TOXICITY AND MODE OF ACTION STUDIES

OF COPPER IN SPECIES OF DAPHNIA

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CONTENTS

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ABSTRACT

The object of the project was to study the toxic effects of copper on Daphnia species by a series of experiments starting with a determination of the acute toxicity followed by sequential generation exposure to sub-lethal doses of copper to induce a tolerant strain for radiochemical, histochemical and pathological studies in order to characterize tolerance and elucidate its mechanism. The 24 hour LC50 of copper against 1st instar D. magna was 15.2ug Cu^{++}/1. To overcome the analytical problems which this low level posed, a solution of 0.009 m M EDTA added to the culture medium gave a 24 hour LC50 value of 169.1 ug Cu^{++}/1 which was amenable to analysis by the technique of ion concentration and solvent extraction. A continuous flow system was constructed for the administration of copper to daphnia. It incorporated the mass culture of the feeding organism Chlorella vulgaris and the cells and copper solutions were simultaneously relayed to daphnia. Background levels of copper in the system were consistently high. This was unfortunate and since the minimum background corresponded with the maximum safe dosage of copper for administration to daphnia this part of the study had to be discontinued and a modified programme was devised.

Studies on the influence of copper on the respiratory rate of daphnia showed that concentrations of 5.0, 0.5 or 0.25mg Cu^{++}/ℓ caused significant increases in oxygen uptake compared with the non-copper control. At a concentration of 0.125 mg Cu^{++}/ℓ the rate of oxygen uptake did not differ significantly from the control. The elevated rates of respiration would deplete the energy reserves and it is postulated that this effect represented one of the main toxicological routes of copper. Experiments with $^{64}Cu^{++}$ showed that after 1 hour's exposure to a concentration of 3.80^{-1} μ Ci $^{64}Cu^{++}$/mℓ. radioactive copper accumulated to 0.72^{-3}μg. $^{64}Cu^{++}$ per single daphnid. After six to nine hours' exposure daphnia excreted a high proportion of the absorbed radioactive copper. A single experiment tentatively indicated the absence of a metallothionein binding system in daphnia.

CHAPTER 1

INTRODUCTION

1.1 A Review of copper tolerance (and associated metal tolerance) in various bioassay organisms

This review is concerned with copper, and other metals only as relevant.

1.1.1 Animals

Tolerance to heavy metals has been studied in several species of marine invertebrates and some freshwater species and various theories have been advanced to account for the way the organisms have become adapted to tolerate low levels of some heavy metals, in particular, zinc, lead and copper. For example, Brown (1977) in studies with a copper-tolerant strain of Asellus meridianus obtained from sites receiving mine-drainage in Cornwall found that the main storage organ for copper was the hepatopancreas and showed that tolerant animals accumulated copper from experimental solutions and from metal-enriched food; this was in contrast to non-tolerant strains which showed no evidence of accumulation. Histochemical analysis of the hepatopancreas showed spherical inclusions 5 - 10 um in diameter in the caecae of copper-tolerant animals. Sections of the hepatopancreas viewed by transmission electron microscopy revealed dense spherical inclusions bounded by a membrane and around these structures there were distended mitochondria which had lost their cristae. X-ray microanalysis demonstrated significant amounts of sulphur in these structures only in the tolerant strain. In an earlier study the same author (Brown, 1976) showed that tolerance to lead persisted in the laboratory in the F_2 generation which indicated that tolerance was under genetic control.

The above reference to the presence of sulphur in copper-tolerant Asellus is of interest since it supplements the studies

by Holm-Jensen (1948) who reported that the toxic effects of silver and copper to Daphnia magna were diminished by the addition of cysteine at a concentration equivalent to that of the heavy metal.

By x-ray photometric examination, Wieser (1961) showed that terrestrial isopods contained high levels of copper. Of eight species analysed copper levels in term of dry weight varied from 0.022 to 0.118%. Up to 1961 the occurrence of the copper-based pigment haemocyanin had been proved only in stomatopods and decapods and Wieser comments that it was reasonable to suppose that isopods would contain this pigment in which case the hepatopancreas might be a store for the synthesis of haemocyanin. In a later publication (1967), Wieser states that in decapods copper may be stored in the form of pseudo-crystals or as large refractive bodies in special copper cells of the hepatopancreas; in isopods it can be found throughout the hepatopancreas as small granules in an easily dissociable state that can be complexed with chelating agents. In marine and intertidal species copper can move freely from cell to cell while in terrestrial isopods copper is nearly always confined to specialised cells which are held between large secretory cells. When copper is being mobilised, at the moulting cycle or during periods of stress, a protein-rich compound synthesized in the hepatopancreas picks up the free copper converting it to a tightly bound complex. At the end of such a cycle of activity the protein-rich compounds break down liberating the copper which may reappear in the hepatopancreas there to be concentrated in the storage cells.

Studies by Coombs (1974) with zinc and copper complexes in the Oyster (Ostrea edulis) indicated the presence of a soluble component in the soft tissues and an insoluble part in the cell

membrane residues. The soluble material which constituted some 40% of the total zinc and copper fractionated on Sephadex G - 25 and the metals were shown to be weakly complexed to the small molecular weight compounds taurine, lysine, ATP and possibly homarine. The soluble complexes were said to be a mobile reservoir of the metals for metal-dependent enzyme systems.

According to Saltman (1965) the mechanism of uptake of heavy metals is poorly understood. He suggested that endogenous or exogenous ligands or chelating agents combined with the metal ions to form soluble and low molecular weight complexes and in this form are transferred into the cell. This is an interesting observation in view of subsequent studies by George and Coombs (1977) on the uptake and accumulation of cadmium by Mytilus edulis. They concluded that prior complexation of cadmium with EDTA, humic and alginic acids or pectin doubled the rates of cadmium accumulation, and eliminated the initial lag period in initial uptake. They suggested that ionic cadmium must first be complexed before uptake can occur.

The involvement of metallothionein in cadmium and zinc-induced tolerance to cadmium toxicity was studied by Leber and Miya (1976) in the mouse in attempts to determine the mechanisms conferring tolerance. The discovery of a cadmium-metalloprotein in equine kidney by Margoshes and Vallee (1957) suggested a mechanism to explain the long half-life for cadmium in the body. Subsequent work showed that metallothionein has a low molecular weight, approximately 6500, and that it contains zinc and copper in addition to cadmium, and that a high proportion of its amino acid residues (33%) are capable of chelating metal ions in a tridentate complex (Kagi et al., 1974). Leber and Miya (ref. cit.) found that mouse-derived

metallothionein consisted of two distinct cadmium-binding proteins with similar absorption and metal-binding properties. Mice exhibited tolerance to cadmium toxicity after pre-treatment with cadmium or zinc acetate. Treatment with cadmium yielded a liver metallothionein containing cadmium and zinc, while treatment with zinc produced metallothionein which contained only zinc. Mice treated with cadmium or zinc followed by a later treatment with cadmium resulted in displacement by cadmium of zinc from both cadmium- and zinc- metallothionein. The conclusion from the study was that tolerance to cadmium in pre-treated mice was conferred by the displacement of zinc from metallothionein and the chelation of cadmium to metallothionein.

The concentrations of zinc and copper in eighteen species of freshwater and marine decapods were reported by Bryan (1968) to lie between 20 and 35 ug/g for both metals, the apparent consistency being attributed to a system of regulation operating in the tissue and body fluids. The blood proteins of the different species were separated by starch-gel electrophoresis and it was found that the majority of proteins were haemocyanins, with a consequent linear relationship between the solids content of the blood and its concentration of copper. When large amounts of zinc were absorbed from the stomach only a fraction of this appeared in the blood apparently because the hepatopancreas in addition to being a route for absorption can re-absorb and store excess zinc from the blood thus reducing the risk of toxic action.

The influence of different nutritional levels, from feeding to starvation, on levels of copper in the blood and hepatopancreas and on the blood proteins of Crangon vulgaris has been investigated by Djangmah (1970). He reported that the blood proteins of Crangon

consisted of haemocyanin (60 - 93% of total protein) and minor proteins characterized as esterases and glycoproteins on starch-gel electrophoresis. These proteins may vary greatly in concentration between different seasons and during the moulting cycle. During starvation the hepatopancreas accumulated copper from the blood and the copper was held in the form of copper vesicles.

Bernard and Lane (1961) reported greenish-black deposits of copper in various tissues or organs of planktonic cyprids, in the connective tissues, the anteroventral region, in epithelium lining of the stomach, and in the epithelium lining of the posterior two-thirds of the hind gut.

A comparison of copper levels in brown bullhead fish in acute and accumulation studies by Brungs et al. (1973) showed that the copper concentration in the gills, operculum, liver and kidney did not differ between the 6-day acute test and the long term exposures of one to 20 months. The exposure levels were from 6.5 to 104 ug/l. Copper levels in the red blood cells and plasma in the 20-month exposure period did not differ from the untreated controls.

Saliba and Ansanullah (1973) exposed Artemia salina for three weeks to 0.1 ppm copper and found that the median lethal time of the exposed animals to 1 ppm copper was approximately double that of the non-exposed controls.

Using ^{64}Cu Bryan (1974) found that copper was absorbed more rapidly by tolerant than non-tolerant Nereis diversicolor. This was considered to be due to copper-tolerant worms having a greater capacity for binding and detoxifying copper in the epidermal cells. "The rapid absorption of copper from non-toxic concentrations seemed

to reflect the presence of material for binding the metal but does not necessarily reflect the existence of copper tolerance". This is a curious comment since the presence of a binding material might well be the factor protecting the organism in the first place from the "non-toxic concentration".

In their study on the mode of action of copper and mercury toxicity in three species of Crustaceans, Corner and Sparrow (1956) found that the rate of penetration was an important factor, an observation which was also made by McLusky and Phillips (1975) who suggested that the rate of uptake of copper was more decisive than the concentration of the accumulated metal in causing mortality in the polychaete Phyllodocea maculata. Corner and Sparrow (ref. cit.) suggested that mercury compounds mainly act internally but toxic effects may also be initiated at surfaces. Thus Artemia treated with a sub-lethal dose of mercuric chloride became more sensitive to poisoning by copper an effect which was appreicably reduced if the mercury-treated animals were suspended for a few minutes in cysteine or in reduced glutathione. To determine whether Artemia was sensitized to mercury poisoning by prior exposure to copper, animals immersed for 1 hour in sea water containing 1 g Cu^{++}/l of copper citrate and subsequently suspended for five minutes in sea water alone and in sea water containing 0.01 M cysteine and 0.01 M glutathione were then exposed to 250, 125 and 62.5 mg Hg^{++}/l in sea water. The animals treated with copper and then washed in sea water died at the same enhanced rates in the mercury solutions, while copper-treated animals suspended in cysteine or glutathione showed a lower rate of mortality. Thus some of the copper which lowered the resistance of Artemia to mercury poisoning was located

on the surfaces of the animals and this attached copper was removable with cysteine or glutathione but not with sea water. The authors, however, did not take into account the possibility that a proportion of the cysteine or glutathione was absorbed by the animals and as strong chelating agents, mercury would be partly immobilized and pass through the gut unabsorbed.

In their studies on the effect of copper and mercury on the respiration rate and motility of Artemia larvae, Corner and Sparrow (ref. cit.) showed that the normal respiratory rate of 1.01 to 1.15 ul O_2/mg wet weight per hour was markedly and rapidly decreased by 1 g Cu^{++}/l and considerably reduced though more slowly by 250 mg Hg^{++}/l. By contrast mercury caused a more rapid reduction than copper in the motility of the larvae. The authors conclude that the effect of copper on the animals appeared to be one of specific inhibition of the respiratory mechanisms.

Hellerman, Chinard and Deitz (1943) working on urease, and Stoppani et al. (1953) working on enzyme-inhibition in yeast carboxylase found that the inactivation of these enzymes by small amounts of copper, mercury and various mercaptide-forming substances derived from mercury could be partly reversed by thiol compounds such as cysteine. Their findings indicated that the heavy metal poisons inhibit these enzymes by attaching to the surfaces at sulphydryl groups responsible for catalytic activity.

R. A. Peeters (1965) studying the genesis of convulsions found that 0.1 m M Cu^{++} was apparently very toxic to mitochondria in pigeon brain but he had reservations concluding that the copper was attacking the mitochondria in vivo since the action was immediate and there did not seem time for diffusion to take place unless there

were mitochondria in very close contact with the brain membranes. Further experiments carried out on brain slices showed that copper was much less toxic than with the isolated mitochondria, thus ruling out the simple mitochondrial hypothesis and pointing to the possible involvement of membrane enzyme ATP-ase. Labelled studies with ^{64}Cu demonstrated the effect of Cu^{++} in reducing the amount of phosphate split from ATP by the membrane ATP-ase present. The ATP-ase appeared to have an - SH component and the author found that the addition of the thiols penicillamine dimercaptopropanol and diethyldithiocarbamate induced increases of 25% in the activity of the enzyme. The hypothesis was advanced that copper induced convulsions by interfering with an - SH containing group in a membrane ATP-ase. In vivo studies suggested that the compound which copper forms with the tissue constituent was stable and once the initial toxicity occurred it could not be reversed by any of the - SH substances. The author tentatively concluded that the convulsions induced by the toxicity of copper in the brain were due to an inhibition of the membrane transport of ions.

When given orally ^{64}Cu reached peak concentration in humans in 1 to 2 hours according to G. W. Evans (1973). Absorption of copper into blood probably occurred in the stomach or upper intestine and the results suggested that the copper was eventually bound in the intestinal cells to a low molecular weight protein of about 10,000. This protein was said to be similar to metallothionein, a protein rich in sulphydryl groups that binds cadmium, copper and zinc ions.

In their review of copper in medicine the International Copper Research Association (1975) report that ascorbic acid and

molybdenum compounds may interfere in the absorption and utilization of copper in humans. Copper might inhibit a series of important enzymes by forming inert complexes with thiol ligands.

A recent study by S. G. George et al. (1978) on the ultrastructural location of copper and zinc in the oyster Ostrea edulis demonstrated by electron microprobe x-ray analysis that copper and zinc were present in vesicles bounded by a membrane and the copper was associated with sulphur, which confirms the observation made by Brown (1977) and zinc was associated with phosphorus. Further studies are in progress on the characterization of the vesicle to determine whether the copper is present naturally as inorganic copper sulphide or is bound to a protein sulphydryl group. In the processing of the tissues, the use of H_2S - saturated buffer solution at fixation was essential to retain the copper which otherwise would have been lost by diffusion through the tissue block.

1.1.2 Plants

Currently there is no evidence of constitutional tolerance to copper in plants, although in Silene vulgaris evolutionary resistance has always occurred when mine habitats are colonized. Some species of grasses are able to evolve tolerance, others are unable to do so even if high populations occur in the vicinity of metal-contaminated soils. In Agrostis tenuis tolerance was selectively induced in one generation. The surviving plants (1 in 7,000) from seeds sown on copper mine soil plus loam were transferred to normal soil and tested for tolerance and they were shown to have significantly greater tolerance than the original population (Antonovics et al., 1971).

