in the labyrinth are somewhat similar to those described following exposure of the crayfish to copper, (Hubschman, 1967), and the american lobster to phosphorus (Aiken and Byard 1972). Unfortunately the information of these changes is very scant, simply being that initial vacuolation is followed by complete cellular distintegration. Hubschman does not mention any effect on the coelomosac, while Aiken and Byard found that changes in the coelomosac were less common than the changes in the labyrinth.

Also, following injection of mercuric chloride into Carcinus maenas, to give body levels of 5 ppm, Thiriot-Quiévreux (1966) found similar changes after two and four days, ie swelling of the labyrinth cells but with less effect on the coelomosac cells, just a slight increase in vacuolisation. She also found that internal areas of the labyrinth tended to be less affected than the periphery, as in this study.

The 'brick-red lesion'

The changes found in the coelomosac in the first series, particularly the development of the 'brick-red lesion' are most interesting and have not been described before. There do, however, appear to be

similar findings described in the literature that are likely to be related to the lesion. These will be reviewed here briefly to add strength to the interpretation of this lesion given in the results section; that is, that the lesion is formed by cell necrosis resulting in the deposition of lipofuscin that is progressively walled off by the action of hemocytes.

The first reference to a similar phenomenon is given in work by Cuénot (1898). He found that both indian ink particles injected into the body of the oligochaete, Allolobophora terrestris, and infection of the worm with parasites, particularly nematodes, caused the production of what he called 'nodules phagocytaires'. He interpreted these as secretions of the phagocytes that surrounded the invading particles or organisms effectively walling them off in a capsule.

Hollande (1920) describes a similar finding in the caterpillar of Galleria mellonella, in response to the presence of bacteria and inorganic particles such as talc, chalk and carmine, and Goodrich (1928) describes the formation of 'chitinoid bodies' in Gammarus pulex L following infection by yeasts. The description that Goodrich gives of the 'chitinoid' nodules is very similar to the finding of this study, when the impression of the 'brick-red lesion' being a chitinous growth was very strong.

While Goodrich thought that the nodule was composed of chitin, Hollande put forward a quite different interpretation of the similar nodules found in the caterpillar. He suggested that it was formed by the oxidation of substances in the blood by diastase enzymes leading to the formation of a melanin type pigment. Goodrich criticised this on the grounds that crustacean blood does not oxidise to give a dark pigment when exposed to air, as Hollande said that insect blood does,

which was an essential pre-requisite for his hypothesis.

However, Goodrich tested the 'chitinoid nodules' for chitin and using histochemical stains, including the two stains used in this study, obtained negative results and concluded the substance is 'not identical with true chitin'. Additional evidence that Goodrich used for its being chitin is that the 'chitinoid' substance was insoluble in caustic potash (30-40%) even when heated and was also insoluble in dilute acids. Solubility was not tested in this study, but melanin is also extremely insoluble, being insoluble in most solvents and resistant to all but the severest acid and base digestion (Edelstein 1971). Goodrich also gave further evidence for the 'chitinoid nodules' being formed of melanin when she described how they became progressively darker in colour. Melanin characteristically becomes darker in colour with progressive oxidation.

The chitinous nature of the lesion found in this study was very obvious, however, being very hard and Goodrich said that such nodules are commonly found in other invertebrates such as worms and echinoderms but they are in these cases soft and compressible. There is therefore the possibility that crustacea are producing a melanin of slightly different form.

Similar nodules have been described more recently in the brown shrimp, Penaeus aztecus, following wounding of the integument by Petersen disc tags (Fontaine and Dyjak 1973), and also in the white shrimp, Penaeus setiferus, following injection of carmine particles into the body. The 'brown nodules' are seen to form around necrotic cells that have ingested carmine.

Evidence for the interpretation of Hollande (1920) being largely correct is provided by Poinar, Leutenegger and Gotz (1968) who demonstrate

the formation of a melanin capsule around invading nematodes by the hemocytes of the third stage larvae of the beetles of Diabrotica sp. in an electron microscope study. They confirmed the presence of melanin histochemically by Masson' Fontana, (negative in this study), and melanin bleach (positive in this study).

Similarly, Babu and Hall (1974) demonstrated a similar event in the mayfly followiing infection by a helminth. Positive histochemical results for the composition of the capsule being melanin were also given by melanin bleach, and Masson' Fontana method but results were negative for the Ziehl-Neelson stain that was positive in this study.

It seems likely therefore that what has been observed in his study has been a normal cellular response to invasion by foreign material but in this case provoked by cell breakdown. The study of Poinar, Leutenegger and Gotz (1968) suggests how this might happen. Following infection with the nematode, the hemocytes that initially make contact with the parasite, lyse and liberate their cytoplasm over the cuticle of the nematode, and this layer becomes melanised. Further hemocytes are seen to attach to this initial layer and present typical features of cell necrosis, with clumping of chromatin in the nucleus, and vacuolation of the cytoplasm etc. Several layers of cells form and the outer layers are very flattened and elongated (possibly as seen in plate 20 bc). The majority of the cells do not actually lyse but pigmented inclusions form within the cells, the frequency of these inclusions decreasing towards the outside of the cpasule, until on the outside the cells are normal. Hemocytes continually attach to the layers around the capsule until free-floating cells recognise the outer layers as normal.

A similar process has very recently been described in crustacea. Solangi and Lightner (1976) found that following introduction of the

the pathogenic fungus, Fusarium sp. into the shrimps, Penaeus aztecus and Penaeus setiferus, the macroconidia of the fungi became surrounded by a single layer of hemocytes. This was followed by melanization of the hemocyte-macroconidia capsules occurring within twenty eight hours after injection, being pale-yellow to light-brown in colour. By forty eight hours the capsules were dark brown and the macroconidia inside were lysed. This mechanism is also obviously part of the immune system of crustacea, phagocytosis being already recognised as important in this system (Sindermann, 1971; Paterson, Stewart and Zwicker, 1976).

It is easy to see how a similar reaction could occur around necrotic cells, the inital lysis around the invading body being replaced by lysis due to the action of mercury and thereafter provoking the normal responses. It seems very likely that it is a similar reaction that causes the large 'brick-red lesion' and the granuloma cores.

The reaction in the lobster coelomosac following exposure to mercury is somewhat different, however, in that it is accompanied by an intense inflammatory reaction that has not been described before in other crustacean tissue. It is, however, very similar to the reactions provoked by the injection of talc into the oyster (Pauley and Sparks 1967) and of talc and methylcholantrene into the cockroach (Schlumberger 1957). This may be due to the extensiveness of the action of mercury whereas invading bodies will cause more localised effect. A similar accumulation of fibrous tissue to that found in this chronic inflammatory reaction was seen in thebrown shrimp following wounding of the exoskeleton with the Peterson Disc tag, when fibrous tissue formed around the tag pin consisting of fibrocytes (sic) in close association with many collagen-like fibres (Fontaine and Dyjak 1973). This suggests that the action of mercury

causes formation of scar tissue, following massive cell death.

Possible beneficial result of formation of the melanin deposit

While it is likely that the formation of the melanin deposit is just an inevitable result of cell necrosis, there is a possibility that its presence, once formed, can act beneficially. Melanin is known to bind many small cyclic organic molecules and also to act as cationic ion exchange resin absorbing sodium and other cations (Edelstein 1971). It may be possible for it to absorb the mercury. This would be an extremely difficult hypothesis to prove or disprove but it is an interesting possibility.

Another interesting fact of this formation of melanin comes from Taylor (1969) who has suggested that the formation of melanin by the polyphenol-phenoloxidase system is an almost universal defence system, being a primitive system capable of killing microorganisms, isolating foreign bodies, sealing and repairing wounds, providing protective colouration and hardening tissues.

Difficulties of cell nomenclature

Since so little work has been done on invertebrate pathology in general, and crustacean pathology in particular, the nomenclature of the cells has presented difficulty. Ellis, Munroe and Roberts (1975) point out that there is general confusion over cellular components of the defence system in fish because terms used in mammalian haematology have been applied to fish with no evidence of functional similarities.

This problem is even more likely to be true for the invertebrates. One example of this problem is that reference has been made to fibroblast type cells in this study. Masson's Trichrome stain showed that they did in fact produce collagen. However, Schlumberger (1952) describes the engulfment of the particles of talc and methylcholanthrene injected into the cockroach, by hemocytes. These hemocyte cells are then seen to elongate and begin to resemble connective tissue of vertebrates.

Therefore while a variety of cell types are present in this inflammatory reaction in the coelomosac, in the absence of more detailed knowledge of crustacean cell types, no attempt has been made to name them or explain their presence.

This is clearly a very interesting development and a problem worthy of further study. Whatever the changing seasonal effects leading to the development or non-development of this inflammatory, granulomatous reaction, it is also clear that inorganic mercury at both 10 ppb and 100 ppb causes clearly disruptive and then destructive changes in the cells of the green glands. Unfortunately, it is not known how quickly these effects become apparent but Thiriot-Quiévreux did find immediate changes of the type found in the labyrinth of the lobster green gland in this study, although the levels she used were very much higher. If the changes did occur rapidly it could account for the change in rate of excretion of mercury found in two of the lobsters in the urine experiment. In any case the changes found over the minimum period used in this study, that is thirty days, could account for the two phased loss of mercury found in Cancer magister, (Sloan, Thompson and Larkin,1974), and for one of the reasons they postulated (see page 14).

More importantly if the exposure to mercury continues the necrosis becomes so severe that urine production ceases altogether and death then ensues.

Thus histological examination provides a very useful technique to study long term sub-lethal effects of pollutants and could usefully be used a great deal more in order to understand how particular pollutants damage tissue and to determine the levels at which this damage will ensue.

f) Part 2 An electron microscope study of the green gland following exposure to inorganic mercury

It has been said in the General Introduction (page21) that electron mciroscope studies have been used to clarify cellular changes due to exposure to pollutants.

For this reason, an electron microscope study was made of the green glands after exposure to 100 ppb and 10 ppb inorganic mercury for thirty days and fifty days respectively. It was hoped that more information on the way in which mercury was affecting this organ would be forthcoming from such an approach.

Only the labyrinth was studied in this way because cellular effects on this tissue were fairly uniform and it was thought it might be possible to gain further understanding of these changes from this approach. The variability of the response in the coelomosac made it unlikely that much extra clarification would be obtained without further

histological studies being made first.

Materials and methods

Small pieces of green glands were taken from freshly killed lobsters kept for thirty days in sea water, thirty days in 100 ppb mercury, and fifty days in 10 ppb mercury respctively. They were fixed in glutaraldehyde (2.5% glutaraldehyde in Millonig's phosphate buffer at pH 7.2) for two hours. They were then washed in several changes of buffer and post-fixed in osmium tetroxide at 4° C for one hour. This was followed by dehydration in a graded alcohol series to propylene oxide and the samples were then embedded in an Araldite-Epon mixture.

The orientation of the tissues in the Araldite-Epon Blocks was determined by cutting the 2-4μ sections on an LKB Pyramitome and viewing in a Zeiss phase contrast microscope. Thin sections were then cut of the labyrinths of the green glands, using a Reichert Ultramicrotome and mounted on uncoated grids. Sections were stained for fifteen minutes in a 1% uranyl acetate and 10% potassium permanganate solution. The sections were then viewed in a Jeol 100C electron microscope.

Results

The results in fact only add depth to the findings from the light microscope studies.

Thus the control sample shows a clear microvillal border (mv) on the luminal surface (the brush border of light microscopy), large nuclei,

(n) in the rectangular shaped cells (plate 22) Mitochondria (m) are distributed through the cells and have ill-defined christae. Secretion granules (s) can be seen to form in the plasma membrane above the blood space (b). The intricate folding of this membrane is very reminiscent of the processes of the podocytes of the renal corpuscle of vertebrate kidney (Bloom and Fawcett, 1975).

After fifty days exposure to 10 ppb there is a dramatic increase in vacuolation (v) of the cells and the brush borders are seen to be lost in areas apparently due to extrusion from the cell (plate 23). Mitochondria cannot be identified with any degree of certainty.

In the 100 ppb lobster, the microvillal border has been lost. Vacuolation as such is not apparent but the cell appears to be more densely packed with secretion granules (s) (plate 24).

In some areas, (plate 25) while 'podocyte' -like processes are still clearly making contact with the blood space (b) the structure of the cell above is completely disordered and here vacuolation (v) on a large scale is obvious. However, the difficulty of the interpretation of the significance of such appearances is illustrated by reference to plate 26 which shows a similar area but from a control gland. There is vacuolation apparent here too but possibly the formation of the secretion granules is more ordered.

Discussion

This study has been greatly hampered by the lack of other work on the electron microscopy of this gland with which to make comparisons.

Plate 22 (X 6000)

Electron micrograph of control labyrinth: showing the border of microvilli (mv) nuclei (n), blood space (b) at base of cells, and with much folded basement membrane reminiscent of podocyte processes found in vertebrate kidney (p). Mitochondria (m) lacking clearly defined christae can be seen throughout the cells. secretion granules (s).

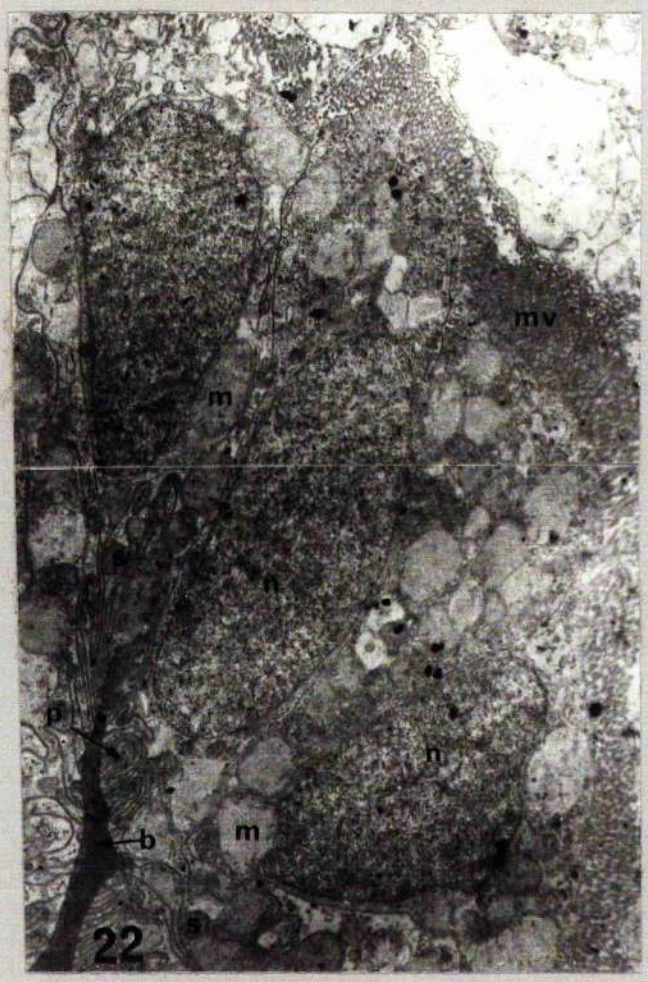

mv
m
n
p
n
b
m
s
22

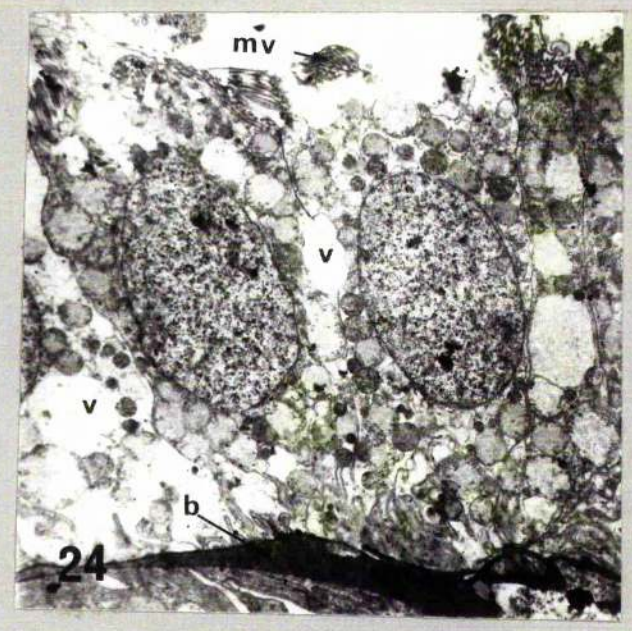
Plate 23 (X 4000)

Electron micrograph of labyrinth cells after exposure to 10 ppb. mercury for fifty days: showing the vacuolisation of the cells (v) with large numbers of secretion granules (s). There is partial loss of the microvillal border due to extrusion of cytoplasm. A blood cell (w) can be seen in the blood space (b)


mv
v
s
v
s
b
w
23

Plate 24 (X 4000) Electron micrograph of labyrinth cells after exposure to 100 ppb. mercury for thirty days: showing extensive vacuolisation (v) and loss of microvillal border.

Plate 25 (X 4000) As plate 24: showing membrane contacts on the blood space (b) and extensive vacuolisation.


mv
v
v
b
24

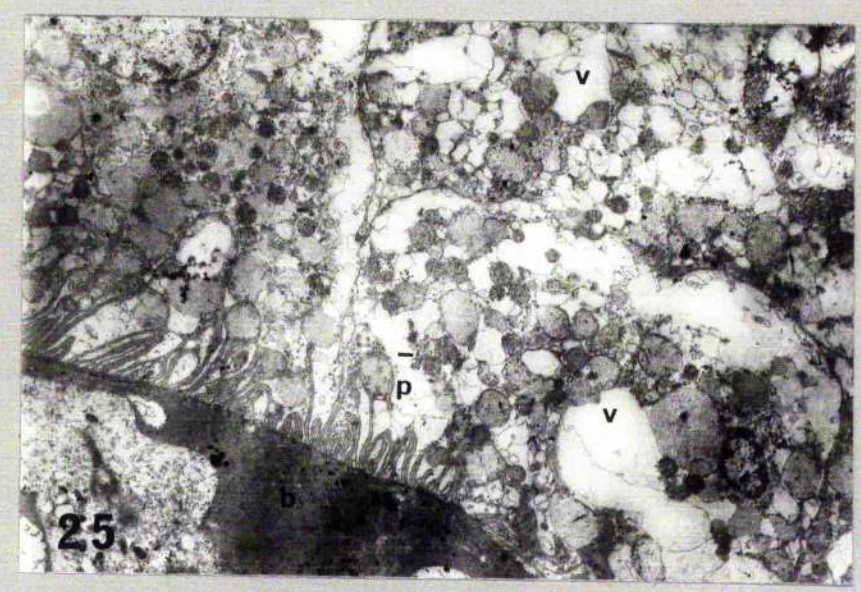

v
p
v
b
25

Plate 26 (X 4000) Electron micrograph of labyrinth cells of control tissue: blood space (b) podocyte- like structures (p)


n
b
26

There do not appear to be any electron microscope studies of the green gland of the lobster, and the studies of the glands of related crustacea are not numerous: for example, Schmidt-Nielsen, Gertz and Davis (1968) made an ultrastructural study of the green gland of the fiddler crab, Uca mordax, and Anderson and Beams (1956) made a similar study of the green gland of Cambarus sp. (freshwater crayfish). It was not possible therefore to note any major change that had not already been apparent with light microscopy.

The original intention of the electron microscopy study was to determine if it was possible to detect changes within cells that might be of significance in the long term, yet not detectable by light microscopy. The basis for this aim was work by Trump, Jones and Sahaphong (1975). They describe five stages in cell necrosis in the kidney tubule of the flounder following exposure of the isolated tubules to various concentrations of methyl mercuric chloride, parachloromercuribenzene sulfonate, and mercuric chloride. Of these stages, the second and third (the first being the normal cell) are reversible while the latter two are the final stages of necrosis. The reversible changes are such events as loss of cell volume regulation, with cell swelling, vacuolization, fatty accumulation, increased numbers of autophagic vacuoles, etc. These are known to be compatible with continued survival of the cell if the stimulus is removed. The final stages involve membrane changes especially in the mitochondria of which some denature and swell, while others contract, and changes to the nuclear membrane. These final stages are said to be undetectable with light microscopy for up to eight hours after they have occurred.