According to Antonovics (ref. cit.) studies on the genetics

of metal tolerance in plants are cursory and inconclusive. He lists possible mechanisms for tolerance including differential uptake of ions, removal of metal ions from metabolism by deposition in vacuoles or removal by pumping from the cell, or modification to an innocuous form. There could be alternative metabolic pathways by-passing a sensitive site or an increase in the concentration of a metabolite that antagonises the inhibitor or a decrease in the permeability of cell or sub-cellular units to metal ions. Non-protein sulphydryl groups can exert a protective effect against zinc, lead and copper toxicity.

The first demonstration of a metallothionein in a vascular plant was described by Rauser and Curvetto (1980). They described metallothioneins as low molecular weight proteins with high contents of half cystinyl residues (up to 33%) which can bind metals such as zinc, copper, cadmium, mercury or silver in mercaptide complexes. They may, therefore, play an important role in detoxification mechanisms.

1.1.3 Fungi

Antoine (1965) demonstrated the existence of copper-tolerant races of yeast, and Ashida (1965) showed that copper-tolerant yeasts deposit excess copper as sulphide at the cell periphery as a consequence of cysteine activity.

An example of a metal-complexing mechanism responsible for heavy metal tolerance was shown by Ashworth and Amin (1964) in their studies on Aspergillus niger. They suggested that mercury tolerance was due to a pool of non-protein sulphydryl groups that protected the enzyme systems by forming complexes with mercury as it entered the thallus.

1.1.4 Algae

Foster (1977) concluded from her experiments with Chlorella vulgaris that copper exclusion was the mechanism of tolerance to the metal. The growth rate of the copper tolerant culture and the non-tolerant as a function of the amount of copper accumulated was practically identical indicating that the two strains responded identically to the same amount of cellular copper. Once the metal reached a threshold level in the tolerant cells no further accumulation occurred and a mechanism operated to exclude the further intake or uptake of the metal.

1.1.5 Bacteria

The resistance to copper of oxidising and reducing strains of bacteria has been examined by Booth and Mercer (1963). Sub-cultures of pure strains of sulphur-oxidising and sulphate-reducing bacteria were grown on media containing a known concentration of copper ions added as $CuSO_4$. When growth of the sub-culture occurred a second sub-culture was made into medium containing double the concentration of copper. This procedure was continued until a concentration of copper was reached sufficient to inhibit growth. Six successive sub-cultures were then made at the next lower copper concentration before a second attempt was made to obtain growth at the high concentration. If growth occurred the process was repeated. If there was still inhibition the lower concentration was taken as the limiting concentration of growth. The limiting concentration for the sulphur-oxidising bacteria was 10,000 ppm Cu^{++}, and 20 ppm for the sulphate-reducing bacteria. The sulphur oxidising bacteria showed a remarkable resistance to copper but the authors do not advance any hypothesis on the mechanism leading to resistance.

1.1.6 Conclusion

The literature search has demonstrated an apparent relationship between sulphur or sulphur derivatives or sulphydryl groups and tolerance to copper.

In studies in which the test animals were exposed to external solutions of copper, elemental sulphur was demonstrated in the copper tolerant strain of Asellus and was absent in the non-tolerant strain. In the oyster, x-ray probe analysis illustrated that sulphur was again associated with copper and further studies are in progress to determine if the copper is bound to a protein sulphydryl group or exists as inorganic copper sulphide. Copper-tolerant yeasts have been shown to deposit excess copper as the sulphide in the cell wall in response to cysteine activity. Metallothioneins have been implicated with metal tolerance in several studies and the high contents of half cystinyl residues may bind copper or other metals as part of a detoxification process. Finally, the sulphur association has been demonstrated by the unusually high resistance of sulphur-oxidising bacteria to copper.

1.1.7 Summary of review exclusive of metals other than copper

1) Copper tolerant strains of some organisms have demonstrated a capacity for more rapid absorption and accumulation of copper than when tolerance is absent.
2) The toxicity of copper may be caused by an inhibition of the membrane transport of ions or by a loss of natural ions.
3) The rate of uptake of copper was more important than the amount of metal accumulated in the organism in influencing acute toxicity.
4) Sensitivity to copper poisoning may be enhanced by prior

exposure to a metal, for example mercury.

5) Copper tolerance has been induced in the laboratory by exposing Artemia salina for three weeks to 0.1 ppm copper.

6) Sulphur has been identified in an organism tolerant to copper and in a species exposed to copper. Its presence may signal the initiation of a defence mechanism associated with the binding of copper to sulphydryl groups or to Cu-metallothionein protein.

1.2 The Biology of Daphnia

1.2.1 General

The genus consists of some fifty species distributed over a wide geographical area from the temperate zones to the tropics and the near Arctic regions. It is a common inhabitant of ditches, ponds or lakes. It feeds on algae, bacteria and detritus which are sieved by means of bristle-fringed thoracic limbs and are channelled towards the mouth by water currents formed by the action of two pairs of limbs. In favourable conditions the female lays thin-shelled eggs which develop parthenogenetically, but in adverse conditions two thick-shelled resistant eggs are produced which require fertilization and which develop inside the specially thickened cuticle of the brood pouch, the ephippium. The eggs remain in the ephippium until diapause is broken by a return to higher temperatures or more favourable conditions for hatching. Invariably female nymphs are hatched from the ephippial eggs.

In normal parthenogenesis each female produces several broods, the number of nymphs hatched and the frequency of brood production varying with nutritional status, temperature and density

of population. At temperatures of 10 - 15°C a female may live for 40 to 50 days and in that time produce five to six broods consisting of from two to three to 100 nymphs. The nymph takes about eight days to reach adult status to continue the cycle of reproduction.

1.2.2 Anatomy

The body is bilaterally compressed and consists of a head and thorax which is enclosed in two shells constituting the carapace. There is no clear definition between the head and the thorax. The head is flexed ventrally and carries a single compound eye which vibrates continually and is ennervated by one of two nerves originating from the cerebral ganglia. The other nerve is directed to the vestigial median eye.

The carapace is large and protects the organism and forms the basis of a channel to canalize the stream of water carrying the food particles. Within each carapace lobe is a large, coiled, maxillary gland which is an excretory structure whose ducts open immediately behind the maxillules. Between the carapace and the thorax there is the brood pouch for eggs or developing embryos which are enclosed by two thoracic spines.

1.2.3 Digestive System

The alimentary system from the oesophagus consists of a narrow foregut opening up into the wider midgut from which arise two unbranched curved diverticula which are probably associated with the secretion of digestive enzymes. The midgut leads to the hindgut which extends to the anus. Waste material and undigested food particles are expelled by the action of the sphincter muscle at the junction of the mid- and hind- gut, activating an anti-peristaltic

PAGE

MISSING

1.2.4 Circulatory System

The heart is a simple thin-walled muscular sac lying in front of the brood pouch, ovoid in shape with a single pair of ostia and a very short aorta. The blood passes through the ostia and is pumped forwards into the heart region, then back through the flaps of the carapace and through the body ventral to the midgut.

An early description of the blood circulation published by Herouard (1905) showed that the circulation was conducted through lacunae and sinuses thus refuting the presence of blood vessels which were earlier assumed to be the means of transport. As the ventricle contracts the blood is forced out in a line parallel to the long axis of the body towards the head where the flow is diverted towards the dorsal part of the optical ganglia and then towards the rostrum. There it changes direction to descend into the ventral cavity in the region of the maxillae and subsequently to the appendages and thence to the carapace. From there the flow descends to the gut cavity and then to the ovary returning via the dorsal cavity to the pericardium.

1.2.5 Physiology

Daphnia is regarded as showing a high sensitivity to toxins in water and for this reason is used as a test organism in the safety evaluation of agrochemicals and other chemicals. Governmental authorities in various countries require that data on the toxicity of chemicals submitted for approval purposes should include a determination of the lethal concentration against first instar nymphs of Daphnia magna. The appropriate size grade is obtained by sieving from a mass culture. An alternative method for producing the nymphs has been reported by Doma (1979) who developed a technique for

breaking the diapause in ephippial eggs by a 24-hour period of air-drying followed by re-hydration. The Daphnia hatched from ephippia are invariably female but the uniformity obtained in this respect for toxicity-testing purposes is probably outweighed by their varied genetic complement (since the ephippial eggs require fertilization by the male), compared with the genetic uniformity in a strain propagated by continual parthenogenesis.

The pharmacological responses of Daphnia magna have been examined by Sollman and Webb (1941) in which the action of various drugs was tested for activity in the heart, peristalsis, striated muscle and on respiration. The authors elected to use Daphnia in view of its highly differentiated organ structure, and its transparency which allowed direct visual observation under a low power microscope of internal and external movements.

The oxygen affinities of certain invertebrate haemoglobin have been studied by Fox (1945) by calculating the percentage of oxyhaemoglobin in Chironomus, Planorbis and Daphnia, and he related these values to the amount of dissolved oxygen in the external water. "When aquatic animals with haemoglobin in their blood are exposed to water containing a small amount of dissolved oxygen there must at each temperature be a limiting low concentration of oxygen in the water below which the pressure gradient through the body wall is insufficient to maintain the haemoglobin in the oxygenated state since oxygen is continuously being removed by metabolic activities".

Studies by Fox (1953) on the factors influencing haemoglobin synthesis by Daphnia revealed that haemoglobin content is increased in water that has a low content of dissolved oxygen, and a high temperature results in the synthesis of more haemoglobin than a low temperature in waters of the same low oxygen content. The increased haemoglobin synthesis is probably due to a higher metabolic

rate at a high temperature and consequent lower oxygen concentration within the body and to the lower oxygen affinity of haemoglobin at a high temperature resulting in a reduced oxygen supply to the tissues.

Radioactive tracer studies by McMahon (1970) on the ingestion and cycling of iron in D. magna showed that about 60% of the iron was present as gut contents. Daphnia assimilated iron at the rate of 3.8×10^{-3} ug per mg dry wt per hour. After feeding on ^{59}Fe-labelled Chlorella for an extended period, Daphnia assimilated only 1.3% of the ingested iron. In the course of these studies it was reported that a single daphnid filtered 29.8 ml of water in 47 hours.

Studies on iron excretion by Daphnia during haemoglobin loss (Smaridge, 1954) showed that when haemoglobin is being lost inorganic iron accumulates in the excretory organs. Iron was found in the caeca when haemoglobin was being synthesized, and when haemoglobin was being lost inorganic iron was found in the fat body which may be the site of the breakdown. In a subsequent paper Smaridge (1956) found that Daphnia losing haemoglobin had loosely bound iron in the walls of the gut caeca, in the fat cells and in the excretory shell glands.

It is not known precisely how a low oxygen pressure increases the synthesis of haemoglobin in Daphnia or how a high O_2 pressure decreases the content of haemoglobin in the blood. As stated earlier the fat cells are involved in the synthesis of haemoglobin; they may also be associated in its breakdown.

1.3 Aims and Objectives of Study

The main object of the study was to investigate the factors involved in the induction of copper tolerance in Daphnia, including the rate of uptake of the metal and its distribution at a tissue and organ level. Exposure of successive generations of Daphnia to an appropriate concentration of copper might confer tolerance to the metal and this would generate a further series of studies on differences between tolerant and normal strains illustrated by histochemical techniques and electron microscopy.

Initially, however, the investigation would include a method of culturing Daphnia to provide a ready flow of specimens for toxicity tests with copper and this would be followed by the successive generation exposure. Several generations of Daphnia exposed to copper would be compared against normal Daphnia to determine if tolerance had been induced. These tests would be highly replicated to determine precisely the lethal concentration of the metal which caused 50% mortality of the populations at various intervals up to a maximum of 48 hours.

The use of Daphnia as a laboratory animal for studying the effects of metal toxicity was mainly based on the fact that large numbers of genetically homozygous animals derived from a single parthenogenetic female can be reared within the period of a few weeks permitting if necessary repeat experiments in a short space of time combined with a large replication of treatments for statistical analysis. One of the weaknesses in mammalian toxicology has been the introduction of various genetic strains which has led to innumerable problems in the interpretation of results from different laboratories reporting studies with the same chemical compound tested

against the same 'strain' of laboratory animal. If inheritance studies were contemplated with Daphnia, a modification of the environmental medium by a restriction of food supply, a rapid drop in temperature or an increase in population density causes a change from the normal form of reproduction by parthenogenesis to sexual reproduction. A change in the level of oxygen in the water medium of Daphnia modifies the haemoglobin status of the animal permitting studies on the significance of haemoglobin or oxygen transport in a controlled environment.

The disadvantage of using Daphnia was the absence of background toxicological profile in terms of documented responses at a tissue or organ level or biochemical lesions in response to metal toxicity.

1.1(a) SUPPLEMENTARY LITERATURE REVIEW

An extended literature review has been made of subjects including toxicity of copper to aquatic organisms, speciation of metals and its influence on toxicity, metabolism of copper and other metals and the importance of bioavailability of trace metals in aquatic organisms and finally a brief reference to resistance of D. magna to metals.

SUMMARY

1) Various aspects of copper toxicity are considered including the effect of the metal on the inhibition of alkaline phosphatase in a marine diatom, the use of chemical equilibrium models to relate the species of copper to toxicity, the importance of analytical methods to determine the fraction of copper which is toxic and the way in which chelation influences the toxicity of copper and other metals.

2) The speciation of trace elements in waters is reviewed in terms of analytical methodology and the effect of ligands on speciation is considered. An additional form of combating metal toxicity by Daphnia is in the synthesis of complexing agents which are released into the surrounding water and in one experiment these complexes had stability constants similar to those for humic and fulvic acids.

3) In studies on the mechanisms of copper toxicity, one experiment has shown that copper sulphide induced breakage of the DNA strand in ovary cells of the Chinese hamster. The primary mode of action in fish experiments was the destruction of the epithelial mucous and chloride cells of the gills leading to suffocation.

4) The vital role of bioavailability of trace elements to aquatic organisms is discussed together with reference to the use of indicator species to illustrate the availability of trace metals in estuaries.

5) The question of genetic resistance of D. magna to copper has not been resolved by multi-generation exposure to copper solutions. A form of 'physiological resistance' is posed as the mechanism to account for the observed resistance.

TOXICITY

The toxicity of 12 freshwater sediments to D. magna and the mayfly nymph Hexagenia limbata Walsh was examined by Maleug et al (1983) in a recycled laboratory system in which mortality was the index of toxicity. The sediments collected from the reservoir, river or spring creek sites, stored at 5°c until used in the bioassay some 7 to 44 days after collection. A river water was used in the recycling bioassays which were carried out in aerobic conditions with an air pump to aerate and circulate the water in the apparatus which had a total volume of 7000ml with water sediment volume ratio of 9.5. Daphnia was a more sensitive indicator of toxicity than Hexagenia.

The response of 4 species of Daphnia to copper toxicity has been examined by Winner and Farrell (1976) in acute and chronic bioassays in pond water. The 72 hour LC 50 for D. magna was 86.5 ug. Cμ/l., and 86.0 ug. Cμ/l. for D. pulex which differed significantly from 72.0 ug. Cμ/l. for D. parvula and 67.7 ug. Cμ/l. for D. ambigua. The difference in toxicity was attributed to the larger size of the 2 former species. In the chronic bioassays D. magna showed a decrease in the instantaneous rate of population growth at concentrations of copper greater than 60 ug. Cμ/l. while decreases were observed in the other species at 40 μg. Cu/l.

A comparison of body length, number of young and longevity has been made as indices of the toxicity of copper and zinc to D. magna by Winner (1981). He found that reductions in body length and in longevity were more sensitive as indicators of toxicity than brood number.