In this study, however, the inital stages of vacuolisation and

cell enlargement were clearly apparent with light microscopy while definite changes in the mitochondria were not even apparent in the electron microscope study. (One reason for this may have been due to poor fixation of the tissue. Comparison with the study of Beams and Anderson (1956) when the mitochondria are not unlike those seen in the control tissue in this study makes this unlikely. In both this, and the study of Beams and Anderson, however, the mitochondria are different from the very clearly defined and often elongated mitochondria shown in the study of Schmidt-Nielsen, Gertz and Davis (1968)). The final stages of necrosis were also clearly seen and it was immaterial to this study at what precise time they had occurred.

There may in fact be an important difference between the study of Trump, Jones and Sahaphong (1975) and the work described here, in that in the former study the levels of mercury are very low and the changes are occurring over a long period of time. It may be that as the changes occur slowly, they are in fact visible with light microscopy as they happen. In the other study the levels of exposure are very much higher (from 200 ppm to 2 ppm) and the mercury at these levels is being applied direct to the isolated tissue, with the result that the final stages of necrosis are being reached in six hours, and even in half an hour after exposure to mercuric chloride at the two highest concentrations. To achieve accuracy of measurement at this rate of change it is likely thatelectron microscopy is essential.

The other instance in which electron microscopy may be of great value is when there are changes detectable with light microscopy that are not readily explicable. Such a case is illustrated by the study of Fowler (1972) when feeding rats on low doses of methyl mercury resulted in the presence of spherical masses visible with light microscopy. With electron microscopy these were found to be developments of the smooth

endoplasmic reticulum, which was consistent with the hypothesis that demethylation occurred, since the smooth endoplasmic reticulum is associated with detoxification activity. No such developments were observed in this study.

Electron microscope studies in pollution studies may also have a role in the study of tissues already well known ultrastructurally. This is not the case for the crustacean green gland and probably many other invertebrate tissues.

On the whole these criteria given above rule out there being any value to the electron microscope as a tool for use in the study of pollutant effects on invertebrate tissue. The little extra information on the changes readily observable with the relatively simple light microscopy techniques is not worth the extra time and expense entailed with the electron microscopic techniques.

General Discussion

The overall aim of this study has been to illustrate the value of approaching pollution studies from several different vantage points, and in this way to gain an understanding of the way a pollutant behaves in and affects a particular species and to enable possible stresses in the animal from the interaction to be highlighted. The aim has also been to show the value of relatively simple techniques. It must be remembered that the study of pollution is an 'applied science' and so the approach to it should be governed by other considerations than those of pure research. The approach used in this study has been to find a logical method that can be used in a practical way. It should be possible using the techniques used successfully in this study, to determine the minimum level that will cause harmful effects in a given species. In this way it could be possible to determine minimum permissable levels for many of the major pollutant substances.

Not all the techniques used have been successful, or have given enough additional information for their use to have been worthwhile. The use of neutron activation analysis, with radiotracer experiments, and long term histological studies however, has enabled an understanding to be gained of the way the lobster takes up and accumulates mercury, of how it is able to control the levels, and of the long term effects of exposure to the metal.

The value of such a multiple approach has already been shown by Fujiya (1965) who studied the effects of Kraft pulp mill waste on fish. In his study, physiological effects were found before histopathological changes could be observed in visceral organs. The histopathological changes were however, still found at levels very much lower than the median tolerance limit for Kraft pulp mill effluent. That is, cytochemical symptoms such as accelarative secretion of gill mucus, or decrease in RNA and glycogen in the liver, were detected at levels just below 10ppm. while histological effects on the kidneys and gills were found at levels of just less than 30ppm. The median tolerance limit is approximately 400ppm. In the same study, effects on bivalves were also studied and in these, the minimum changes detected were histopathological, rather than physiological changes. Whilst it may be difficult to assess the significance of the physiological changes, the presence of cell necrosis must indicate a harmful effect. The physiological parameters measured in fish may also be of more significance in higher animals, while histological investigations are more suited to the study of invertebrates where individual variations in physiological parameters will be far more marked and less easy to relate directly to the effect of the pollutant. This therefore confirms the value of histological investigation of the effects of pollutants on invertebrates.

In the study of Fujiya (1965) there is however, no measurement of the levels of the pollutant within the tissues where the effects are measured. This is one factor in the approach used in this study that has been shown to be of great value. That is, the localisation of the main areas of accumulation and, even more importantly, of control, in order to determine likely sites of cellular damage. This was shown to mark the green glands as areas of susceptibility to mercury. The gills were areas of accumulation but it was not known if they were also areas of active control, although evidence made this seem unlikely. The finding of acute cellular necrosis in the green glands with no sign of damage in the gills added weight to this interpretation. There is evidence that crabs gills are involved in the transport of mercury and it would be interesting in view of the hypothesis put forward in this study, ie that damage due to mercury at low levels of exposure only occurs at sites of active control, to know if crab gills show any necrosis after exposure to low levels of mercury.

The hypothesis may only be valid with respect to mercury because it is possible that in some cases it can be the presence of the metal alone that can cause histological damage. For example, Aiken and Byard (1972) found extensive effects on both the green glands and digestive glands of the american lobster following exposure to yellow phosphorus. Fletcher (1971) found that yellow phosphorus accumulated to the greatest extent in the digestive gland and then the ovary of the american lobster (green glands were not measured). As this was in proportion to the lipid content of these organs it was assumed that phosphorus accumulation depended on lipid content.

A similar effect was also found in cod (Dyer et al. 1970). If the accumulation of phosphorus is simply in proportion to lipid content it is unlikely to be actively controlled in these sites, and therefore it must just be the presence of the metal that is causing the cell damage. But, as it has been said before, the levels of exposure in the study of Aiken and Byard (1972) were not known. If the levels of exposure were high, then their findings may not invalidate the hypothesis for phosphorus either, since there was clear damage found in the green glands.

It can be said here that while the use of neutron activation analysis was of great value for the background level determinations, it is much more accurate than is really required for this type of preliminary localisation of accumulation and control sites. Radioactive isotopes could easily be used to locate these sites, as the preliminary to histological investigations.

While it is important to locate the sites of likely damage from exposure to a pollutant, it is more important to identify the parts of the life cycle that are more susceptible to the pollutant. Damage to the excretory organs at one level of exposure becomes unimportant if the species is failing to survive at all due to far greater susceptibility to a lower level of pollutant at a different stage of the life history.

In this study, lobster larvae were investigated, and with comparisons from other work (Connor 1972), it seemed that lobster larvae, while more susceptible possibly than adults, were not as susceptible as larval forms of other species.

Another point that makes the study of lobster larvae possibly less important is that they have such a high mortality in any case. Herrick (1895) assessed that for Homarus americanus only two out of 10,000 must survive for adult numbers to be maintained. Kensler (1970) gives a figure of 99% for the mortality of the first four stages, again in the american lobster. There are two possible arguments here; on one hand since mortality is so high, pollutants may add the additional factor that undermines their ability to survive entirely; on the other, since mortality is so high due to predation the effect of the pollutant will be to ensure that those that are predated will be the ones most affected by the pollutants (because they will become more conspicuous as they become more unhealthy see page94) while those resistant larvae will still survive. So little is known about the survival of the lobster larvae in the natural environment, these two possibilities cannot be investigated at the moment. It may be more sensible in anycase to investigate the effects of pollutants on species of which more is known of larval forms, and their survival rates and patterns of behaviour, such as some fish larvae.

What is a much more important finding of this study is the suggestion that pre-moulting animals are less resistant to the presence of mercury. This possibility has not been investigated as such, but during the course of the investigation evidence that this was so, accumulated.

In the urine experiment (d) carried out mainly with pre-moult lobsters there was a much reduced resistance to mercury, and the lobsters rapidly became anuric and died.

In the histological investigation (f) there was far greater cellular damage found in the coelomosacs of the green glands in the first series of experiments than in the second. The lobsters were not in the pre-moult condition but it may be that there are changes in the excretory patterns over a longer time than the immediately moulting period, and that, as the animal has to excrete more, the presence of mercury imposes a greater strain on the coelomosac. Travis (1955) found in the spiny lobster, Panulirus argus Latreille that calcium levels in the blood rose in the pre-moult stage (D). The change in the urine to blood ratio for calcium was however, only found three to four days prior to moulting. Therefore in this study while it may be calcium excretion that caused the greater mortality found in the urine experiment, it cannot account for the greater damage found in the coelomosac cells in the histological studies. Maluf (1940) did however find that the coelomosac cells were involved in the secretion of calcium. Riegel (1966b) found localisation of the orange pigment occurred in the coelomosac cells in stage D crayfish. It appeared to be accumulated and eliminated by the coelomosac cells. It could be such changes, or others that account for the increased mortality and increased cellular damage.

Additional evidence that moulting crustacea may be more susceptible to mercury is provided by Wilson and Connor (1972) who found in long term exposure to mercury that shrimps were more susceptible to the metal in the immediately post-moult stage.

Also, Thiriot-Quiévreux (1966) found that crabs of the species Carcinus maenas, injected with mercuric chloride to give body concentrations of 5ppm. would die very soon after injection in stages A, B, and $C_1 - C_3$ of the moult cycle, while up to eighty-two percent would survive in stages C_4 and D.

This is a very important finding since it is possible that while levels as low as 10ppb. of mercury cause damage to normal adults over long periods of time, even lower levels may cause damage to pre-moult and post-moult and even other stages in the moulting cycle. This is definitely a problem worthy of more detailed investigation.

Levels of mercury used in this study

It is relevant here to comment on the levels used in this study. While background levels in the oceans are taken to be on average 0.1ppb. (Hammond 1971) the levels used in this study were 10ppb. and 100ppb. It is worth reiterating that levels in the experimental tanks would have been lower than this. For example, Coyne and Collins (1972) found loss of 42% from a 50ppb. solution of mercury made in distilled water and stored in polyethylene containers.

A similar solution made up in 'creek water' lost 96% of the mercury within three hours. Loss from open tanks with air stones was possibly even higher since, if it is volatility of mercury that causes the loss, then continual agitation in an open system will increase this.

It therefore is unfortunate that it was not possible to obtain a measure of this loss. It is also not known whether it is the average level of exposure that is important or the maximum level even if this is experienced for only a short time.

Cause of death from mercury

While this study has been mainly concerned with sub-lethal levels of mercury, lobsters have died during the course of the study. This is a convenient place to consider the cause of death from mercury poisoning.

In levels of 1ppm. mercury, lobsters were found to die in a characteristic way; the carapace would be swollen away from the abdomen by an accumulation of clotted blood, (this is on the basis of four observations). This manner of death is similar to that described due to phosphorus poisoning in Homarus americanus, (Zitko et al. 1970 Aiken and Byard 1972) in that the blood becomes clotted, although the swelling of the abdomen is not mentioned by these workers. This swelling is characteristic for death in dilute sea water, ie from osmotic stress (Goggins 1960).

The mode of death due to phosphorus poisoning is also described as being similar to that due to asphyxiation except that then the blood does not clot. (Aiken and Byard 1972 Zitko et al. 1970)

This mode of death may suggest that both yellow phosphorus and mercury can act on the blood to cause clotting. It is possible that at high levels of exposure mercury could compete with copper for sites in the haemocyanin molecule. Corner (1959) in his work with highly lethal levels of mercury (10ppm) in Maia squinado although he does not describe the mode of death, does hypothesise that there is an interaction with copper in haemocyanin. The levels of copper and mercury measured in his study were 55.5ppm. and 25.1ppm. respectively, and so such interactions as he suggests; that is partial displacement of copper from haemocyanin, or attachment of mercury to sites on the protein for which copper has no affinity are both more likely to occur at this level of exposure. This is especially so, since Corner was able to identify the mercury as being associated with the protein fraction of the blood. At lower levels of exposure this action on the blood is less likely to occur as the level of mercury, in comparison with the copper, will be very small.

Aiken and Byard (1972) suggest however, that with phosphorus the haemolymph clotting is not triggered by phosphorus directly but is an indirect result of damage to the cells of the antennal gland and hepatopancreas. This however seems an unlikely explanation in view of the results of this study since when there was acute green gland damage and anuria causing the death of lobsters in this study no such appearance as found with the acute levels of exposure was ever found.

Corner (1959) also suggests that the pressure on the green glands to excrete both the average amount of copper a day (20µg) as well as mercury are likely to cause damage to these glands. Corner and Rigler (1958) also suggests that mercury as a poison acts by interfering with the excretory system. These ideas are more in accord with the findings of this study at the lower levels of exposure, but also, the swelling of the carapace at higher levels may suggest osmotic stress due to acute failure of the excretory system.

It has been mentioned before (page68) that it was noticed that non-urine producing lobsters rapidly succumbed on exposure to mercury. (This is based on only a few observations since once it was noticed only urine producers were used in the study) Burger (1957) noticed that american lobsters, taken from commercial pounds had variable abilities to produce urine; some were completely anuric. This anuria appeared to be related to blood protein content since the injection of serum from urine producing lobsters stimulated urine production in anuric lobsters. It is not known if lobsters are ever anuric in the wild. Burger (1957) says that they can live for up to a month without producing urine. If this condition of anuria does occur in nature then certain lobsters may be more susceptible to mercury poisoning.

Since the gills are the major site of mercury accretion it may be surprising that no cellular damage is found in them, but it has already been mentioned that such damage may well be found in the gills of crabs.

Other workers have suggested that gill concentrations of heavy metals in the gills of various species as being related to the cause of death. Mount and Stephan (1967) working on cadmium poisoning in the fishes the bluegill, Lepomis macrochirus, and the brown bullhead, Ictalurus nebulosus, put forward the hypothesis that there is a critical level of cadmium in the gills above which death is inevitable. Following from this, Vernberg and O'Hara (1972) showed that translocation of mercury from the gills to the digestive gland in Uca pugilator, was higher at higher temperatures. High levels of mercury remained in the gills at low temperatures and they suggest that this is the cause of the higher mortality found at lower temperatures.

O'Hara (1973b) found a similar critical level of cadmium also in the gills of the fiddler crab, Uca pugilator, and that this level was about 110ppm. He says that with both mercury and cadmium, mortality is related to accumulation of the metal in the gills with associated breakdown in osmoregulatory or respiratory function. It could also be that this cause of death is due to cell damage by the attempted excretion of the metals by the gills and it would be interesting to investigate this possibility.

Concentrations of mercury were not measured in the gills at levels at which death occurred but at the levels dealt with in this study the cause of death was clearly not dependant on the gills, and this difference is very probably due to the different emphasis on method of excretion in the lobster and crabs (Bryan 1966).

Hunter (1949) suggested from the results of his studies of poisoning by copper and mercury that poisoning can act on different levels according to the dose. Hubschman found with the crayfish that exposure to levels of copper of 1ppm. and above that respiratory enzymes were rapidly inhibited, while at lower levels it was cellular damage to the green glands that eventually caused death. A similar phenomenon appears to occur in the lobster with mercury. At low levels, it is clearly failure of the excretory organ that causes death. At higher levels of exposure, there is probably some action of the blood which brings about death by asphyxiation, possibly with accompanying acute excretory breakdown.

While it can be seen from this study that cell necrosis is a clear reason for failure of the green glands, it is less apparent why mercury should act in this way. Trump, Jones and Sahaphong (1975) suggested from the effects seen in the isolated kidney tubule of the flounder that the important effect of mercury is that it binds to sulfhydryl groups on surface membrane proteins. They suggest that this may have a variety of effects including inhibition of ATP ase activity, but the overall result is a permeability change leading to an inward leak of sodium and outward leak of potassium and subsequent volume shifts.

Renfro et al. (1974) showed a complete inhibition of Na-K-ATP ase in bladder homogenates also of the flounder and this was at levels of 2ppm. mercuric chloride and higher, as well as other effects on sodium transport and this therefore gives support to the hypothesis of Trump, Jones and Sahaphong (1975). Such effects could account for the changes seen in the green gland with the initial swelling of the cells.

The affinity of mercury for sulfydryl groups could also account for binding of mercury in the gills of the lobster.

The findings of work on isolated tissues must however, be treated with caution as Renfro et al. (1974) points out, since it has been shown in other studies that action of mercurials demonstrated in in vitro studies do not occur in whole animals.

This further highlights the value of histological techniques as tools for studying the effects of pollutants since the action on the whole animal can be studied, even if the mechanism cannot be fully understood.

The state of understanding achieved by this approach to the study of mercury in the lobster can briefly be reviewed here. When lobsters are exposed to mercury at levels of 100ppb. and 10ppb. mercury is taken up mainly by the gills although very limited amounts may enter via the stomach and pass through the digestive gland to the blood. From evidence of the lack of cell destruction and slow rate of loss from the gills when the lobster is returned to fresh sea water, it is suggested that most mercury taken up binds to the cell walls of the gills but a proportion gradually enters the blood. This mercury passes in the blood to the green gland where it is taken up and excreted. This excretion may take place at a high initial rate but possibly due to cell destruction this rate drops. Over a long period cell destruction may prevent urine production completely and then death is inevitable. If blood levels are raised then the level in the digestive gland may rise but not to the level found in the green glands or gills. Since no tissue damage is found this is probably a passive process. If exposure is stopped then the green gland continues to excrete mercury at a low steady rate and the concentration in the green glands tends to fall followed by a much more gradual drop in the gills. If the mercury is bound in the cuticle then the mercury will be lost at moulting. If the gills, on the other hand continually lose mercury from the cells to the blood stream there will be a continuing long term demand on the excretory powers of the green glands. It is not known which is the case. If however, when exposure ceases if the gills do not continue to lose mercury into the blood it is likely that tissue damage to the green glands will recover because cell damage appears

to progress from the outside of the labyrinth inwards, so if exposure is not prolonged the tissue may recover. There is also the likelihood that long term exposure to levels as low as 10ppb. may be more harmful than acute exposure. Thiriot-Quiévreux (1966) for example found acute effects in the green glands of Carcinus maenas, after injection with levels of mercuric chloride such as to give body mercury levels of 5ppm. Crabs that survived the procedures of injection and operation on the carapace to expose the green gland on one side, and that showed initial inflammation of the labyrinth would often show complete recovery within four days.

Evidence was also found that background levels of mercury in the lobster could be high. When considered with the other measurements made on both Homarus gammarus caught in Scotland, and Homarus americanus caught in Canada, it also seems that tail muscle levels become high in comparison with other tissues (MAFF 1971, 1973 Holden and Topping 1972 Freeman et al. 1974) (Although gills and green glands were not analysed in these other studies). This suggests that it may be possible to deduce the history of exposure to mercury in the lobster in the same way as Mount and Stephan (1967) suggest may be possible with cadmium in fish. From their results they found that acute exposure to cadmium caused high accumulation in the gills with virtually no uptake in the liver, whilst prolonged sub-lethal exposure caused considerable accumulation in the liver. They suggested that high cadmium content in the liver (300-400ppm.) indicates a past history of exposure to cadmium, while levels of 75--100ppm. would mean that cadmium exposure had been short or at very low water concentrations.

In the same way it is possible that the relationship of the tail muscle concentrations of mercury on the lobster to other tissues may impart similar information; that is, when tail muscle levels are higher than the levels in the gills or the green glands uptake from background levels or very low levels is suggested while raised levels in the gills of the green glands suggest that accumulation is occuring faster than the animal can control it. It would be very interesting to know what this level would be in the external environment because it must be lower than the levels used in this study. It would therefore be most useful to have more results on background levels in all tissues, and it is possible that the lobster could act in this way as an indicator species.

Since it is the muscle that is of relevance with regard to human consumption however, this lack of information is unlikely to be rectified.

Clearly not all questions have been answered. The most important of these is what levels of mercury do not cause long term effects on the green glands found in this study, and even more importantly, what levels of mercury are lethal, or cause long-term damage to pre- and post-moult lobsters; that is, to what extent is the range of susceptibility to mercury extended around the moulting time. This must be the most important area for future investigations.