The toxicity of various metals has been measured against D. magna in acute and chronic exposures in an extensive series of tests by Biesinger and Christiensen (1972). In addition determinations were made on the effect of the metals on the total weight of Daphnia, and total protein and glutamic oxalacetic transaminase have been measured. A concentration of 22 ug. Cμ/l. caused a 16% decrease in the number of young produced, and in a 3 week test the LC 50 was 44 μg. Cu/l. which also reduced total protein by 5% and glutamic oxalacetic transaminase by 10%.

Biesinger et al (1974) have demonstrated that the toxicity of NTA (Nitriloacetate) to D. magna and of NTA combined with various metals was influenced considerably by the hardness of the water. At a water hardness of 220 mg/l the 3 week LC 50 of NTA was 650 mg/l compared with 145 mg/l when hardness was 45 mg/l. The 3 week LC 50 of copper 0.044 mg/l while in the presence of 2 mg/l Na_3 NTA this was reduced to 0.26 mg Cu/l. The toxicity of zinc was 3 times less when combined with NTA than as the non-chelated ion. By contrast the toxicity of iron was not affected by chelation.

The capacity of sea water to form complexes with copper and the resultant toxicity of copper to shrimp larvae (Pandalus danae) have been investigated by Young et al (1979). They used differential pulse anodic stripping voltammetry (asv) to analyse the amount of labile copper which was defined as 'electroactive copper which includes ionic copper and easily dissociable copper complexes'. The analysis suggested that at nominal copper concentration of 5 or 10 μg. Cu/l. seawater, the amount of labile copper was 9 to 12% of the totalcopper, while at nominal concentrations of 20 and 50 ug. Cμ/l., labile copper constituted 40-46%. The results indicated a relationship between the level of labile copper and toxicity although the definition was not clear between the mortality results from 50 and 20 μg. Cu/l. The authors suggest further study is required to establish if a relationship exists between asv labile copper and mortality.

A computer study by Pagenoff et al (1974) on six bioassays of copper on fish has shown some interesting relationships between complexation and toxicity. A chemical equilibrium model was applied to water hardness and complexation by inorganic carbon and hydroxide. A water system in which inorganic carbon is present is capable of complexing copper ions, a capacity which increases with higher levels of alkalinity and pH. The equilibrium calculations showed that there were 5 possible copper species viz $CuCO_3$, $Cu(CO_3)_2^{2-}$, $CU_2(OH)_2^{2+}$, $CuoH^+$ and Cu^{2+} of which Cu^{2+} emerged as the major toxic species. The toxicity of copper can be influenced by the indirect action of calcium and magnesium complexing carbonate and bicarbonate thus reducing their availability for complexation of copper.

One of the aspects of copper toxicity, its effects upon the inhibition of alkaline phosphatase in the marine diatom Thalassiosira pseudonanna has been reported by Rueter (1983) in in-vitro tests with the purified enzyme, in cultures with phosphate-starved diatoms and in-vivo with natural populations of phytoplankton. Alkaline phosphate was sensitive to copper within the range of cupric ion activity for sea-water, 10^{-12} to 10^{-96}M. The rapidity of the inhibitory reaction suggested that cupric ion was in direct contact with the cellular enzyme which is found at the cell surface and is thus vulnerable to inhibition by the cupric ion. The sensitivity of alkaline phosphatase to copper suggests the possible use of the enzyme inhibition as bio-chemical marker for bioassay purposes.

SPECIATION OF METALS

The speciation of trace elements in waters is reviewed by Florence (1982) who considers its influence on bioavailability and toxicity, and evaluates current methodology in studies on speciation in waters including sampling techniques, filtration and storage. The importance of classifying an element into its various physico-chemical forms or species is now recognised in trace metal studies in water systems since the total

concentration expression fails to provide information on bioavailability or metal interaction with sediments or suspended particles. Changes in the form of an element can greatly influence its toxicity, for example alkyl compounds of mercury because they are lipid-soluble are much more poisonous than the inorganic metal and while chromium (III) is an essential element, chromium (VI) is highly toxic. Studies on the toxicity of common heavy metals to fish have shown that the metal ion is the most toxic form, in addition hydroxy compounds of copper are also considered to be toxic although to a lesser extent than the free ion (Magnusson et al (1979) referred to by Florence). The technique commonly used for speciation analysis of heavy metals in water is anodic stripping voltammerty (asv) and the asv-labile fraction which is the part of the metal determined by asv at the natural pH or in mildy acid conditions may approximate to the bioavailable fraction. Computer-modelling programmes suggest that the predominant inorganic forms of copper in sea water are $Cu(OH)_2$ (40%) $CuCO_3$ (50%), and in a typical river $CuCO_3$ (95%). In sea water up to 50% in total copper may be associated with organic matter and a high percentage combined with organic colloidal particles. In sea water the asv-detectable fraction of copper is usually much less than 10%. Humic acid and fulvic acid are of great importance in trace metal speciation in fresh water and possibly in sea water.

Pursuing his studies on the effect of ligands on the speciation of metals, Florence et al (1983) have reported on experiments to relate the levels of labile copper determined by asv and chemical methods to the toxic fraction of copper determined by assay with the test organism Nitzschia closterium. In addition they tested the toxicity of natural and synthetic copper complexes to the algae, the assays being carried out in natural sea water in order to avoid difficulties in interpreting results using sea water enriched with the usual nutrients for algal media. To test the effect of the algae on asv labile copper, 5 concentrations of copper were added to Nitzschia. An initial cell density of 2-4 x 10^4 cells/ml produced an exudate which complexed a maximum of about 20 µg. Cu/l. which was a direct response to copper exposure since filtrates from the algae with no added copper had no complexing capacity. Leaching of copper - contaminated algae with fresh sea water removed 40-50% of the adsorbed copper suggesting this fraction of copper was loosely bound. A calibration graph of algal growth against total added copper was not linear in contrast to growth against asv - labile copper which was linear. Among the natural and synthetic copper ligands tested for toxicity the copper-fulvic acid complex almost completely inhibited the toxicity of copper while copper-lecithin reduced copper toxicity considerably and copper nitrilotricecetic acid complex caused an 80% reduction in toxicity compared with ionic copper. Florence (Ref. Cit.) concludes that asv with a low deposition potential (-0.6V) may give an estimate of the toxic fraction of copper in sea water.

The characterization of copper-complexing agents released by D. magna has been studied by Fish and Morel (1983). The excretions from Daphnia were analysed by copper titration techniques for the presence of ligands, cupric ion activity associated with the titration technique was determined by a radiometer selectrode coupled to a reference electrode and ion analyser meters. A titration of filtered medium in which 110 Daphnia had been incubated in 110ml for 72 hours revealed that a substantial degree of complex exudate hadbeen generated by the Daphnia. The complexes were modelled as a mixture containing a large proportion of a weak ligand and a smaller proportion of a moderately strong ligand. The composition of the complexes was not elucidated but the stability constants were similar to those obtained for humic and fulvic acids.

The interaction of speciation and toxicity of copper to the Daphnid Simocephalus serrulatus has been examined by Giesey et al (1983) in two types of water which were classified according to binding capacity (ie the total amount of copper complexed by water) and speciation. The forms of copper after addition of 10 µg. Cu/l. to pond water consisted of 36% as Cu^{++}, 63% as Cu-organic, 0.5% as $CuSO_4$.
The copper complexed as Cu-organic caused a reduction of toxicity and accumulation of copper in Daphnia compared against an equivalent amount of copper expressed as Cu^{++}.

Experiments to test the toxicity of cupric ions to the 4 species of marine phytoplankton by Hawkins and Griffiths (1982) using the technique of Sundra and Guillard (1976) have demonstrated again the importance of relating toxicity to chemical speciation. Copper was supplied as $CuSO_4 . 5H_2O$ and the cupric ion activity (pCu) was calculated from the equation devised by Sundra (1975). The exposure of the 4 species of algae was done in a highly chelated sea water medium in which the free cupric ion activity was systematically varied though different concentrations of total copper, chelator [Tris (hydroxymethyl) - amino methane] and pH. Among the 4 species tested Stichococcus becillaris Nägeli (Chlorophyceae) exhibited a high level of tolerance to copper and the authors suggest that further studies should enquire into an intracellular sequestration mechanism which may possibly account for this characteristic.

METABOLISM

Radio-labelled studies on the accumulation and excretion of nickelin D. magna and in 5 component tissues or organs after exposure to doses of 50, 250 and 750 µg/l nickel in solution have been reported by Hall (1982). To study the rate of uptake at near equilibrium, Daphnia were first exposed for 59 hours to a stable solution of nickel followed by a 6 hour exposure to a radioactive solution and the activity was recorded on entire specimens and on the carapace, eggs, filtering apparatus, gut and body fluids. The rates of excretion were studied in Daphnia and in the components after exposure first to radioactive nickel for 59 hours followed by 20 hours exposure in a stable solution. The accumulation studies with 50 and 750 µg Ni/l were incomplete due to moulting in Daphnia which occurred earlier than anticipated. It was shown that moulting significantly reduced the total body content of nickel. The uptake experiment with exposure at 250 µg Ni/l was completed satisfactorily. It was shown that the rate of uptake was 1.32 µg Ni/mg Daphnia per hour. Among the component parts the most rapid increase in uptake occured in the body fluids. In excretion experiments the nickel contents in the entire Daphnia did not decline to zero but proceeded towards a state of equilibrium which suggested the presence of a reservoir of nickel in the body.

Robinson et al (1982) studied the effect of copper sulphide and various metal compounds on the DNA of Chinese hamster ovary cells in order to explore at a molecular level the effect of known mutagens or carcinogens. Cultured ovary cells with $[^3H]$ thymidine-labelled DNA were exposed to a concentration of 10 µg/ml for 24 hours of copper sulphide and various other metals. Cell lysates were subjected to alkaline sucrose gradient centrifugation and DNA average molecular weight was determined in these cells. Copper sulphide induced breakage of the DNA strand and caused a large reduction in the average molecular weight of DNA.

In their study of mechanisms of copper toxicity in aquatic animals Miller and MacKay (1983) noted that in rainbow trout fingerlings copper caused a loss of equilibrium with the fish finally adopting an inverted position and undergoing convulsive contractions of the locomotor muscles. In order to determine if copper had a direct influence on neuromuscular systems and a direct effect upon muscle they exposed the rectus abdominus muscle of a pithed frog in a muscle transducer connected to a electrophysiograph, to 10^{-5}M acetylcholine and combined with 10^{-6}M copper which was also tested singly. It was found that copper had little effect on its own but a mixture of copper and acetylcholine caused larger contractions than the acetylcholine and after 10-15 minutes the mixture caused spontaneous spasmodic contractions. After the muscle was bathed in copper-Ringer's solution the subsequent contraction caused by aectylcholine-copper solution led to rapid convulsive activity which indicated an impairment of the contractile mechanism inside the cell or at the neuromuscular junction. From a consideration of their own work and studies by others the authors

conclude that the toxic action of copper is caused by damage to the liver and kidney together with impaired nervous and muscular activity. At the higher concentrations of copper associated with 48 or 96-hour LC 50 tests the primary mode of action is against the gills including separation of the epithelial tissue, destruction of the epithelial mucous and chloride cells ultimately leading to suffocation.

Sharma (1983) investigated the forms of cadmium, zinc and copper in the New Zealand oyster (Ostrea lutaria) after it was observed that higher than normal levels of cadmium were found in oyster fishermen. Homogenates of oyster were chromatographed on Sephadex G-75 columns and fractions were collected and analysed for cadmium, zinc and copper. The sedimented particulate fractions obtained after centrifugation of the homogenates were also analysed for the above metals. Within the oyster tissue cadmium was equally distributed between the particulate and soluble fractions and amounted to a total of 5.8 µg. Cd/g. wet weight while in the case of zinc and copper 70% and 80% were respectively present in the cytosol and the total in the whole tissue amounted to 67.0 and 14.1 µg. /g. wet weight. Approx. 60% of the cystolic cadmium and 40% of cystolic zinc and copper was bound to high molecular weight proteins. Some 32% of the cystolic cadmium was bound to protein with a molecular weight range of 6000-12000 daltons which was defined as the metallothionein fraction. Less than 1% of zinc was associated with this fraction in contrast to some 20% copper. Within the cell, metallothionein exerts a protective function against the toxicity of cadmium (Rugstad and Norseth (1975) referred to by Sharma) while extra-cellularly cadmium-metallothionein seems to be several times more toxic than the cadmium ion (Valberg et al (1977) referred to by Sharma). The studies continue in an effort to elucidate the apparently paradoxical role of metallothionein in the toxicity of cadmium.

Lewis (1983) refers to the studies by Gibbs, Bryan and Ryan (1981) on copper accumulation by the polychaete Melinna palmata as follows; "The roles played by copper in organisims are many ranging from an enzyme co-factor to a possible anti-predation mechanism. The distribution of the metal within the organism also varies as a function of the role of the metal (eg Bryan and Gibbs (1980) ". Reference is also made by Lewis (ref. cit) to the extensive studies by Bryan et al (1980) on the use of biological indicators of heavy metal contamination in estuaries. The authors comment that "Although the concentration in sediments reflects their input and retention by estuaries, they do not necessarily reflect the biological availability of sediment-bound metals as indicated by the concentration in the sediment-dwelling species".

BIOAVAILABILITY

The vital role of bioavailability of trace elements to aquatic organisms is reviewed by Luoma (1983) who considers the subject under metal uptake and metabolism, mechanisms of uptake from solution and from food availability from sediments, field studies on uptake and environmental processes affecting the uptake. One of the problem areas lies in the estimation of metal bioavailability in natural waters and of the methods currently availabe for example anodic stripping voltammetry, ion specific electrodes, thermodynamic models and measurement of complexation capacity there is not one which is satisfactory in all situations. The difficulties in reproducing metal availability studies in the laboratory are summarized and include the need to determine the distribution coefficient of metal between the sediment and the water, which may not be the same as occurs in the natural state. The studies by Bryan (1974) referred to by Luoma showed that concentrations of copper, lead and cadmium in sediments from the sediment-water interface from several estuaries correlated strongly with the levels in N. diversicolor. Luoma refers to the influence of metal interaction on the availability of metals to organisms including the antagonism which can at times influence the availability of metals.