Summary

1) The aim of this study has been to approach the study of inorganic mercury in the lobster, Homarus gammarus, from various standpoints, in order to gain an overall understanding of the uptake, accumulation, and excretion, and long term effects of exposure to low levels of inorganic mercury. The aim has also been to use relatively simple techniques that could be widely applied as these are more appropriate to pollution studies.

2) The initial approach was to measure background levels in different organs of the lobster. These can be higher than average values for other marine animals. Also, to measure the concentration of mercury in the different organs following exposure to 100ppb. and 10ppb. mercury for varying periods of time. These measurements were made using neutron activation analysis.

3) These results were amplified by radioisotope tracer studies so that besides identifying site of major accumulation the route of uptake could be determined.

4) The ability of the lobster to excrete mercury was also investigated using radioisotope tracers.

5) After identification of the major sites of uptake and control, histological examination of the organs likely to be affected by mercury, being either principal sites of uptake, concentration, or excretion, was carried out. This was amplified in part by electron microscopical examination.

6) By these techniques it was found that mercury was taken up mainly by the gills where the greater part was probably bound in the cuticle or cells. A small proportion passed into the blood stream and it was taken up from the blood into the green glands from where it was gradually excreted.

Long term exposure caused severe damage to the green glands at 100ppb. and less severe at 10ppb. although longer exposure would probably lead to death. Since no cell damage was found in the other organs examined (the gills and digestive gland) it is suggested that damage only occurs at sites of active control.

7) It was though important to look at possible weak links in the life history that might be more susceptible to mercury. Measurement of mercury uptake into lobster larvae using X-ray microanalysis was unsuccessful and experiments on the effect of mercury on the survival of the larvae were inconclusive. It was thought that evidence that accumulated through the course of the study, that moulting animals may be far more susceptible to mercury, was far more important and worthy of further study.

REFERENCES

Abelson, P.H. 1970. Methyl mercury. Science, N.Y. 169: 237.

Ackefors, H., G. Lofroth, and C-G. Rosén. 1970. A survey of the mercury pollution problem in Sweden with special reference to fish. Oceanogr. Mar. Biol. Ann. Rev. 8: 203-224.

Aiken, D.E. and E.H. Byard. 1972. Histological changes in lobsters (Homarus americanus) exposed to yellow phosphorus. Science, N.Y. 176: 1434-1435.

Anderson, E. and H.W. Beams. 1956. Light and electron microscope studies on the cells of the labyrinth in the "green gland" of Cambarus sp. Proc. Iowa Acad. Sci. 63: 681-685.

Andersson, A. 1967. Mercury in soil. Grundförbättring (quoted in Saha, 1972). 26: 95-.

Anonymous, 1965. Kvicksilverfragen i Sverige, Kvicksilverkonferensen 1965, 1964 års Naturrersutredning, Jordbruksdepartmentet, Stockholm, 208 pp. (quoted in Ackefors, Lofroth, and Rosen, 1970.)

Anonymous, 1971. Study group on mercury hazards. Environ. Res. 4: 4-69.

Aoki, H. 1970. Environmental contamination by mercury (Hg series no. 14). 3rd Report. Inorganic and organic mercury in human hair and marine fish. Jpn. J. Hyg. 24: 556-562.

Babu, J.P., and J.E. Hall. 1974. Histochemical staining of melanin formed in a mayfly in response to helminth infection. J. invertebr. Pathol. 23: 124-12 .

Bache, C.A., W.H. Gutenmann, and D.J. Lisk. 1971. Residues of total mercury and methyl mercury in lake trout as a function of age. Science, N.Y. 172: 951-952.

Backstrom, J. 1969. Distribution studies of mercury pesticides in quail and some fresh water fishes. Acta pharmac. tox. 27; supp. 3: 1-103.

Baker, J.T.P. 1969. Histological and electron microscopical observations on copper poisoning in the winter flounder, (Pseudopleuronectes americanus). J. Fish. Res. Bd Can. 26: 2785-2793.

Barber, R.T., A. Vijayakumar, and F.A. Cross. 1972. Mercury concentrations in recent and ninety-year-old benthopelagic fish. Science, N.Y. 178: 636-639.

Beard, R.L. 1942. On the formation of the tracheal funnel in Anasa tristis induced by the parasite Trichopoda pennipes. Ann. Entomol. Soc. Am. 35: 68-74.

Berg, W., A. Johnels, B. Sjöstrand, T. Westermark. 1966. Mercury content in feathers of Swedish birds from the past 100 years. Oikos 17: 71-83.

Berglund, F., M. Berlin, G. Birke, V. Von Euler, L. Friberg, B. Holmstedt, E. Jonsson, C. Ramel, S. Skerfving, A. Swensson, and S. Tejning. 1971. Methyl mercury in fish. A Toxicological-epidemiologic evaluation of risks. Report from an expert group. Nord. Hyg. Tidskr. Suppl. 4.

Berlin, M. 1972. Present levels of mercury in man under conditions of occupational exposure. In "Mercury Contamination in Man and His Environment". International Atomic Energy Agency, Vienna. Technical Reports, Series no. 137.

Bertilsson, L. and H.Y. Neujahr. 1971. Methylation of mercury compounds by methylcobalamin. Biochemistry N.Y. 10: 2805-2808.

Bloom, W. and D.W. Fawcett. 1975. A textbook of Histology. 10th Edition. W.B. Saunders Company. Philadelphia, London, New York.

Bolles Lee, A. 1921. A Microtomist's Vade-Mecum. J. and A. Churchill, London.

Bryan, G.W. 1964. Zinc regulation in the lobster, Homarus vulgaris. 1, Tissue zinc and copper concentrations. J. mar. biol. Ass. U.K. 44: 549-563.

Bryan, G.W. 1966. The metabolism of Zn and ^{65}Zn in crabs, lobsters and fresh-water crayfish. Symp. Radio ecological Concentration Processes. Stockholm, Sweden, 1005-1016. Oxford, Pergamon Press.

Bryan, G.W., 1967. Zinc concentrations of fast and slow contracting muscles in the lobster. Nature, Lond. 213: 1043-1044.

Bryan, G.W. 1968. Concentration of zinc and copper in the tissues of decapod crustacea. J. mar. biol. Ass. U.K. 48: 303-321.

Bryan, G.W. 1971. The effects of heavy metals (other than mercury) on marine and estuarine animals. Proc. R. Soc. Lond. B. 177: 389-416.

Bryan, G.W. and E. Ward. 1965. The absorption and loss of radioactive and non-radioactive manganese by the lobster Homarus vulgaris. J. mar. biol. Ass. U.K. 45: 65-95.

Buller, A.T., J. McManus, and D.J.A. William. 1971. Investigations in the estuarine environments of the Tay. Physical aspects: an interim report. Research report no. 1. Tay Estuary Research Station.

Burger, J.W. 1957. The general form of excretion in the lobster, Homarus. Biol. Bull. mar. biol. Lab., Woods Hole 113: 207-223.

Burger, J.W. and C. McC. Smythe. 1953. The general form of circulation in the lobster, Homarus. J. cell. comp. Physiol. 42: 369-383.

Cappell, D.F. and J.R. Anderson. 1974. Muir's Textbook of Pathology. Ninth Edition. Edward Arnold. London.

Clarkson, T.W. 1969. Isotope exchange methods in studies of the biotransformation of organo mercurial compounds in experimental animals. In "Chemical Fallout. Current Research on Persistent Pesticides." (edited by M.W. Miller and G.B. Berg). C.C. Thomas. Illinois, U.S.A.

Cocoros, G., P.H. Cahn, and W. Siler. 1973. Mercury concentration in fish, plankton, and water from three Western Atlantic estuaries. J. Fish Biol. 5^{6}: 641-649.

Cole, W.H. 1941. A perfusing solution for the lobster (Homarus) heart and the effects of its constituents ions on the heart. J. gen. Physiol. 25: 1-6.

Connor, P.M. 1972. Acute toxicity of heavy metals to some marine larvae. Mar. Pollut. Bull. 312: 190-192.

Corner, E.D.S. 1959. The poisoning of Maia squinado (Herbst) by certain compounds of mercury. Biochem. Pharmac. 2: 121-132.

Corner, E.D.S. and F.H. Rigler, 1957. The loss of mercury from stored sea water solutions of mercuric chloride. J. mar. biol. Ass. U.K. 36: 449-458.

Corner, E.D.S. and F.H. Rigler, 1958. The modes of action of toxic agents. III. Mercuric chloride and n-amylmercuric chloride on crustaceans. J. mar. biol. Ass. U.K. 37: 85-96.

Corner, E.D.S. and B.W. Sparrow. 1956. The modes of action of toxic agents. I. Observations on the poisoning of certain crustaceans by copper and mercury. J. mar. biol. Ass. U.K. 35: 531-548.

Corner, E.D.S. and B.W. Sparrow. 1957. Modes of action of toxic agents. II. Factors influencing the toxicities of mercury compounds to certain crustacea. J. mar. biol. Ass. U.K. 36: 459-472.

Coyne, R.V. and J.A. Collins. 1972. Loss of mercury from water during storage. Analyt. Chem. 44: 1093-1096.

Crandall, C.A. and C.J. Goodnight. 1963. The effects of sub-lethal concentrations of several toxicants to the common guppy, Lebistes reticulatus. Trans. Am. microsc. Soc. 82: 59-73.

Cuénot, L. 1898. Études physiologiques sur les oligochaètes. Archs Biol., Liège. 15: 114-124.

Dall'Aglio, M. 1968. In "Origin and Distribution of Elements". pp. 1065-1081. (O.H. Ahrens, ed.). Pergamon Press. New York.

D'Amelio, V., G. Russo, and D. Ferraro. 1974. The effect of heavy metal on protein synthesis in crustaceans and fish. Rev. Int. Océanogr. Méd. 33: 111-114.

De Coursey, P.J. and W.B. Vernberg. 1972. Effect of mercury on survival, metabolism and behaviour of larval Uca pugilator (Brachyura). Oikos 23 [2]: 241-247.