Trace metal availability in estuaries by sensitive aquatic organisms Fucus vesiculosus (L) and Littorina littoralis (L) as an alternative to analysis of sea water which frequently poses problems in sampling and in low level detection is considered by Bryan (1983) in a study which comprises laboratory and field experiments. Samples of both organisms collected from coastal sites and estuaries mainly in S.W. Britain and samples of surface sediments were analysed for Ag, Cd, Co, Cr, Ni and Pb. Experiments on the accumulation of metals by Fucus growing in filtered English Channel sea water in laboratory were conducted with Ag, Cd, Cu, Pb and Zn. Individual plants were analysed and concentration factors based on the total dissolved concentration of the metals were calculated by dividing the net increase in the metal concentration of the weed by the mean concentration of the added metal. The use of concentraction factors (CFs) meant that valid comparisons could be made on the behaviour of the different metals within and between the field and laboratory experiments. To quote an example, after 16 days exposure in the laboratory experiment to 6.7 and 88 µg Zn/l. the respective CFs in Fucus were 53.3 and 16.6 which were significantly different while exposure levels of 0.84 and 8.5 µg Zu/l. gave very similar CFs viz. 26.0 and 27.8 and in the field study the same trend was observed with the two metals. The CFs in the laboratory experiment were higher than in the field and apart from the fact that the laboratory plants were continuously exposed to light, water and a salinity of 17.5% a contributory factor for the difference in CFs was the use of 0.45 um filtered sea water thus reducing the levels of organic ligands which would otherwise lower the level of available metal ions. Bryan continues with the observation that Cu has a high affinity for organic ligands (referring to Mantoura et al (1978))and would have a lower CF in the field than in the laboratory which in fact was the case. Among other factors influencing CFs reference was made to the binding capacity of polyphenols for metals in Fucus (Ragan et al (1979)). Bryan concludes that "analysis of F. vesiculous probably gives a good indication of the average bioavailability of Ag, Cd, Cu, Pb and Zn in the water as modified by factors including inorganic and organic complexation, competition from other dissolved metals, and the presence of some particulate metals" provided due attention is paid to sampling time and technique. L. littoralis was regarded as a "less useful" indicator of environmental availability of metals with the exception of Cd.

RESISTANCE OF D. MAGNA TO METALS

An extensive study on D. magna has been carried out by Le Blanc (1982) to determine if pre-exposures to copper, lead or zinc or sodium lauryl sulphate affect the subsequent acute toxicity values through the induction of "physiological resistance"; multi-generation exposures to copper were examined in attempts to induce resistance in Daphnia and efforts were made to distinguish between "physiological resistance" and genetic resistance. Daphnids pre-exposed for 20 hours to copper, lead or zinc exhibited significant resistance to these metals in subsequent acute tests but in the case of sodium lauryl sulphate pre-exposure did not confer any resistance and in fact multi - generation exposures increased its toxicity to Daphnia. Exposure of successive generations (12) of Daphnia to copper induced a significant resistance to the metal but when offspring from the resistant parents were cultured in copper-free water and tested they were found to exhibit no resistance and the earlier result was therefore classified as a form of "physiological resistance". This is an illuminating piece of work and illustrates some of the problems associated with genetical aspects of resistance to metals.

A series of comprehensive reviews on the biological importance of copper has been completed by Lewis (1983, 1984, 1985) who includes in a bibliography of some 4000 references several aspects of the subject from metabolism to analytical methods, speciation, a survey of copper levels in many aquatic and terrestrial organisms, and the use of organisms as indicators of metal availability.

CHAPTER 2

THE ACUTE TOXICITY OF COPPER IN *Daphnia magna*

In this chapter and subsequently the generic and specific name Daphnia magna is abbreviated to the common name daphnia as a more convenient reference to the organism.

2.1 Introduction

The difficulties in acute toxicity tests with daphnia have been summarized by Andrew et al. (1977). They point out the main problem arises from the formation of stable copper complexes with the hydroxides of other metals which constitute the culture medium for daphnia. If an absolute value for the lethal concentration of copper to daphnia is required it would first be necessary to solve various equations in aqueous chemistry and determine by chemical or radio-chemical analysis the levels of copper absorbed by daphnia in a defined time scale. These refinements were not considered necessary in the following experiments which were designed to identify the relative lethal toxicity of copper to daphnia in order to ascertain the exposure levels to use in the culture medium for the induction of copper tolerance. The experiments were regarded as uncomplicated bioassays and the most relevant study in the literature is that by Holm-Jensen (1948) who found that the 24 hour LC50 of copper in daphnia maintained in a medium of ocean water diluted 100 times was between 0.010 and 0.032 mg Cu^{++}/l.

2.1.1 Objective of Experiments

The objective of the initial test was to determine the concentration of copper which was lethal to 50% of the daphnia population at the end of 24 hours exposure.

2.1.2 Experimental

A stock of D. magna obtained from the Department of Biology,

The University, Newcastle-upon-Tyne was transferred to 1 litre beakers containing a culture medium constituted as follows:

	$MgSO_4.7H_2O$	$CaCl_2.2H_2O$	$KHCO_3$	$NaNO_3$	K_2HPO_4	$NaHCO_3$
mg/l	100	200	100	10	100	200

The daphnia were fed with a proprietory compound, 'Liquifry' which had been developed for feeding juvenile fish but which was reported by laboratories in the U.K. to be a satisfactory diet for daphnia. The rate of feeding was 0.2 ml Liquifry/800 ml culture medium at 2-day intervals. The cultures were maintained in well aerated water and the daphnia in this environment did not synthesize haemoglobin, oxygen transfer within the organism being effected by diffusion and convection.

Following customary procedure the test was carried out on 1st instar daphnia which were obtained by screening the mass culture through two screens of mesh size 800 um and 500 um. The organisms for the test passed through the larger sieve and were retained on the smaller screen. Ten daphnia were transferred to each bioassay vessel which contained the concentrations of Cu^{++} as $CuSO_4$ shown in Table 2.1. There was a 5-fold replication of treatments. The pH of the solution in the bioassay vessels was 8.3 and the dissolved oxygen content was 8.8 mg O_2/l. During the test the bioassay vessels were maintained in a water bath at a constant temperature of $20^{\circ} \pm 2^{\circ}C$. at the end of 24 hours the numbers of daphnia which had sunk to the bottom of the bioassay vessels were counted and recorded as dead although in some cases the heart was still beating and there was occasional movement of the appendages. In the absence of the vital swimming function

it was considered that survival would be limited to a short period of time, probably not more than a few minutes. This criterion of death has been used throughout all experiments.

2.1.3 Results

The effect of the different concentrations of copper on the mortality of daphnia is shown in Table 2.1.

TABLE 2.1 Mortality of daphnia (out of 10) after 24 hours exposure to different concentrations of copper

Treatment Conc. of Cu^{++} (ug/l)	Mortality of Daphnia Replicate I	II	III	IV	V	Total
450	10	9	10	10	10	49
150	9	8	7	8	8	40
50	6	6	7	7	6	32
17	4	7	5	4	5	25
5.5	5	3	3	3	4	18
1.8	2	3	2	4	1	12
0.5	0	0	0	1	2	3
0.0(Control)	0	0	0	0	0	0

The above results and also those in the subsequent toxicity test have been analysed by Mr. I. Pell, Huntingdon Research Centre, in the standard computer programme for determination of the LC50 values.

The 24 hour LC50 was 15.2 ug Cu^{++}/l with 95% confidence limits of 10.5 and 21.9 ug Cu^{++}/l.

Simultaneously with the preparations for this experiment determinations of the exposure levels of copper were attempted by atomic absorption spectroscopy as reported in Chapter 3. Initial tests indicated that the detection limit of the instrument was

well above the 24 hour LC50 value and it was thus necessary to modify the culture medium by introducing a chelating agent to decrease the toxicity of copper which would then be more amenable to chemical analysis. A different diet for daphnia was also required since Liquifry failed to provide sufficient energy for a normal reproduction rate and the brood numbers were small. An alternative food source, Chlorella, cultured on agar slopes and transferred to daphnia vessels, has frequently been recorded in the literature as a satisfactory diet. For the purpose of continuous exposure of daphnia to copper in order to induce a tolerant strain of daphnia, an automatic system of feeding Chlorella to daphnia was required which would necessitate the use of a common culture medium compatible to both organisms. The constituents of various culture media reported for Chlorella included EDTA (the di-sodium salt of ethylene diamine tetra-acetic acid) which would satisfy the requirement of a chelating agent referred to above. A combination of a literature search and some small scale tests to confirm the findings in particular of Taub and Dollar (1964) who made comparisons of various metals and salts in their search for a compatible culture medium for daphnia and Chlorella resulted in the following constituents as a basis for examination:

	$MgSO.7H_2O$	$CaCl_2$	$NaHCO_3$	$NaNO_3$	$KHCO_3$	K_2HPO_4
mg/l	66	100	100	85	66	66

	NaCl	$FeSO_4.7H_2O$	EDTA
mg/l	117	5	3.5

To assess the suitability of this medium for daphnia, 10 first instar nymphs which had no food during 24 hours before the test were placed in 4 specimen tubes containing the above constituents

and observations on mortality were taken at 2, 4, 8, 24, 32 and 48 hours. There was total survival at 32 hours and only a single mortality at 48 hours.

In the medium, copper was expected to be less toxic than in the first experiment, due to the presence of EDTA and also to 2 mM NaCl which has been shown by Holm-Jensen (1948), and confirmed by a replicated test at Stirling, to afford some protection against copper poisoning. The protective influence of NaCl, and also the toxicity of nitrate, as single salts of KNO_3 or $NaNO_3$, to daphnia reported by Taub and Dollar (ref. cit.) were of considerable interest. The daphnia used by Taub and Dollar were cultured in a simulated fresh water medium and the organisms would, therefore, contain no haemoglobin. Nitrate toxicity in mammals is well documented and is caused by the strong oxidising action of NO_3 removing an electron from the central iron atom in haemoglobin reducing the metal from the ferrous to the ferric state (methaemoglobin) and thus the oxygen-binding property is lost. The nitrate toxicity in haemoglobin-free daphnia is thus of particular interest but since this effect was counterbalanced by the presence of other salts in the medium, this observation was outside the immediate scope of the present experiments.

2.1.4 Determination of the Toxicity of Copper to Daphnia in a Culture Medium Compatible to Daphnia and Chlorella

The copper solutions were prepared by serial dilution of a stock solution of 1000 mg Cu^{++}/l obtained by dissolving 3.93 g $CuSO_4$ in 1000 ml distilled water, and were dispensed into the above culture medium in 40 ml capacity specimen tubes. Five first instar daphnia were transferred into each tube and there were 10 replicates of 10 treatments shown in Table 2.3. Observations on mortality

TABLE 2.2 Survival of Daphnia, out of 5, in five concentrations of Cu^{++} at 12, 15 and 24 hours

Block No.	Time (Hours)	Concentrations of Cu^{++} (ug/l)					Time (Hours)			Block Total
		0	500	50	5	0.5	12	15	24	
	12	5	4	5	5	5	24			69
I	15	5	4	5	5	5		24		
	24	5	2	4	5	5			21	
	12	5	3	5	5	5	23			
II	15	5	3	5	4	5		22		66
	24	5	3	4	4	5			21	
	12	5	4	5	5	5	24			
III	15	5	4	4	4	5		22		66
	24	5	2	4	4	5			20	
	12	5	3	4	5	5	22			
IV	15	5	3	5	5	5		23		63
	24	5	0	4	4	5			18	
	12	5	4	5	5	5	24			
V	15	5	4	5	5	5		24		70
	24	5	3	4	5	5			22	
	12	5	5	4	5	5	24			
VI	15	4	3	5	5	5		22		65
	24	4	2	4	4	5			19	
	12	5	4	4	5	5	23			
VII	15	5	3	4	5	5		22		65
	24	4	2	4	5	5			20	
	12	5	3	5	5	5	23			
VIII	15	5	3	5	5	5		23		64
	24	5	1	3	4	5			18	
	12	5	5	5	5	5	25			
IX	15	5	4	4	5	5		23		70
	24	5	3	4	5	5			22	
	12	5	3	5	5	5	23			
X	15	5	3	4	5	5		22		65
	24	5	1	4	5	5			20	
	12	50	38	47	50	50	235			
Total	15	49	34	46	48	50		227		663
	24	48	19	39	45	50			201	

were made at 12, 15 and 24 hours. The concentration 5000 ug Cu^{++}/ml caused total mortality within 12 hours and it is excluded from Table 2.2. There was a high survival rate at 50 ug Cu^{++}/l and in the lower concentrations and in order to facilitate the analysis of variance the results are shown as numbers of surviving daphnia which otherwise would be listed as successive zeros if expressed in terms of mortality.

2.1.5 Results

The observations on survival are in Table 2.3.

The data have been analysed according to Snedecor and Cochran (1972).

$$\text{Correction factor} = \frac{(663)^2}{150} = 2930.46$$

$$\text{Total S.S.} = 3063 - 2930.46 = 132.54$$

$$\text{S. S. Blocks} = \frac{44013}{15} - 2930.46 = 3.74$$

TABLE 2.3 Summary Table, Concentration x Time

Time	Concentration x Time					
(Hours)	0	500	50	5	0.5	Total
12	50	38	47	50	50	235
15	49	34	46	48	50	227
24	48	19	39	45	50	201
Total	147	91	132	143	150	663

$$\text{S.S. Concentrations} = \frac{(147)^2 + \text{---} (150)^2}{30} - 2930.46$$

$$= 78.31 \text{ (4 d.f.)}$$

$$\text{S.S. Time} = \frac{(235)^2 + (227)^2 + (201)^2}{50} - 2930.46$$

$$= 12.64 \text{ (2 d.f.)}$$

Interaction Concs. x Time = (S.S. Concs. x S.S. Time) - (S.S. Concs + S.S. Time)

$$= 12.69 \text{ (9 d.f.)}$$

Analysis of Variance

Treatments	d.f.	s.s.	m.s.	F.	Fst 0.05
Blocks	9	3.74	0.42	2.10	2.76
Concs.	4	78.31	19.58*	97.90	5.66
Time	2	12.64	6.32*	31.60	19.49
Interaction					
Conc. x Time	9	12.69	1.41*	7.05	2.76
Residuals	126	25.16	0.20		

The F Test shows that there were significant differences in concentrations and time and in the interaction concentration x time. The significant differences between the mean treatments are considered below.

TABLE 2.4 Mean Survival of Daphnia in five concentrations of copper at 12, 15 and 24 hours

	0			500			50			5			0.5			L.S.D.
Time (Hours)	12	15	24	12	15	24	12	15	24	12	15	24	12	15	24	
Mean survival	5.0	4.9	4.8	3.8	3.4	1.9	4.7	4.6	3.9	5.0	4.8	4.5	5.0	5.0	5.0	0.392

The least significant difference (L.S.D.) for application to the above mean survival values has been calculated as follows:

$$\text{L.S.D.} = \sqrt{\frac{0.20 \times 2}{10}} \times t \text{ (1.96 at P = 0.05)}$$

$$= 0.392$$

The concentration 500 ug Cu^{++}/l caused a significant drop in the mean population of daphnia at 12, 15 or 24 hours compared with the control or with 50 ug Cu^{++}/l. At 50 ug Cu^{++}/l there was no significant reduction in the daphnia population at 12 or 15 hours compared with the untreated control, but there was a significant drop at 24 hours.

The 24 hour LC50 value was 169.1 ug Cu^{++}/l with 95% confidence limits of 106.3 and 271.7 ug Cu^{++}/l.

2.1.6 Conclusion

It is clear that this culture medium, constituted as a basis for compatability for daphnia and Chlorella caused a considerable reduction (169.1 ug Cu^{++}/l compared to 15.2 ug Cu^{++}/l) in the toxicity of copper to daphnia and it was felt that this would lessen the difficulties in the determination of copper levels by atomic spectroscopy. The value 169.1 ug Cu^{++}/l did not take into account the background level of copper present in the distilled water used in the medium. It was anticipated that the background contribution would be determined by chemical analysis.

Summary

1. The object of the toxicity tests of copper on daphnia was to establish the acute toxicity of the metal in order to indicate the exposure levels to use for the induction of copper-tolerance in daphnia.

2. In the first culture medium tested, the 24 hour LC50 value of copper in 1st instar daphnia was 15.2 ug Cu^{++}/1.

3. Since the low level of copper toxicity created difficulties in chemical analysis and in view of the need for a replacement of the Liquifry diet by a proposed Chlorella diet, a different culture medium with constituents compatible to daphnia and Chlorella, containing EDTA and NaCl which would lessen the toxicity of copper to daphnia, was examined. The culture medium was safe for daphnia, and the 24 hour LC50 value of copper in daphnia was increased to 169.1 ug cu^{++}/1.

CHAPTER 3

ANALYSIS OF TRACE AMOUNTS OF COPPER BY ATOMIC ABSORPTION SPECTROPHOTOMETRY

3.1 Introduction

Analytical methods for the determination of trace elements in water have been summarized by Danielsson et al. (1978). They comment that several attempts to analyse unpolluted water with electrothermal analyses and atomic absorption spectrophotometry have not generally been successful due to an inadequate sensitivity of the techniques. The only direct method with sufficient sensitivity was Anode Stripping Voltammetry (a.s.v.) which has been used successfully by Young et al. (1979). Recently electron probe analysis has been developed as a satisfactory technique but as in the case of a.s.v., instrumentation is specialised and expensive. In several laboratories techniques to concentrate the trace metals by chelation followed by extraction with an organic solvent and determination of the concentration by an atomic absorption spectrophotometer preferably equipped with a graphite furnace are in common use. A Perkin Elmer atomic absorption spectrophotometer was available in the Department of Biology, University of Stirling, and the determination of trace amounts of copper by the concentration - extraction technique has been evaluated.

3.1.1 Objective of Experiments

In short term toxicity tests or in long term exposures of daphnia to copper, a proportion of the metal in the dosing solution is lost by adsorption into the glass vessels, or increased in concentration by evaporation losses. It was obviously desirable to identify the available levels of copper and the objective of the initial analyses was to evaluate the technique of ion concentration in determinations of trace amounts of copper in the dosing solutions.

3.1.2 Experimental

The Spectrophotometer was a Perkin-Elmer Model 373 equipped with a standard single head burner for air-acetylene. The light source generating the sharp line spectrum of copper was a single hollow cathode tube for Cu^{++} analysis and it was set at a continuous operating current of 15 m A. The limit of sensitivity of the instrument was stated by the manufacturers to be 20 ug Cu^{++}/l but in practice this was found to be optimistic and the lowest level of detection was 50 ug Cu^{++}/l. To determine the relationship between absorbance of copper and solution concentrations of copper in experiments not involving the ion-concentration technique, a stock solution of 1000 mg/l was prepared by dissolving 3.930 g $CuSO_4.5H_2O$ in distilled water and diluted to give the concentration shown below.

TABLE 3.1 Concentration of Cu^{++} and Absorbance Values in A.A.S.

Conc. of Cu^{++} (ug/l)	Absorbance
5100	0.25
2500	0.12
500	0.02
100	0.004
50	0.001

The relationship between concentration of copper and absorbance is clearly linear as illustrated in Figure 3.1.

The essence of the ion concentration -extraction technique consisted of using ammonium pyrrolidine dithiocarbamate (APDC) to chelate the copper ions into complex forms from which the metal was extracted by the solvent methyl isobutyl ketone (MIBK) and

measured in the spectrophotometer. Several preliminary tests were made to become familiar with the procedure. All glassware was cleaned in chromic acid, and polyethylene in 1:1 HNO_3 + H_2SO_4, followed by several rinsings in distilled or deionized water and drying in a warm air cabinet. In initial tests distilled water was used but subsequently to obtain better quality this was replaced by deionized water or distilled-deionized. In several of the tests the levels of copper found after concentration were high and variable suggesting low quality water or contamination of the glass specimen tubes or polyethylene bottles which contained the samples before aspiration into the spectrophotometer. Samples of the water shown in Table 3.2 added to specimen tubes and polyethylene bottles were tested for conductivity in a Phillips Conductivity Meter.

TABLE 3.2 Conductivity Readings of Deionized and Distilled-Deionized Water in Sample Containers

Type of Water	Sample Vessel	Conductivity Reading (u ohms)
Deionized	Glass specimen tube	1.07
Distilled-deionized	" " "	1.15
Deionized	Polyethylene spec. bottle	112.0
Distilled-deionized	" " "	42.0
Tap water	Glass specimen tube	47.0

It is obvious from the above readings that deionized or distilled-deionized water had low conductivity in the glass specimen tubes, while identical samples of water in the polyethylene had readings which were equivalent to or poorer than tap water. On the basis of this single test, glass specimen tubes were used for all subsequent samples, and conductivity readings were routinely taken as a measure of water quality.

3.1.3 Recovery and Analysis of two Prepared Samples of Cu^{++} in deionized Water by Concentration of the Metal and Solvent Extraction

As a preliminary to calibration of the spectrophotometer with copper standards in the above techniques, a 3-replicated test has been carried out to determine if there were significant differences between the levels of copper concentrated and extracted from prepared solutions and between these levels and a reference standard consisting of deionized water containing no added copper.

A volume of 30 ml MIBK was added to 630 ml of the prepared solutions containing 0.5 or 5.0 ug Cu^{++}/l. This was followed by the addition of 10 ml of 1% (w/v) solution of APDC. The same volumes of MIBK and APDC were dispensed into 630 ml deionized water. The three vessels were shaken for 30 minutes in a mechanical shaker at 100 revs/min. The solutions were then transferred to separatory funnels where there was a rapid differentiation into an aqueous layer and an organic layer. The organic layer was filtered through a Whatman No. 1 filter paper, measured and aspirated directly into the spectrophotometer.

3.1.4 Results

The volume recorded from each separatory funnel was stopped at 18.0 ml and the spectrophotometer readings from the recoveries are shown in Table 3.3.

TABLE 3.3 Levels of Cu^{++} (ug/l) Recovered From Concentration and Extraction of Cu^{++} in Prepared Solutions

Conc. of Cu^{++}(ug/l) in Prepared Solutions	Replicate I	II	III	Total
5.0	850	910	920	2680
0.5	830	910	830	2570
0.0	590	700	710	2000
Total	2270	2520	2460	7250

$$C.F. = \frac{(7250)^2}{9} = 5840277.7$$

Total S.S - C.F. = 104822.3

S.S. Blocks - C.F.= 11355.3

S.S. Concs. - C.F.= 88822.3

Analysis of Variance

Source	d.f.	s.s.	m.s.	F.	F(0.05)
Blocks	2	11355.3	5677.7	1.22	6.94
Concs.	2	88822.3	44411.2	9.56*	6.94
Error	4	4644.7	1161.2		

The F test has shown significant differences among the concentrations and these are considered below for the mean values.

TABLE 3.4 Summary Table of Mean Levels of Cu^{++} Extracted from Prepared Solutions

Conc. of Cu^{++}(ug/l) in Prepared Solution	Mean Level of Cu^{++} in Extract	L.S.D.
5.0	893.3	
0.5	856.7	77.2
0.0	666.7	

S.E. of difference between 2 means $= \sqrt{\frac{2 \times 1161.2}{3}} = 27.82$

Value of t for 4 d.f. (error d.f.) = 2.776 (P = 0.05)

Least Significant Difference (LS.D..) = 27.82 x 2.776 = 77.2

It is clear from the above mean results that there was no significant difference between the concentrated values of copper extracted from the prepared solution of 5.0 and 0.5 ug Cu^{++}/l, although each was significantly greater than the background level of copper in the control.

To enquire further into the reasons for the lack of significance it is of interest to quantify the actual levels of copper present in the volume of material recovered from the separatory funnels.

In the control, the concentration of Cu^{++} determined in the sample of 18.0 ml = 666.7 ug/l.

Wt. of Cu^{++} in 18 ml $= \frac{666.7}{1} \times \frac{18}{1000} = 12.0$ ug

12.0 ug Cu^{++} was concentrated from a total volume of (630 + 30 + 10) = 670 ml

Expressed as ug/l = 17.91 ug Cu^{++}/l

The results for 0.5 and 5.0 ug/l starting solutions have been calculated as above and they are shown below.

TABLE 3.5 Summary Table - Concentration of Copper (ug/l) Extracted from the Original Prepared Solutions

Nominal Conc. of Cu^{++} (ug/l) in Prepared Solution	Conc. of Cu^{++} (ug/l) Extracted from Original Solution
5.0	24.0
0.5	23.0
0.0	17.91

There was a high background of copper in the control and correspondingly high levels of copper in the nominal concentrations of 5.0 and 0.5 ug/l. Deduction of background from the 5.0 ug Cu^{++} starting solution leaves a concentration of 6.09 ug Cu^{++}/l. However, the 0.5 ug/l prepared solution minus background amounts to 5.09 ug Cu^{++}/l. The similarity between the two recoveries suggested a dilution error during sample preparation but this was most unlikely since great care was taken to prevent error. It is more likely that a trace of contamination occurred during the preparatory steps or in the measurement in the spectrophotometer. The relatively high background was a cause of concern and after examining all possible sources of contamination it was concluded that the most likely source originated from the solvent MIBK or from the deionized water. The MIBK was claimed to contain less than 20 ug Cu^{++}/l but tests showed that the level was 160 ug Cu^{++}/l which would then

contribute approximately 25% to the background level of copper.

During the course of these experiments it was evident that the concentration technique as practised was not sufficiently sensitive to detect the low levels of copper associated with the 24 hour LC50 test of copper in daphnia. One of the principles of the technique, that is chelation of copper ions, was applied to the culture medium for daphnia by including the chelating agent EDTA which contributed to raising the 24 hour LC50 value to 169.1 ug Cu^{++}/l which was within the detection range of the spectrophotometer. However, analysis of the levels of copper in Chlorella and in daphnia in the continuous flow system described in Chapter 4 was still required and since trace amounts were involved it was necessary to employ a concentration technique. Discussions to obtain information on the subject were held with chemists at the Institute for Marine Biochemistry, Aberdeen, where determination of trace elements by a variety of sophisticated techniques is routinely performed. They found that extraction with MIBK was unreliable and they had discarded the solvent in favour of Freon TF (1,1,2-trifluoroethane) and also included diethyl ammonium dithiocarbamate (DDTC) together with ammonium pyrrolidinedithiocarbamate (APDC). The procedure has been described by Danielsson (ref. cit.). Preliminary tests have thus been done with this technique to determine the levels of copper in Chlorella vulgaris following the procedure for the extraction of copper from the algal cells adopted by Foster (1977) in her study on copper exclusion as a mechanism of heavy metal tolerance in a green alga.

The results with Chlorella vulgaris appeared promising and several preliminary experiments to determine the trace levels of copper in culture medium suggested that this technique was a considerable improvement on the previous method and it was proposed to carry out further evaluations when the continuous flow system was fully operational.

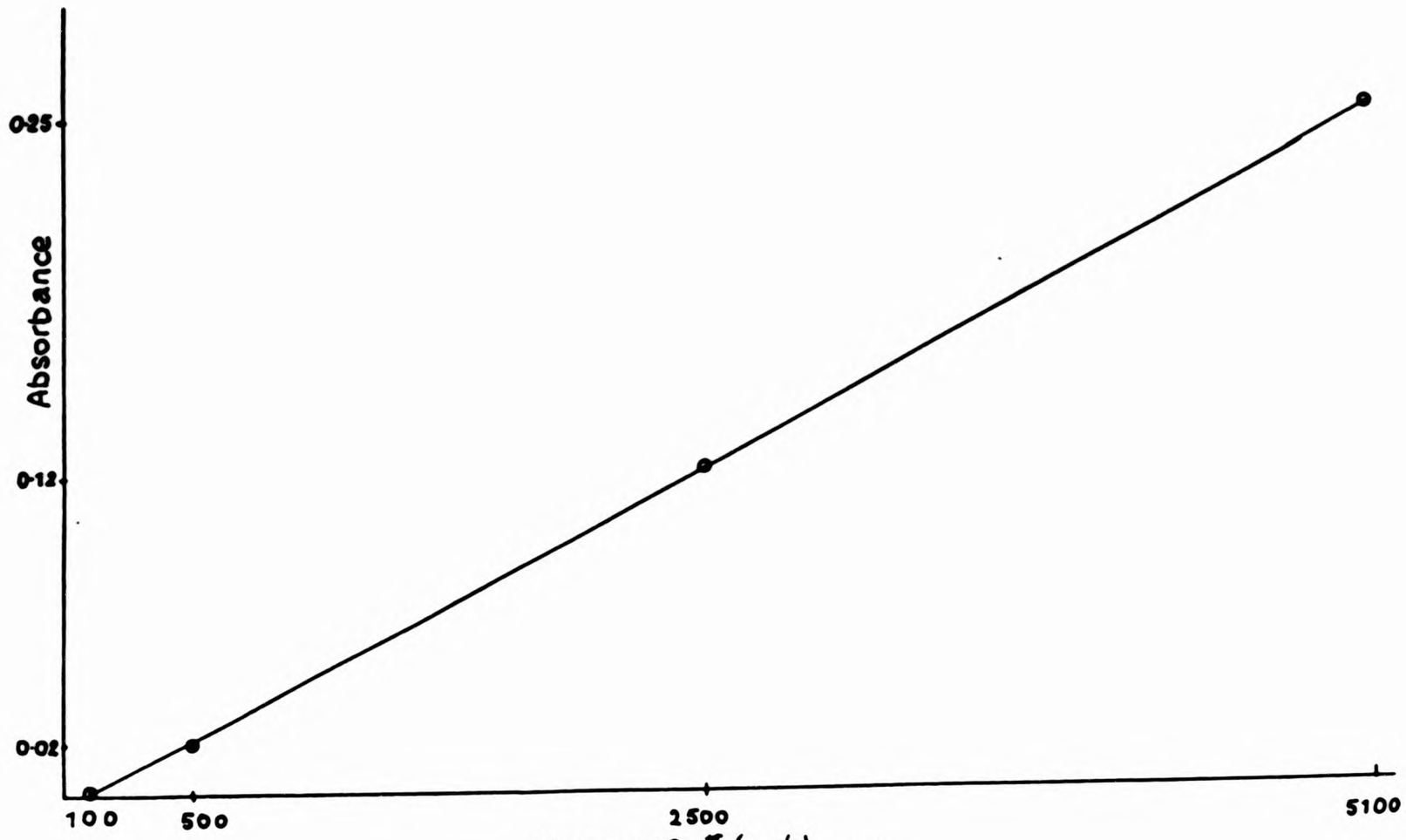
Summary

1. Analysis of trace amounts of copper by ion concentration and solvent extraction has been examined using APDC as the chelating agent and MIBK as the solvent followed by determination of the concentration of the metal in an atomic absorption spectrophotometer. Several tests indicated that the technique as applied was not sufficiently sensitive to distinguish between trace amounts of copper in aqueous solutions.

2. An alternative method using APDC + DDTC as the chelating agents and Freon as the solvent was evaluated in preliminary tests to detect the levels of trace amounts of copper in culture medium and in Chlorella vulgaris. This technique appeared to be more efficient than the previous method and it was proposed to evaluate it fully when the continuous flow system was established.

Fig. 3.1

Conc. of Cu^{++} (ug/l) and Absorbance in A.A.S.