Drach, P. 1930. Étude sur le système branchiale des crustacés décapodes. Arch. anat. microsc. morphol. exp. 26: 83-133.

Dyer, W.J., D.I. Hiltz, R.G. Ackman, J. Hingley, and G.L. Fletcher. 1970. In vivo assimilation by cod muscle and liver tissue of elemental phosphorus from polluted sea water. J. Fish. Res. Bd Can. 27: 1379-1384.

Edelstein, L.M. 1971. Melanin: A unique polymer. In "Pathobiology Annual" (ed. H.L. Ioachim) Butterworth, London.

Eisler, R., G.E. Zarogian, and R.J. Hennekey, 1972. Cadmium uptake by marine organisms. J. Fish. Res. Bd. Can. 29: 1367-1369.

Ellis, A.E., A.L.S. Munroe, and R.J. Roberts, 1975. Defence mechanisms in the fish. I. A study of the phagocytic system and the fate of intraperitoneally injected particulate material in the plaice, (Pleuronectes platessa L) J. Fish. Biol. 7: 67-78.

Feick, G., R.A. Horne and D. Yeaple. 1972. Release of mercury from contaminated freshwater sediments by the run-off of road de-icing salt. Science, N.Y. 175: 1142-1143.

Fletcher, G.L. 1971. Accumulation of yellow phosphorus by several marine invertebrates and seaweeds. J. Fish. Res. Bd. Can. 28: 793-796.

Fontaine, C.T. and R.C. Dyjak. 1973. The development of scar tissue in the brown shrimp, Penaeus aztecus, after wounding with the Peterson disk tag. J. invertebr. pathol. 22: 476-477.

Fontaine, C.T. and D.V. Lightner. 1974. Observations on the phagocytosis and elimination of carmine particles injected into the abdominal musculature of the white shrimp Penaeus setiferus. J. invertebr. Pathol. 24: 141-148.

Formanek, H. and S. Formanek. 1970. Specific staining for electron microscopy of murein sacculi of bacterial cell wall. Eur. J. Biochem. 17: 78-84.

Fowler, B.A. 1972. Ultrastructural evidence for neuropathy induced by long term exposure to small amounts of methy mercury. Science, N.Y. 175: 780-781.

Freeman, H.C., D.A. Horne, B.McTague, and M. McMenemy. 1974. Mercury in some Canadian Atlantic coast fish and shellfish. J. Fish Res. Bd Can. 31: 369-172.

Fujiki, M. 1963. Studies on the course that the causative agent of Minamata disease was formed, especially on the accumulation of the mercury compound on the fish and shellfish of Minamata Bay. J. Kumamotc med. Soc. 39: 494-

Fujiki, M. 1972. The transitional condition of Minamata Bay and the neighbouring sea polluted by the factory waste water containing mercury. Advan. Water Pollut. Res. Proc. Int. Cong. 6: 905-917.

Fujiya, M. 1965. Physiological estimation on the effects of pollutants upon aquatic organisms. Advances in Water Pollution Research. Proc. 2nd Internat. Conf. Tokyo 1964. Pergamon Press 3: 315-331.

Furukawa, K., T. Suzuki and K. Tonomura. 1969. Decomposition of organic mercurial compounds by mercury resistant bacteria. Agric. Biol. Chem. 33: 128-130.

Ganther, H.E., C. Goudie, M.L. Sunde, M.J. Kopecky, P. Wagner, Sang-Hwan Oh, and W.G. Hoekstra. 1972. Selenium: relation to decreased toxicity of methyl mercury added to diets containing tuna. Science, N.Y. 175: 1122-1124.

Gardner, D. and J.P. Riley, 1973a. The distribution of dissolved mercury in the Bristol Channel and Severn Estuary. Estuarine coastal mar. sci. 1^2: 191-192.

Gardner, D. and J.P. Riley. 1973b. Distribution of dissolved mercury in the Irish Sea. Nature, Lond. 241: 526-527.

Gaskin, D.E., K. Ishida, and R. Frank. 1972. Mercury in harbour porpoises (Phocaena phocaena) from the Bay of Fundy region. J. Fish. Res. Bd Can. 29: 1644-1646.

Gaskin, D.E., G.J.D. Smith, P.W. Arnold, M.V. Louisy, R. Frank, O.M. Holdrinet and J.W. McWade. 1974. Mercury, D.D.T. Dieldrin and PCB in two species of Odontoceti (Cetacea) from St. Lucia Lesser Antilles. J. Fish. Res. Bd Can. 31: 1235-1239.

Getsova, A.B. and Volkova, G.A. 1964. Accumulation of radioactive isotopes of phosphorus, yttrium, iodine, and mercury by larvae of aquatic insects. (translated from Zoologischeskii Zhurnal 43^7. 1077.) Fedn. Proc. Fedn Am Socs exp. Biol. 24: 683.

Goggins, P.L. 1960. (reissued 1967). The Storage of Live Lobsters in Recirculated Refringerated Tanks. Dept. of Sea and Shore Fisheries. State House. Angusta, Maine.

Goodrich, H.P. 1928. Reactions of gammarus to injury and disease, with notes on some microsporidial and fungoid disease. Q. Jl Microsc. Sci. 72: 325-353.

Guarino, A.M., J.B. Call, J.B. Pritchard, P.D. Briley, and D.P. Rall. The fate of radiocarbon methyl mercury in the lobster. Bull. Mt. Desert Isl. biol. lab. 11:26-28.

Gurba , J.B. 1970. Mercury situation in Alberta. Proc. 18th Annual Meeting and Conf., Can. Agr. Chem. Assoc. Jasper Alta.

Halcrow, W., D.W. Mackay and I. Thornton. 1973. The distribution of trace metals and fauna in the Firth of Clyde in relation to the disposal of sewage sludge. J. mar. biol. Ass. U.K. 53: 721-739.

Hamilton, W. 1971. Some ecological factors affecting the uptake by fresh-water organisms. Paper presented at 34th American Society of Limnology and Oceanography Meeting. (quoted by Cocoros, Cahn, and Siler, 1973).

Hammond, A.L. 1971. Mercury in the environment: natural and human factors. Science, N.Y. 171: 788-789.

Herrick, F.H. 1895. The American Lobster: A Study of its Habits and Development. Extracted from Bulletin of the U.S. Fish Commission for 1895: 1-252. Published, Government Printing Office, Washington.

Holden, A.V. and Topping, G. 1972. Occurrence of specific pollutants in fish in the Forth and Tay Estuaries. Proc. R. Soc. Edinb. (B) 71: 189-197.

Hollande, A.-Ch. 1920. Le formation du pigment brun-noir (mélanine) au cours de la phagocytose chez les Insectes. C.r. Séanc Soc. Biol. 83: 726-727.

Hosohara, K. 1961. Mercury content of deep sea water. J. chem. Soc. Japan, 82: 1107-1108.

Hubschman, J.H. 1967. Effects of copper on the crayfish Orconectes rusticus (Girard). Crustaceana 12: 141-150.

Hughes, J.T., R.A. Shleser, and G. Tchobanoglous. 1974. A rearing tank for lobster larvae and other aquatic species. Progre Fish Cult. 36^3: 129-133.

Hunter, W.R. 1949. The poisoning of Marinogammarus marinus by cupric sulphate and mercuric chloride. J. exp. Biol. 26: 113-124.

Hutcheson, M.S. 1975. The effect of temperature and salinity on cadmium uptake by the blue crab, Callinectes sapidus. Chesapeake Sci. 15^4: 237-241.

Imura, N., S.-K. Pan and T. Ukita. 1972. Methylation of inorganic mercury with liver homogenate of tuna fish. Chemosphere 5: 197-201.

Imura, N., E. Sukegawa, S.-K. Pan, K. Nagao, S-Y. Kim, T. Kwan, and T. Ukita. 1971. Chemical methylation of inorganic mercury with methyl cobalamin, a vitamin B_{12} analog. Science, N.Y. 172: 1248-1249.

Irukayama, K. 1967. The pollution of Minamata Bay and minamata disease. Adv. Water Pollut. Res (Munich) Proc. Int. Conf. 3: 153-180.

Irukayama, K., T. Kondo, F. Kai and M. Fujiki. 1961. Kumamoto med. J. 14: 157.

Irukayama, K., M. Fujiki, F. Kai and T. Kondo. 1962. Kumamoto med. J. 15: 1.

Irukayama, F. Kai, M. Fujiki and T. Kondo. 1962. Kumamoto med. J. 15: 7.

Jarvenpaa, T., M. Tillander, and J.K. Miettinen. 1970. Methyl mercury: half-time of elimination in flounder, pike, and eel. Suomen. Kem istil B. 43: 439-444.

Jensen, S. and A. Jernelov. 1969. Biological methylation of mercury in aquatic organisms. Nature, Lond. 223: 753-754.

Jernelov, A. 1968. Conversion of mercury compounds. In Chemical Fall-out. (M.W. Miller and G. Berg, eds). Charles C. Thomas, Springfield, Ill.

Johansen, O. and E. Steinnes. 1969. A simple neutron activation method for mercury in biological material. Int. J. appl. Radiat. Isot. 20 : 751-755.

Johnels, A.G., T. Westermark, W. Berg, P.I. Persson and B. Sjostrand. 1967. Pike (Esox lucius L.) and some other aquatic organisms in Sweden as indicators of mercury contamination in the environment. Oikos 18: 323-333.

Jones, A.M., Y. Jones, and W.D.B. Stewart. 1972. Mercury in marine organisms of the Tay Region. Nature, Lond., 238: 164-165.

Kendall, P.A. 1965. Labelling of thiolated antibody with mercury for electron microscopy. Biochim. biophys. Acta 97: 174-176.

Kennedy, W.A. and M.S. Smith. 1972. Sable fish culture - progress in 1971. Fish. Res. Bd. Canada Tech. Report 309.

Kennedy, W.A. and M.S. Smith. 1973. Sable fish culture - progress in 1972. Fish. Res. Bd. Canada Tech. Report 388.

Kensler, C.B. 1970. The potential of lobster culture. The American Fish Farmer, Oct. 1970. 8-27.