ANALYSIS OF TRACE AMOUNTS OF COPPER IN WATER

3.2 (a) Introduction

In an attempt to resolve the problems encountered in analysis of trace amounts of copper in solution as discussed in 3.1.3. and in the Chlorella / Daphnia media (4.2) contact was made with The Institute For Marine Biochemistry, Aberdeen and a visit arranged to participate in a determination of trace amounts of copper in water, using the specialized equipment in their laboratory. The trace amounts analysed were 1, 2 and 4 μg. Cu^{++}/l. For this co-operation I wish to record my thanks to Dr. S.G. George, Department of Biochemistry at the Institute.

3.2.1 (a) Procedure

The spectrophotometer was the AA 575 Varian model fitted with a graphite atomiser. The source of copper was BDH Standard Solutions for Atomic Spectrophotometry and solutions of 1, 2 and 4 μg. Cu^{++}/l. were prepared in double distilled water together with concentration of 5, 10 and 15 μg. Cu^{++}/l. for calibration of the instrument. After the calibration curve was completed a 5 ul. volume of each sample for analysis was injected into the instrument, dried by exposure for 60 seconds at 150^{o} C, ashed at 400^{o} C for 30 seconds and atomised at 2000 C for 2 seconds.

3.2.2 (a) Results

The results of the analysis are shown in the table 4.3. (a).

Table 3.3 (a) Calibration Curve Standards and Recoveries of 3 concentrations of Copper in Double Distilled water.

Nominal Conc. of Cu^{++} (μg./l.)	Level of Cu^{++} (μg./l.) determined by analysis
Calibration Standards	
0	1.2 $\pm$ 0.2
5	4.7 $\pm$ 0.2
10	11.2 $\pm$ 0.6
15	15.0 $\pm$ 2.3
Test Conc. of Cu^{++}	
1	1.4 $\pm$ 0.3
2	3.1 $\pm$ 0.4
4	3.9 $\pm$ 0.3

The above values are the mean of 5 determinations. Compared with the results from the earlier experiments, this analysis has demonstrated that a reasonable level of accuracy has been obtained and that the method is capable of analysing copper down to its background level.

There are probably several reasons for the divergence of the results at Stirling compared with the above analysis. Firstly the use of a more sophisticated instrument fitted with a graphite atomiser ensured greater accuracy and reproducibility of results and this may have been the vital factor causing the lack of success at Stirling. Secondly the low background contrasted greatly with the figure at Stirling which was some 15 times higher and again this could be partly attributed to superior instrumentation for analysis and for the production of high quality water suitable for analysis of trace amounts of metals.

It is apparent that the limitations on the analysis of trace amounts of copper in water at Stirling also reflected on the levels of copper contamination reported in the culture medium prepared for Chlorella and Daphnia. The absence of an accurate analytical procedure would have compounded the error in the analysis of copper in the culture medium and in the level of copper contamination recorded in the controls.

Summary

1) Analysis of 1,2 and 4 µg./l. Cu^{++} in double distilled water, carried out at the Institute For Marine Biochemistry, Aberdeen, in a Varian spectophotometer fitted with a graphite atomiser showed high accuracy compared with earlier results at Stirling and the procedure used was demonstrated to analyse copper down to its background level.

2) It was concluded that sensitive instrumentation, low background, and high quality dilution water were the main features giving high analytical capability at Aberdeen, and their absence at Stirling contributed to less accurate analysis of copper in water and may have overstated the level of copper contamination recorded in the culture medium for Chlorella and Daphnia.

CHAPTER 4

DEVELOPMENT OF A CONTINUOUS FLOW SYSTEM FOR CULTURING *Chlorella vulgaris* AND FEEDING *Daphnia* AND ATTEMPTS TO INDUCE COPPER TOLERANCE IN *Daphnia*

4.1 Introduction

In an initial programme to develop a copper-tolerant strain of daphnia, nymphs and adults were exposed to two concentrations of copper and a reference control and successive generations were recorded and transferred to the same concentrations in which they originated. The concentrations were 0.0, 0.5 and 1.8 ug Cu^{++}/l. representing approximately $\frac{1}{30}$ and $\frac{1}{8}$ of the 24 hour LC50 value. There were five replicates of treatments with five adults or five first instar nymphs in each treatment, giving a total starting population of 75 adults and 75 nymphs. The organisms were maintained in 40 ml specimen tubes and solutions were changed twice weekly. The diet was 'Liquifry' at 2-day intervals. When sufficient numbers of F_1, F_2, F_3 and F_4 became available it was proposed to compare the acute toxicity of copper in each generation against the control daphnia. The exposures continued for several weeks but the rate of reproduction was too low to provide sufficient daphnia for toxicity testing at any stage during the exposure either in the control or in the treatments containing copper. It was concluded that 'Liquifry' was an inadequate diet to sustain a normal reproduction rate and it was decided to change the diet to Chlorella vulgaris which has been used successfully in many laboratories. It was proposed to introduce levels of copper into the Chlorella culture for continuous administration to daphnia and this necessitated the development of an appropriate system for the mass culture of Chlorella. The developed analytical method would be used to analyse the level of copper in Chlorella and in the daphnia culture medium and ultimately in the daphnia. The volume of Chlorella cells available for consumption would be determined by cell counts in

the Coulter counter. Progeny testing of daphnia for tolerance to copper would proceed as described above.

4.1.1 Experimental Methods

Development of a Continuous Flow System for Chlorella and Daphnia

Several methods for the mass culture of algae have been described in the literature and the most appropriate was the system used by Bourne (1969) in his study on the carbon transfer from C. vulgaris to D. magna. Essentially it consisted of growing the algal culture in a glass cylinder 76 cms long by 8 cms internal diameter and filtering sterile air into the cylinder to maintain the cells in a suspended state in the medium. The air was introduced into the medium through an air-stone which was fitted into a rubber stopper at the base of the cylinder. When this system was tested it was found unsatisfactory because the air bubbles varied in size to such an extent that a large number of the cells failed to remain in suspension and they accumulated at the bottom of the cylinder. To obtain a uniform flow of air through the medium, the Shared Technical Services Department, University of Stirling fitted a 2 mm diameter glass tube, which had several apertures drilled at various points around the tube, across the culture cylinder at a few cms from the base. One end of the tube was fused into the inside of the cylinder and the other end protruded through the cylinder to be fitted on to an air line, the air having first been rendered near sterile by filtration through a 0.45 u filter which was enclosed in a metal holder adapted for use in an air line. A photograph of the cylinder and fittings is in Figure 4.1. The air currents from the apertures in the glass tube maintained a high proportion of the Chlorella cells in suspension but after

a few hours some of the cells of this sedementary species of algae accumulated on a few apertures rendering them inoperative. This failure was attributed to slight variations in the diameter of the apertures and in order to utilize a system with precision-drilled apertures, the glass tube was replaced by a manufactured gas distribution tube with a domed sintered glass bulb. This proved to be very effective and five such cylinders were constructed. In order to obtain a flow of Chlorella cells through the cylinder, the base was converted into a funnel the end of which was connected with rubber tubing leading to a 1 litre beaker containing the daphnia. The flow of cells was controlled by a screen clip fitted round the rubber tubing. The inflow to the beaker was balanced by an outflow through a u shaped suction tube the inner arm of which was positioned at the base of beaker and the outer terminating in a screw clip for appropriate volume adjustment led to waste. To remove the moulted skins of daphnia, excess algal cells and egested food which accumulated at the base of the beaker, the base was tapered into a funnel terminating in an elongated stem fitted with a stop-cock to discharge the debris. The nutrient for the system was introduced into the cylinder through a thin-bore piece of glass tubing fitted through a rubber stopper at the top of the cylinder. An adjacent piece of glass tubing acted as an air vent and a piece of cotton wool was inserted in both glass tubes to prevent aerial contamination of the culture.

A study by Ryter (1954) on the inhibitory effects of phytoplankton on daphnia demonstrated that senescent, non-dividing cells of C. vulgaris produced a substance termed chlorellin which inhibited the growth of daphnia and after 11 - 13 days caused high

levels of mortality. When cultured on actively growing cells, daphnia grew rapidly and maintained a high rate of reproduction. A synchronous culture of C. vulgaris with the vast majority of cells in the exponential growth phase can be obtained by a combination of photoperiod variation, daily dilution of the cells and daily addition of nutrient, while a continuous culture in a steady state of growth can be achieved by continuous addition of fresh nutrient without the necessity to vary the photoperiod (Kuhl and Lorenzen, 1964). It was obviously desirable to aim for a high proportion of cells in the exponential growth phase and this was largely achieved by a photoperiod of 16 hours illumination : 8 hours darkness combined with frequent addition of nutrient to balance the amount used in the metered outflow to daphnia. However on occasions, aggregation or clumping of the cells occurred from time to time particularly in cultures containing added copper. In order to separate the aggregates into individual cells for counting, the samples were transferred to specimen tubes and exposed for 3 seconds to high speed ultrasonics from a probe sonicator.

The development of the automatic system for culturing Chlorella and feeding daphnia took many weeks to complete and what initially appeared to be a straightforward exercise in devising a method to maintain actively growing Chlorella cells in a state of suspension for relay to daphnia presented several unforseen difficulties all of which were eventually solved to provide a versatile unit capable of sustaining a wide variety of experimental procedures with a high degree of reproducibility and precision.

In a test run for one week the system operated well, the Chlorella developing a deep green colour and the daphnia grew

actively.

4.1.2 Calibration of the Coulter Counter for Counting Cells of Chlorella vulgaris

In order to determine the total intake of copper in daphnia exposed to solutions of copper in the relayed culture medium and feeding on cells which contain accumulated copper, it was necessary to quantify the number and volume of cells in the medium in addition to chemical analysis of copper in the cells and in the medium. The Coulter counter has frequently been used for the purpose of enumeration and several reports on the efficiency of the instrument have been issued on a number of different species of algae. A suspension of algae is introduced into an electrically conductive solution (NaCl) which is forced to flow through a small aperture with an immersed electrode on either side, and a cell passing through the aperture changes to resistance between the electrodes. This produces a voltage pulse of short duration and of magnitude proportional to the cell volume. The pulses are scaled and counted electronically. The flow of suspension through the aperture is controlled by an external vacuum source and a mercury manometer. This is so arranged that when the vacuum is cut off the return of the mercury to its original level maintains a constant flow of the suspension and successively activates and stops the counter by contact with two electrodes (Sheldon and Parsons, 1967). The instrument can be employed to count a variety of cells defined by the aperture diameter, and the diameter (d) of the cells can be calculated from the expression:

$$d = K \sqrt[3]{t.I.A.}$$

where K is the diameter calibration constant

t is the threshold scale value

I is the aperture current

A is the amplification

The diameter calibration constant of the instrument was determined by using 15.0 u diameter latex particles according to the recommended procedure and it was found to be 2.7 u. By application of the constant to the above expression, the diameter of cells of various sizes can be determined.

In order to assess the efficiency of the instrument, microscope counts have been made on 100 cells of Chlorella vulgaris and compared with counts by the Coulter counter. The microscope measurements (Table 4.1) were made, under a magnification of 1000, by Miss E. Bowen-Colthurst whose contribution is duly acknowledged.

TABLE 4.1 Microscope measurement of 100 cells of Chlorella vulgaris (1 graticule division = 1.43 u)

Graticule Divisions	No. of Cells	Cell Diameter (u)
1	3	1.43
2	8	2.86
3	11	4.26
4	33	5.72
5	29	7.15
6	12	8.58
7	3	10.01
8	1	11.44
	Mean	6.14

A count of 6000 cells by the Coulter counter set at a threshold value of 3.5, with aperture current at ½ and amplification at 4 gave a mean cell diameter of 5.14 u. Thus there was reasonable agreement between both measurements.

By variations in the threshold value or aperture current or amplification the mean cell volume of a population of C. vulgaris cells can be found by the settings shown in Table 4.2.

TABLE 4.2 Coulter Counter Settings for Determination of Mean Cell Volumes in Chlorella vulgaris

Mean Cell Volume (u^3)	Threshold Value	Aperture Count	Amplification	Sensitivity
2.16	3.5	½	$\frac{1}{8}$	$\frac{1}{16}$
4.31	3.5	½	¼	$\frac{1}{8}$
8.62	3.5	½	½	¼
17.73	3.5	½	1	½
35.45	3.5	½	2	1
70.90	3.5	½	4	2
141.80	7.0	½	4	4
283.60	14.0	½	4	8
567.20	28.0	½	4	16
1134.40	56.0	½	4	32

To obtain the total cell volume for each setting the count of cells is multiplied by the mean cell volume and the sum of this value for all settings gives the total cell volume for each sample.
A measure of the development of samples taken over a period of days can be obtained by comparing the growth constants:

$$K_{10}(\text{days})^{-1} = \frac{\log N_2 - \log N_1}{\text{Interval 2} - \text{Interval 1}}$$

4.2 Discussion

When the calibration of the instrument was completed it was decided to test the toxicity of copper to C. vulgaris in order to ascertain that the dosage levels to be used in the continuous flow system would be safe for use in Chlorella. A fresh culture of C. vulgaris 211/1C was obtained from the Culture Centre of Algae and Protozoa, Cambridge and a 4 ml suspension of cells was introduced asceptically into culture media in the glass cylinders which contained five concentrations of Cu^{++} from 5.0 mg Cu^{++}/l to 5.0^{-4} mg Cu^{++}/l. The media and glassware had previously been sterilized. The counts from the Coulter counter on the day of inoculation showed a reasonably uniform number of cells in the various cylinders and after 24 hours there were marginal increases or decreases in the cell populations. On subsequent days the counts in 0.5 and 0.5^{-1} mg Cu^{++}/l were exceptionally high and approximately double those recorded in the reference control. The counts in 0.5^{-2} and 0.5^{-3} mg Cu^{++}/l were similar and about 30% higher than the reference control which had the same count as 0.5^{-4} mg Cu^{++}/l. In view of these unusual results it was considered that the most likely source of error was caused by variable background noise picked up from adjacent cables in the conduit carrying the power lines. The instrument was tested on a different power line in another part of the laboratory but the results confirmed that the original line was normal. The entire experiment was repeated but the counts were again high and variable and bore no relationship to copper concentration. A microscopic examination of samples from the various cylinders revealed a large number of cell fragments and unidentified non-biological material. Communication with other workers indicated that unless great care

was taken to sterilize equipment and nutrient media, cultures of C. vulgaris rapidly became contaminated and in one laboratory several fresh cultures had to be acquired before a satisfactory technique was developed. A new culture was obtained and a thorough programme of sterilization was carried out, but despite this the counts were very high and inconsistent with concentration of copper. Further microscopic examination again demonstrated the presence of cell fragments and extraneous material. Cell fragmentation could have occurred as a result of cell collisions in the cylinder, particularly when cell numbers became high. The volume of air entering the cylinder was decreased in order to reduce the velocity of the cells but this had no effect on the counts. A typical response of C. vulgaris to copper is the formation of cell aggregates in a proportion of the cell population and since then their volume is very much greater than that of individual cells, the chance of collision between aggregates in constant motion in a column is high. It is possible that as a result of collision between two aggregates, the outermost cells would be damaged and the fragmented pieces would subsequently be recorded in the counts. This feature has not been recorded in the literature probably because this method of evaluating copper toxicity on algae has not been practised.