Khayrallah, N. and A.M. Jones. 1975. A survey of the benthos of the Tay Estuary. Proc. R. Soc. Edinb. B. 75: 113-135.

Kimoto, S. 1972. The scanning microscope as a system. Jeol News 10 no. 2.

Kirschner, L.B. and S. Wagner. 1965. The site and permeability of the filtration locus in the crayfish antenal gland. J. exp. Biol. 43: 385-395.

Kitamura, S., T. Tsukamoto, K., K. Hayakawa, K. Sumino and T. Shibata. 1966. Application of gas chromatography to the analysis of alkyl mercury compounds. (Issue 1) Med. Biol. (Helsinki) 72: 274-282. (quoted in Berglund et al., 1971).

Kiwimäe, A., Å. Swensson, U. Ülfvarson, and G. Westöö. 1969. Methyl mercury compounds in eggs after oral administration of mercury compounds. J. agric. Fd. Chem. 17: 1014-1017.

Klein, D.H. and E.D. Goldberg. 1970. Mercury in the marine environment. Environ. Sci. Technol. 4: 765-768.

Klemmer, H., S.N. Luoma, and L.S. Lau. 1973. Mercury levels in marine biota. Gov. Rep. Announce. 73^{10}. Also in U.S. National Oceanic and Atmospheric Administration Report NOAA - 73022303.

Knapik, M. 1969. The effect of the $HgNO_3$ content in a water medium upon the survival of certain crustacean species. Acta biol. cracov. Ser. Zool. 12: 17-27.

Koeman, J.H., W.H.M. Peeters, C.H.M. Kondstaal-Hol, P.S. Tjioe, and J.J.M. DeGoeij. 1973. Mercury-selenium correlations in marine mammals. Nature, Londi 245: 385-386.

Kramer, H.J. and B. Neidhart. 1974. Accumulation of mercury in fish. Presented at 2nd Conference on Nuclear Methods in Environmental Research. July 1974/University of Missouri - Columbia.

Kulczycki, A. 1968. Incorporation of ^{203}Hg mercury contamination of media by Daphnia pulex (de Geer) under laboratory conditions. Acta biol. cracov ser. Zool. 10: 143-147.

Kurland, L.T., S.N. Faro, and H. Siedler. 1960. Minamata Disease. World Neurol. 1: 320-396.

Leatherland, T.M., J.D. Burton, M.J. McCartney, and F. Culkin. 1972. Mercury in North-Eastern Atlantic ocean water. Nature Lond. 232: 112-113.

Leatherland, T.M., J.D. Burton, F. Culkin, M.J. McCartney and R.J. Morris. 1973. Concentrations of some trace metals in pelagic organisms and of mercury in Northeast Atlantic ocean water. Deep Sea Res. 20: 679-685.

Lillie, R.D. 1957. Ferrous ion uptake: A specific reaction of the melanins. AMA Archs Path. 64: 100-103.

Lofroth, G. 1969 Methyl mercury. (2nd edition). Swedish Natural Science Research Council. Ecol. Res. Comm. Bull. (Stockholm) 4. 1-38.

Majori, L. and F. Petronio. 1972. Marine pollutions by metals and their concentration by biological indicators (accumulation factor). Journées Étud. Pollutions, pp. 103-104. Athènes, C.I.E.S.M. (1972).

Maluf, N.S.R. 1941a. Micturition in the crayfish and further observations on the anatomy of the nephron of this animal. Biol. Bull. mar. biol. Lab., Woods Hole 81: 134-148.

Maluf, N.S.R. 1941b. Secretion of inulin, xylose, and dyes and its bearing on the manner of urine-formation by the kidney of the crayfish. Biol. Bull. mar. biol. Lab., 81: 235-260.

Matsunaga, K. 1975. Concentration of mercury by three species of fish from Japanese rivers. Nature, Lond. 257: 49-50.

Metchnikoff, E. 1892. Lecons sur la Pathologie comparée de l'Inflammation. Bibliotheque des annales de l'institute Pasteur, Paris.

Miettinen, J.K., M. Tillander, K. Rissanen, V. Miettinen, and Y. Ohmomo. 1969. Distribution and excretion rate of phenyl and methyl mercury nitrate in fish, mussels, molluscs and crayfish. Proc. 9th Jap conf. Radioisotopes: 474-478.

Ministry of Agriculture, Fisheries, and Food. 1971. Working party on the monitoring of foodstuffs for mercury and other heavy metals: First Report, and
1973. Supplementary Report.

Mount, D.I. and C.E. Stephan. 1967. A method for detecting cadmium poisoning in fish. J. Wildl. Mgmt 31: 168-172.

Mundkur, B. 1964. Electron microscopical studies of frozen-dried yeast. Expl Cell Res. 34: 155-181.

Newton, D.W. and R. Ellis. 1974. Loss of mercury (11) from solution. J. Environ. Qual. 3^{1}: 20-23.

Nishigaki, S. and M. Harada. 1975. Methylmercury and selenium in umbilical cords of inhabitants of the Minamata area. Nature, Lond. 258: 324-325.

Noren, K. and G. Westöö. 1967. Methyl mercury in fish. Vår Föda 19^{2}: 13-24.

Norseth, T. and T.W. Clarkson. 1970. Studies on the biotransformation of 203-Hg labelled methyl mercuric chloride in rats. Archs environ. Hlth. 21: 717-727.

O'Hara, J. 1973a. Cadmium uptake by fiddler crabs exposed to temperature and salinity stress. J. Fish. Res. Bd. Can. 30: 846-848.

O'Hara, J. 1973b. The influence of temperature and salinity on the toxicity of cadmium to the fiddler crab, Uca pugilator. Fishery Bull. Fish Wildl. Serv. U.S. 71: 149-153.

Ogilvie, R.F. 1967. Histopathology. Sixth Edition. E. and S. Livingstone. Edinburgh and London.

Olson, K.R. and P.O. Fromm. 1973. Mercury uptake and ion distribution in gills of rainbow trout (Salmo gairdneri): Tissue scans with an electron micro probe. J. Fish. Res. Board. Can. 30: 1575-1578.

Parry, G. 1960. Excretion. In The Physiology of Crustacea I. Metabolism and Growth. (T.H. Waterman, ed.) Academic Press.

Pauley, G.B. and A.K. Sparks. 1966. The actue inflammatory reaction in two different tissues of the pacific oyster, Crassostrea gigas. J. Fish. Res. Bd. Can. 23: 1913-1921.

Pauley, G.B. and A.K. Sparks. 1967. Observations on experimental wound repair in the adductor muscle and the leydig cells of the oyster. J. invertebr. Pathol. 9: 298-309.

Peakall, D.B. and R.J. Lovett. 1972. Mercury: its occurrence and effects in the ecosystem. Bioscience 221: 20-25.

Pearse, A.G.C. 1968, 1972. Histochemistry Theoretical and Applied. 2 vols. 3rd edition. Churchill Livinstone. Edinburgh and London.

Pepe, F.A. and H. Feick, 1961. The use of specific antibody in electron microscopy. II. The visualisation of mercury-labelled antibody in the electron microscope. J. biophysic . biochem. 11: 521-548.

Peters, H. 1935. Über den Einfluss des Salzegehaltes im Aussen medium auf den Bau und die Funktion der Exkretionsorgane Dekapoder Crustaceen (Nach Untersuchungen an Potamobius fluviatilis and Homarus vulgaris) Z. Morph. Ökol. Tiere 25: 293-306.

Poinar, G.O., R. Leutenegger and P. Götz. 1968. Ultrastructure of the formation of a melanotic capsule in Diabrotica (Coleoptera) in response to a parasitic nematode (Mermithidae). J. Ultrastruc. Res. 25: 293-306.

Portmann, J.E. 1968. Progress report on a Programme of insecticide analysis and toxicity testing in relation to the marine environment. Helgol. wiss. Meeresunters 17: 247-256.

Raeder, M.G., and E. Snekvik. 1941. Quecksilbergehalt mariner organismen K. norsk. Vidensk. Selsk. Forh. 13: 169-172.

Renfro, J.L., B. Schmidt-Nielsen, D. Millar, D. Benos, and J. Allen. 1974. Methyl mercury and inorganic mercury: uptake, distribution and effect on osmo-regulatory mechanisms in fish. In Pollution and Physiology of Marine Organisms. (F.J. Vernberg and W.B. Vernberg, eds). Academic Press.

Riegel, J.A. 1966a. Micropuncture studies of formed-body secretion by the excretory organs of the crayfish, frog, and stick insect. J. exp. Biol. 44: 379-385.

Riegel, J.A. and L.B. Kirschener. 1960. Excretion of inulin and glucose by the Crayfish antennal gland. Biol. Bull. mar. biol. Lab., Woods Hole, 118: 216-307.

Rissanen, K. and J.K. Miettinen, 1972. Use of mercury compounds in agriculture and its implications. In Mercury Contamination in Man and his Environment. Technical Reports no. 137. Internationa Atomic Energy Agency. Vienna 1972.

Rivers, J.B., J.E. Pearson and C.D. Shultz. 1972. Total and organic mercury in marine fish. Bull. environ. contam. Toxicol. 8: 257-266.

Roberts, R.J. 1975. The effects of temperature on diseases and their histopathological manifestations in fish. In The Pathology of Fishes. (W.E. Ribelin and G. Migaki. eds) University of Wisconsin Press.

Roesijadi, G., S.R. Petrocelli, J.W. Anderson, B.J. Presley, and R. Sims. Survival and chloride ion regulation of the porcelain crab, Petrolisthes armatus, exposed to mercury. Mar. Biol. (Berl) 27: 213-217.

Rucker, R.R. and D.F. Amend. 1969. Absorption and retention of organic mercurials by rainbow trout and chinook and sockeye salmon. Progre Fish Cult. 31: 197-201.

Saha, J.G. 1972. Significance of mercury in the environment. Residue Revs 42: 103-164.

Saha, J.G., A.K. Sumner, and F.M. Atton. Unpublished results. (quoted in Saha, 1972).