Although it has not been possible to determine the toxicity of copper on C. vulgaris, the administration of copper to daphnia through the Chlorella continuous flow system was still considered perfectly viable since the cell aggregates would be trapped by the filter feeding mechanism of daphnia, and a high proportion of cells would remain unicellular for consumption by daphnia.

The continuous flow system was thus used in attempts

to induce a copper tolerant strain of daphnia. A culture of C. vulgaris cells suspended in a very high dose of copper, 500 ug Cu^{++}/l, was transferred to the glass cylinder of the continuous flow system and was relayed to 50 daphnia. After a few days there were no surviving daphnia, and the dose was reduced to 250 ug Cu^{++}/l which again caused total mortality. A dose of 100 ug Cu^{++}/l was then tested on 100 daphnia and compared with a reference control. There were several survivors in the copper treatment but there was no reproduction and after three weeks the population gradually diminished to zero which was in contrast to the control which had moderate survival and a reasonable level of reproduction. It was apparent that a dose of 50 ug Cu^{++}/l or lower would be required for adequate survival of daphnia for induction of copper tolerance but analyses of the control culture medium in the Chlorella cylinder and in the daphnia vessel both of which were nominally copper-free showed that the background level was a minimum of 50 ug Cu^{++}/l. A repeated programme of cleaning all equipment and glassware in Haemosol detergent and chromic acid and several rinsings in distilled and deionized water failed to reduce the background below 50 ug Cu^{++}/l. The main source of background copper was attributed to the glass in the cylinder containing the Chlorella suspensions. The constant agitation of the suspensions and consequent friction at the surface of the glass would remove traces of copper from the surface, leading to the unacceptably high background levels in the system. There seemed to be no practical method to reduce the level of background copper and in view of the high sensitivity of daphnia to copper it was concluded that copper tolerance could not be induced in daphnia in the laboratory.

The failure to develop a copper-tolerant strain of daphnia was unfortunate and the programme of experiments which had originally been planned to characterize copper tolerance had to be modified to one which included studies on the effect of copper on daphnia respiration, rate of uptake of radio-labelled copper, and on the binding of copper by metallo-thionein protein in daphnia as described in the chapters which follow.

Summary

1. A continuous flow system for feeding Chlorella to daphnia has been developed to facilitate the continuous administration of copper to daphnia in an endeavour to induce copper tolerance in daphnia.

2. The system maintained a high proportion of Chlorella cells in an actively growing phase and in an initial test run the daphnia culture exhibited normal growth.

3. In order to ascertain the concentration of copper which could be safely introduced into the Chlorella suspensions, studies to determine the toxicity of copper on Chlorella were inaugurated, using cell counts and cell volumes as the indices of toxicity.

4. In the Chlorella suspensions copper caused the typical formation of cell aggregates to occur in a proportion of the cells and this characteristic led to collision between aggregates in the column with consequent fragmentation of cells. The cell fragments gave erroneous counts in the Coulter counter and the method was, therefore, inadequate for determining the toxicity of copper on Chlorella.

5. A proportion of the Chlorella cells in copper solution remained unicellular and the continuous flow system was still considered feasible for the administration of copper to daphnia.

6. Copper concentrations of 500, 250 and 100 ug Cu^{++}/l were administered through the system but there were no survivors among the daphnia population. A maximum of 50 ug Cu^{++}/l would be required for survival of daphnia but this concentration coincided with the background level of copper and it was not possible to achieve lower background. The experiments to develop a copper-tolerant strain of daphnia were then discontinued.

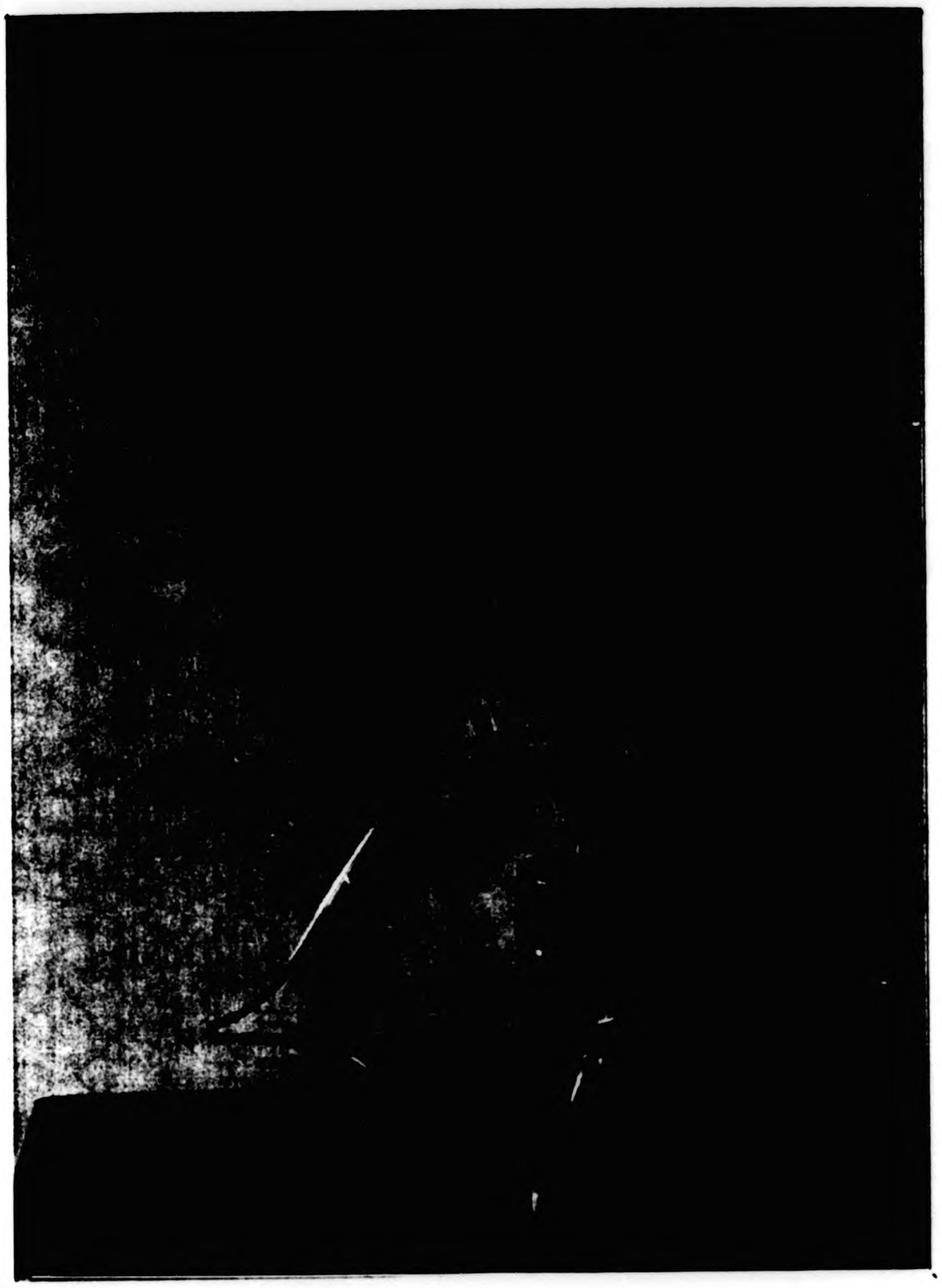


FIGURE 4.1

1. Air vent
2. Nutrient supply tube
3. Suspension of Chlorella vulgaris
4. Daphnia

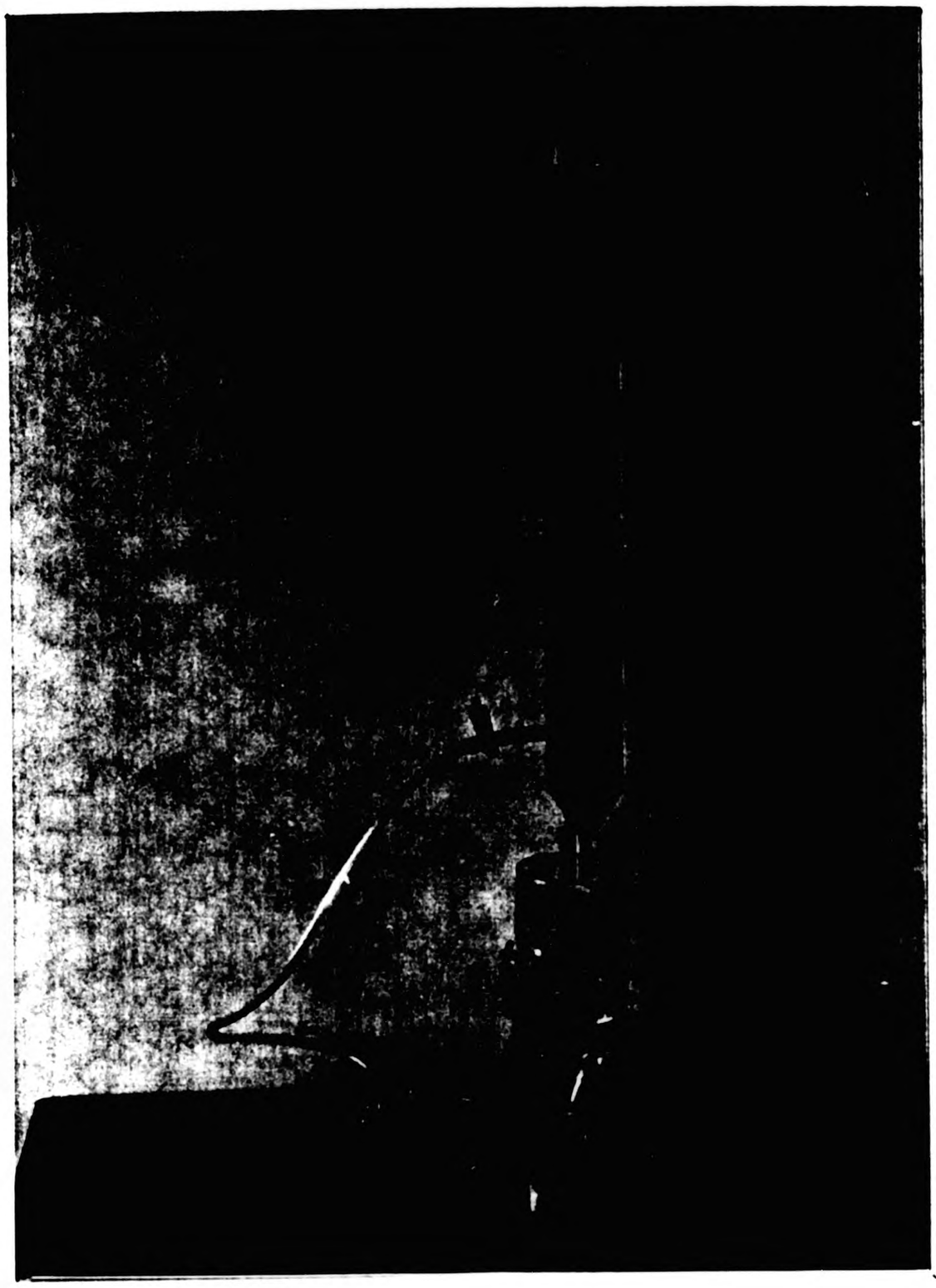


FIGURE 4.1

1. Air vent
2. Nutrient supply tube
3. Suspension of Chlorella vulgaris
4. Daphnia

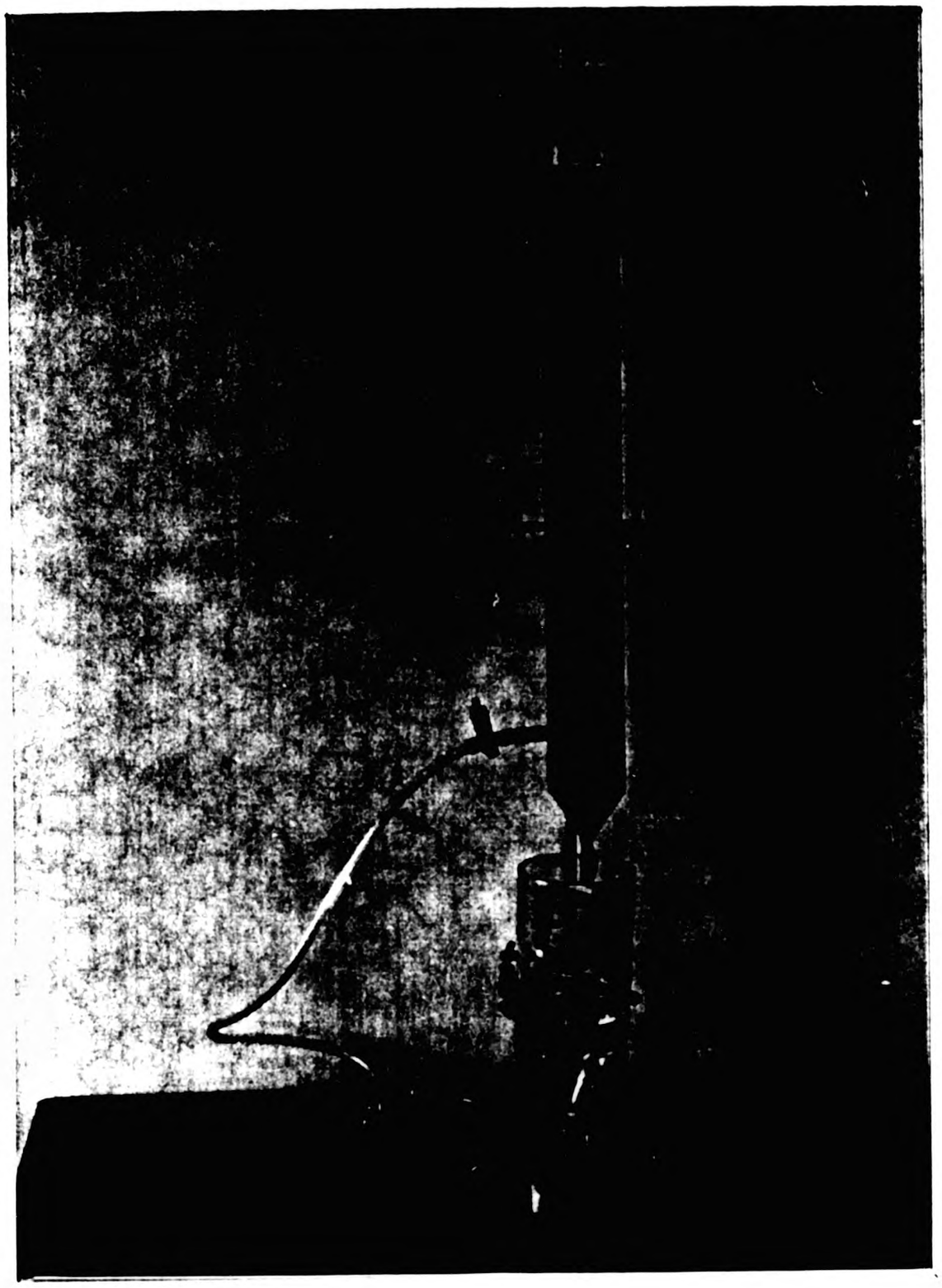


FIGURE 4.1

1. Air vent
2. Nutrient supply tube
3. Suspension of Chlorella vulgaris
4. Daphnia

CHAPTER 5

THE EFFECT OF COPPER ON THE RATE OF OXYGEN UPTAKE BY *Daphnia* Sp.