Saito, N. 1972. Use of mercury and its compounds in industry and medicine. In Mercury Contamination in Man and his Environment. Technical reports no. 137. International Atomic Energy Agency. Vienna.

Schlumberger, H.G. 1952. A comparative study of the reaction to injury. I. The cellular response to methylcholanthrene and to talc in the body cavity of the cockroach (Periplaneta americana). AMA Archs Path. 54: 98-113.

Schmidt-Nielsen, B., K.H. Gertz and L.E. Davis. 1968. Excretion and ultrastructure of the antennal gland of the fiddler crab Uca mordax. J. Morph. 125: 473-496.

Selli, R., M. Frignani, C.-M. Rossi and R. Viviani. The mercury content in the sediments of the Adriatic and the Tyrrhenian. In C.I.E.S.M.M. Journées d'études sur les pollutions marines. 39-40. Monaco. Commission International pour l'Exploration Scientifique de la Mer Mediteranée. 1973.

Seymour, A.H. 1971. The rate of loss of mercury by pacific oysters. In Mercury in the Western Environment. Proceedings of a workshop Portland, Oregon February 1971. A Continuing Education Book.

Sindermann, C.J. 1971. Internal defences of crustacea: A review. Fishery Bull. Fish Wildl. Serv. U.S. 69: 455-489.

Sloan, J.P., J.A.J. Thompson and P.A. Larkin. 1974. The biological half-life of inorganic mercury in the Dungeness crab (Cancer magister). J. Fish. Res. Bd. Can. 31: 1571-1576.

Smith, J.C., T.W. Clarkson, R. Von Burg and F. Lasselle. 1971. The form of mercury in North American fish. Fedn. Proc. Fedn. Am. Socs exp. Biol. 30: 221- abs.

Smith, R.E. and Fishman, W.H. 1969. p-(acetoxymercuric) aniline diazotate, a reagent for visualising the napthol AS-B1 product of acid hydrolase action at the level of the light and electron microscope. J. Histochem. Cytochem. 17: 1-22.

Solangi, M.A. and D.V. Lightner. 1976. Cellular inflammatory response of Penaeus aztecus and P. setiferus to the pathogenic fungus, Fusarium sp., isolated from the californian brown shrimp, P. californiensis. J. invertebr. Pathol. 27: 77-86.

Sparks, A.K. 1972. Invertebrate Pathology: Noncommunicable diseases. Academic Press. New York and London.

Stewart, J.E., J.W. Cornick, D.M. Foley, M.F. Li, and C.N. Bishop. 1967. Muscle weight relationship to serum proteins, hemocytes, and hepatopancreas in the lobster, Homarus americanus. J. Fish. Res. Bd. Can. 24: 2339-2354.

Stock, A. and F. Cucuel. 1934. Die Verbreitung des Quecksilbers. Natur wissenschaften 22: 390-393.

Stoewsand, G.S., J.A. Anderson, W.H. Gutenmann, C.A. Bache, and D.J. Lisk. 1971. Eggshell thinning in Japanese quail fed mercuric chloride. Science, N.Y. 173: 1030-1031.

Suzuki, T., T. Miyama and C. Toyama. 1973. The chemical form and bodily distributionoof mercury in marine fish. Bull. environ. contam. Toxicol. 10: 347-355.

Sumino, K. 1968. Analysis of organic mercury compounds by gas chromatography. Part I. Analytical and Extraction Method of Organic Mercury compounds. Kobe J. med. Sci. 14: 115-130. (quoted in Berglund et al.).

Taylor, R.L. 1969. A suggested role for the polyphenol- phenoloxidase system in invertebrate immunity. J. invertebr. Path. 14: 427-428.

Thiriot-Quiévreux. C. 1966. Note sur une néphropathie expérimentale provoquée par du bichlorure de mercure chez C. maenas. Archs. Zool. exp. gén. 107: 272-282.

Thomas, H.J. 1954. The oxygen uptake of the lobster, (Homarus vulgaris Edw.) J. exp. Biol. 31: 228-252.

Timur , M. 1975. A study of the Carregeenin Granuloma in the Plaice, Pleuronectes platessa. Ph.D. Thesis of the University of Stirling.

Tonomura, K., K. Maeda, F. Futai, T. Nakagami and M. Yamada. 1968. Stimulative vaporization of phenyl-mercuric acetate by mercury resistant bacteria. Nature, Lond. 217: 644-646.

Travis, D.F. 1955. The molting cycle of the spiny lobster, Panulirus argus Latreille. III. Physiological changes which occur in the blood and urine during the normal molt cycle. Biol. Bull. mar. biol. Lab., Woods Hole 109: 484-503.

Trump, B.J., R.T. Jones, and S. Sahaphong, 1975. Cellular effects of mercury on fish kidney tubules. In The Pathology of Fishes. (W.E. Ribelin and G. Migaki. eds.) University of Wisconsin Press.

U.S. Dept. of the Interior. Mercury in the Environment. 1970. J.S. Geological Survey Professional Paper no. 713.

Uthe, J.F., F.A.J. Armstrong and K.C. Tam. 1971. Determination of trace amounts of mercury in fish tissues: Results of a North American check sample study. J. Ass. Offi. Analyt. Chem. 54: 860-869.

Uthe, J.F., J. Solomon, and B. Grift 1972. Rapid semi-micro method for the determination of methyl mercury in fish tissue. J. Ass. Offi. Anal. Chem. 55: 583-589.

Vermeer, K. 1972 The crayfish, Orconectes virilis, as an indicator of mercury contamination. Can. Fld. Nat. 86: 123-125.

Vernberg, W.B., P.J. DeCoursey and W.J. Padgett. 1973. Synergistic effects of environmental variables in larvae of Uca pugilator. Mar. Biol. (Berl) 22: 307-312.

Vernberg, W.B. and J. O'Hara. 1972. Temperature-salinity stress and mercury uptake in the fiddler crab, Uca pugilator. J. Fish. Res. Bd. Can. 29: 1491-1494.

Vernberg, W.B. and J. Vernberg. 1972. The synergistic effects of temperature, salinity, and mercury on survival and metabolism of the adult fiddler crab, Uca pugilator. Fishery Bull. Fish Wildl. Serv. U.S. 71: 149-153.

Vogel, F.S. 1959. The deposition of exogenous copper under experimental conditions with observations on its neurotoxic and nephrotoxic properties in relation to Wilson's Disease. J. exp. Med. 10: 801-810.

Vonk, H.J. 1960. Digestion and metabolism. In The Physiology of Crustacea. I. Metabolism and Growth. (T.H. Waterman, ed.) Academic Press.

Ware, R.A., L.W. Chang, and P.M. Burkcholder. 1974. Ultrastructural evidence for foetal liver injury induced by in utero exposure to small doses of methyl mercury. Nature, Lond; 251: 236-237.

Weiner, I.M., R.I. Levy and G.H. Mudge. 1962. Studies on mercurial diuresis; renal excretion, acid stability and structure-activity relationships of organic mercurials. J. Pharmac. exp. Ther. 138: 96.

Westermark, T. and B. Sjostrand. 1960. Activation analysis of mercury. Int. J. appl. Radiat. Isot. 9: 1-15.

Westermark, T. 1965. Mercury in aquatic organisms. In The Mercury Problem in Sweden. Stockholm. Royal Swedish Ministry of Agriculture.

Westöö, G. 1966. Determination of methyl mercury compounds in foodstuffs. I. Methyl mercury compounds in fish, identification and determination. Acta Chem. Scand. 20: 2131-2137.

Westöö, G. 1967. Determination of methyl mercury compounds in foodstuffs. II. Determination of methyl mercury in fish, egg, meats, and liver. Acta chem. Scand. 21: 1790-1800.

Westöö, G. 1968. Determination of methyl mercury salts in various kinds of biological materials. Acta Chem. Scand. 22: 2277-2280.

Westöö, G. 1968. Comparison between Japanese and Swedish methods for determining methyl mercury compounds in fish flesh. National Institute of Public Health S-104 01 Stockholm 60, Stencilo, (quoted in Berglund et al.).

Westöö. G. 1971. (Personal communication, see Saha, 1972).

Westöö, G, and M. Rydalv. 1969. Kvicksilver och metykvicksilver i fish och krafter. Vår Föda. 19: 13-17.

Westöö. G. and M. Rydalv. 1969. Mercury and methyl mercury in fish and crayfish. Vår Föda. 21: 19-111.

Westöö, G. and M. Rydalv. 1972. Methyl mercury in fish. Vår Föda 23: 179-318.

Wilson, K.W. and P.M. Connor. 1972. The use of a continuous flow apparatus in the study of longer-term toxicity of heavy metals. I.C.E.S. C.M. 1971/E8. Fisheries Improvement Committees. Ref (Hydro. Cttee)

Windom, M., F. Taylor and R. Stickney. 1973. Mercury in North Atlantic plankton. J. Cons. int. Explor. Mer, $35^{(1)}$: 18-21.

Wobeser, G., N.O. Nielson, R.H. Dunlop and F.M. Alton. 1970. Mercury concentrations in tissues of fish from the Saskatchewen river. J. Fish. Res. Bd. Can. 27: 830-834.

Wood, J.M., F.S. Kennedy and C.G. Rosen. 1968. Synthesis of methyl mercury compounds by extracts of a methanogenic bacterium. Nature, Lond. 220: 173-174.

Yoshida, T., T. Kawabata, and Y. Matsue. 1967. Transference mechanism of mercury in marine environment. J. Tokyo Univ. Fish. 53: 73-84.

Zitko, V., D.E. Aiken, S.N. Tibbo, K.W.T. Besch, and J.M. Anderson. 1970. Toxicity of yellow phosphorus to herring (Clupea harengus) Atlantic salmon (Salmo salar) lobster (Homarus americanus) and beach flea Gammarus oceanicus). J. Fish. Res. Bd. Can. 27: 21-29.

Zitko, V., B.J. Finlayson, D.J. Wildish, J.M. Anderson, and A.C. Kohler. 1971. Methyl mercury in freshwater and marine fishes and marine fishes in New Brunswick, in the Bay of Fundy, and on the Nova Scotia Banks. J. Fish. Res. Bd. Can. 28: 1285-1291.