5.1 Introduction

In the experiments reported in the preceding chapters it was observed that daphnia in solutions of copper sulphate initially exhibited hyperactivity characterised by a rapid beating of the antennae and appendages and since the latter organs support the thin-walled well vascularised epipodites which function as gills it was assumed that the increased activity was associated with an initial increase in the rate of oxygen uptake. Subsequently the hyperactivity ceased and there followed a period of lethargic beating of the antennae combined with irregular movements of the appendages consisting of erratic sweeps of varying magnitude. Since the beating action of the appendages initiates the current of water to the filter feeding mechanism of the daphnia it was assumed that the regular flow of food was thereby disrupted as a result of copper intoxication.

This disruption accompanied by an increased metabolic rate caused by the initial hyperactivity would rapidly deplete the food reserves and lead to the death of the organism by starvation. It was postulated that these effects would be regarded as demonstrating acutely toxic symptoms of copper poisoning. Apart from suggestions in the literature (Holm-Jensen, 1948; R. A. Peeters, 1965) that the acute action of copper may be due to an inhibition or deprivation of essential ions of other metals there have been few convincing conclusions on the pharmacology of copper toxicity.

Experiments by Bernard and Lane (1963) showed that a copper concentration of 500 ug/l increased the respiration rate of planktonic cyprids of the barnacle, Balanus amphitrite and only at concentrations greater than 5,000 ug/l were the rates lower than

in the controls. Against sub-tropical Sagitta hispida and Undinula vulgaris and temperate Calcanus plumchrus, Reeve et al. reported by Davies (1979) found that concentrations of copper exceeding the 24-hour LC50 value had little effect upon the respiration rate.

The respiration systems in daphnia have already been referred to in 1, 2, 3, and these references included the change from oxygenation by diffusion and fluid convection to oxygen transport by haemoglobin which occurs when oxygen levels are low in the water medium. The daphnia cultures for the main experiments reported in this chapter were maintained in a well aerated system and thus had no haemoglobin.

The experiments to determine the influence of copper solutions on the rate of oxygen uptake by daphnia were carried out with the laboratory culture of daphnia which was regarded as D. magna and the experiments were recorded under that specific name. However, when the results were eventually studied it was apparent that the weights of daphnia recorded in the experiments were about one-third those observed for a fresh stock of D. magna which had subsequently been acquired. Also a free-hand drawing of the ephippium which was made at a later date of the daphnia used in the respiration experiments very clearly demonstrated that the species was not D. magna. The ephippium was completely typical of D. pulex which is a much smaller species than D. magna. No attempt was made at the time of the experiments to identify the species since it was assumed to be D. magna. Since there is no certainty about the precise species used in these experiments, although it is likely to have been D. pulex, reference is made in this chapter to the generic name only.

5.1.1 Objective of Experiments

The object of the studies was to determine the effect of various concentrations of copper, as copper sulphate solutions in the aquatic medium, on the rate of uptake of oxygen by daphnia.

5.1.2 Experimental Methods

Initial tests were carried out in a 30 ml capacity plastic chamber fitted with a screw-tight cap in the middle of which was a precision-drilled aperture to accommodate the oxygen electrode of a radiometer. The unit is illustrated in the photograph in Figure 5.P.1. As a result of several preliminary tests it was found that 30 daphnia in a volume of 20 ml culture medium survived for 17 hours and during that interval of time all oxygen had been consumed. The volume of the respirometer was accordingly reduced to 20 ml. When the respirometer was filled with the test liquid and the screw cap tightened, the insertion of the oxygen electrode cylinder introduced a bubble of air into the liquid. To eliminate the pocket of air, which if trapped on the polypropylene membrane of the electrode would give erroneous readings of the oxygen levels in the liquid, a piece of glass tubing 2 cm long x 0.5 mm diameter was inserted through the screw cap to penetrate the surface of the liquid. By manipulating the respirometer into a position where the air bubble was immediately below the glass tubing and by marginally raising the temperature of the liquid the bubble was forced through the tubing and when the first few drops of liquid started to overflow the top of the tubing was sealed with plasticine.

No attempt was made to trap CO_2 in the respirometer since in aquatic animals generally, the Bohr effect is of little consequence (Jones, 1975), especially in the case of daphnia which has an open

vascular system.

The rate of oxygen uptake by daphnia in the test liquid in the respirometer was determined with a radiometer PO_2 electrode which measured changes in the oxygen tension in the liquid. The electrode consists of a combined platinum cathode and silver/silver chloride anode fused into a glass rod which is immersed at its base in a solution consisting of a phosphate buffer and KCl. The glass rod fits closely into a cylinder at the base of which is a polypropylene membrane fractionally spaced apart from the tip of the rod and held securely in position by an '0' ring fitted round the circumference of the cylinder.

The radiometer was calibrated at 20°C by immersing the electrode in fully aerated distilled water and setting the scale to the maximum reading 160, and after clearing the water from the membrane, by setting zero PO_2 in a solution of 0.5 g Sodium sulphite crystals dissolved in 25 ml deionized water. The readings were corrected for changes in atmospheric pressure as shown in the following example

Atmos.press. on day of test = 760 mm Hg

S.V.P. at 20°C = 17.5 mm Hg

21% of P - S.V.P. (PO_2) = 155.9 mm Hg (partial pressure O_2)

Radiometer reading of sample = 130 mm Hg

Corrected PO_2 in sample = $\frac{130}{1} \times \frac{155.9}{160}$ = 127 mm Hg

The wet weight on each sample was recorded at the end of the experiment and since this value is not subsequently referred to it is shown here for three of the five samples; the original records for the remaining two samples have been lost but it is

assumed that the weights were within the range of those recorded. It is also assumed that dry weight would be closely correlated to wet weight.

Wet Wt (mgs) of 30 Daphnia

Treatment (mg Cu^{++}/l)	Wet Wt of Daphnia (mgs)
5.0	-
0.5	8.0
0.25	4.0
0.125	9.0
0.0	-

There was a large difference between the weight of the daphnia in the 0.25 mg/l concentration and 0.5 or 0.125 mg Cu^{++}/l and this will have to be borne in mind in the subsequent comparison of the results.

The culture medium used in all experiments is shown below:

	$MgSO_4$	K_2HPO_4	$CaCl_2$	NaCl
mg/l	61.6	87.1	73.6	117.0

Before each experiment the medium and the respirometer were sterilized and the daphnia were rinsed through three changes of distilled water in attempts to remove protozoa or other superficial organisms. Each experiment was conducted in a growth room at a temperature of $20^{o}C \pm 2^{o}$.

Since the experiments consisted of recording the uptake of oxygen by an initial population of 30 daphnia in an enclosed chamber containing 20 ml of the test solution of copper sulphate until such time as mortality intervened due to the acute toxicity

of copper or lack of oxygen, control daphnia were placed in open specimen tubes containing the identical solution as in the respirometer in order to examine the possibility of an interaction occurring between reducing tension and daphnia mortality in the respirometer. Each control consisted of 10 daphnia in 6.6 ml of the test solution and there were three such controls for each experiment to represent the identical ratio of the number of daphnia to the volume of the solution in the respirometer. An additional control was included consisting of 10 daphnia in the culture medium alone in order to ascertain that the population under test was normal.

The results of the experiments have been evaluated by multiple regression analysis and covariance analysis according to Snedecor and Cochran (1972).

5.2 Experiment I

To Determine the Influence of 5.0 mg Cu^{++}/l on the Rate of Oxygen Uptake by Daphnia

A population of 30 daphnia was introduced into the test solution of 5.0 mg Cu^{++}/l in the respirometer and mortality and PO_2 observations were taken at 15 minute intervals. The data are presented in Table 5.1 and in Figure 5.1. Observations on the mortality of daphnia in the reference controls were taken at the same 15 minute intervals as above and are compared with the mortality in the respirometer in Table 5.2.

TABLE 5.1 Daphnia population (X_1) in 5.0 mg Cu^{++}/l in respirometer and PO_2 values (Y) recorded at 15 minute intervals (X_2)
$\hat{Y}$ is the estimated PO_2 value (See Text)

Daphnia Population X_1	Time (mins) X_2	Radiometer Reading	Corrected PO_2 (mm Hg) Y	$\hat{Y}$	$Y - \hat{Y}$
30	0	122	116	114.8	+1.2
24	15	115	109	110.8	-1.8
23	30	112	106	105.0	+1.0
16	45	105	100	101.4	-1.4
7	60	104	99	98.4	+0.6
0	75	100	95	94.7	+0.3
Total 100	225		625		-0.1
Mean 16.7	37.5		104.2		

$\Sigma X_1^2 = 2310$ $\Sigma X_2^2 = 12375$ $\Sigma Y^2 = 65399$

$C = 1666.7$ $C = 8437.5$ $C = 65104.2$

$\Sigma x_1^2 = 643.3$ $\Sigma x_2^2 = 3937.5$ $\Sigma y^2 = 294.8$

$\Sigma X_1X_2 = 2190$ $\Sigma X_1Y = 10827$ $\Sigma X_2Y = 22380$

$C = 3750$ $C = 10416.7$ $C = 23437.5$

$\Sigma x_1x_2 = -1560$ $\Sigma x_1y = 410.3$ $\Sigma x_2y = -1057.5$

$$\text{Regression coefficient } b_1 \text{ of Y on } X_1 = \frac{(\Sigma x_2^2)(\Sigma x_1y) - (\Sigma x_1x_2)(\Sigma x_2y)}{(\Sigma x_1^2)(\Sigma x_2^2) - (\Sigma x_1x_2)^2}$$

$$= -0.344$$

$$\text{Regression coefficient } b_2 \text{ of Y on } X_2 = \frac{(\Sigma x_1^2)(\Sigma x_2y) - \Sigma x_1x_2)(\Sigma x_1y)}{(\Sigma x_1^2)(\Sigma x_2^2) - (\Sigma x_1x_2)^2}$$

$$= -0.405$$

Prediction equation: $\bar{Y} - b_1\bar{X}_1 - b_2\bar{X}_2 = 125.1$

Multiple Regression equation: $\hat{Y} = 125.1 - 0.344X_1 - 0.405X_2$

5.2.1 Results

The multiple regression equation shows that each unit reduction in the daphnia population corresponds with a reduction of 0.344 units of PO_2, and in a time interval of one minute PO_2 is reduced on average by 0.405 units. From the multiple regression equation, the predicted values of Y are shown in Table 5.1 and the deviations in the final column show the capacity of the X_1 and X_2 values to predict Y. In this experiment the deviations were small indicating that a good measure of prediction of oxygen uptake was attainable from the X_1 and X_2 values.

Figure 5.1 shows the rapid decline in the daphnia population in time, and the relationship between a plot of these values and the actual (not to scale) PO_2 readings PO_2 and the predicted readings (on scale). It will be seen from Figure 5.1 that 50% of the daphnia population consumed an estimated (114.8 - 102.5) 12.3 units of PO_2 in 37.5 minutes.

In order to evaluate the effect of copper on daphnia mortality in the diminishing oxygen tension in the respirometer, the mortality readings are compared with those in the open specimen tubes as shown in Table 5.2.

TABLE 5.2 Daphnia Mortality in the Respirometer and in the Reference Standards

Time (mins)	Respirometer (5.0 mg Cu^{++}/l)	Control 3 x 10 Daphnia (5.0 mg Cu^{++}/l)				Control 1 x 10 (0.0 mg Cu^{++}/l)
		a	b	c	Total	
15	6	2	3	3	8	0
30	7	6	6	9	21	0
45	14	6	6	9	21	0
60	23	10	10	10	30	0
75	30	-	-	-	-	-

An initial 't' test showed that the differences between the replicates a, b and c in the control were not significant and the total mortality which occurred at 60 minutes has been compared with the mortality in the respirometer. There appeared to be a higher mortality in the control but the difference between the control and the respirometer was not significant, the theoretical value of 't' for 6 d.f. (n = 3 + 3 pooled) at the 5% level is 2.447 compared with an observed value of 1.25.

5.2.2 To Determine the Influence of 0.5 mg Cu^{++}/l on the Rate of Oxygen Uptake by Daphnia

Precisely the same procedure was used as in the previous experiment and the results are shown in Table 5.3.

TABLE 5.3 Daphnia population (X_1) in 0.5 mg Cu^{++}/l in respirometer and PO_2 values (Y) recorded at 15 minute intervals(X_2)

Daphnia Population X_1	Time (mins) X_2	Radiometer Reading	Corrected PO_2 (mm Hg) Y	$\hat{Y}$	$Y - \hat{Y}$
30	0	145	138	131.9	+6.1
30	15	134	127	126.0	+1.0
28	30	125	119	120.0	-1.1
23	45	115	109	114.4	-5.4
10	60	111	105	109.1	-4.1
8	75	106	101	103.3	-2.3
5	90	102	97	97.5	-0.5
2	105	99	94	91.7	+2.3
0	120	95	90	85.9	+4.1
Total 136	540		980		+0.1
Mean 15.1	60		108.9		

$\Sigma X_1^2 = 3306$ $\Sigma X_2^2 = 45900$ $\Sigma Y^2 = 108786$

$C = 2055.1$ $C = 32400$ $C = 106711.1$

$\Sigma x_1^2 = 1250.9$ $\Sigma x_2^2 = 13500$ $\Sigma y^2 = 2074.9$

$\Sigma X_1 X_2 = 4185$ $\Sigma X_1 Y = 16320$ $\Sigma X_2 Y = 53655$

$C = 8160$ $C = 14808.9$ $C = 58800$

$\Sigma x_1 x_2 = -3975$ $\Sigma x_1 y = 1511.1$ $\Sigma x_2 y = -5145$

Regression coefficient $b_1 = \frac{13500 \times 1511.1 - (-3975 \times -5145)}{1086525} = -0.047$

Regression coefficient $b_2 = \frac{1250.9 \times -5145 - (-3975 \times 1511.1)}{1086525} = -0.395$

$\hat{Y} = 133.3 - 0.047X_1 - 0.395X_2$

This analysis showed that each daphnia mortality corresponded to a decrease of 0.047 units of PO_2 and at each 1 minute interval PO_2 diminished by 0.395 units. The multiple regression of PO_2 on the daphnia population through time is illustrated in Figure 5.2 which shows that 50% of the daphnia population consumed an estimated 23 units of PO_2 in 60.75 minutes.

The daphnia mortality in the respirometer and in the reference controls is shown in Table 5.4.

TABLE 5.4 Mortality of Daphnia in the Respirometer and in the Reference Controls

Time (mins)	Respirometer (0.5 mg Cu^{++}/l)	Control 3 x 10 Daphnia (0.5 mg Cu^{++}/l)				Control 1 x 10 (0.0 mg Cu^{++}/l)
		a	b	c	Total	
30	2	0	0	0	0	0
45	7	0	1	0	1	0
60	20	2	3	0	5	0
75	22	5	6	4	15	0
90	25	7	6	5	18	0
105	28	7	9	8	24	0
120	30	9	9	9	27	0
135		10	10	10	30	0

There appeared to be a higher mortality in the respirometer than in the controls containing copper but the differences were not statistically significant. The value of 't' in the analysis was 1.08 compared with 2.179 at the P = 0.05 level.

Comparison of the Regression Coefficients of PO_2 on Time in 5.0 and 0.5 mg Cu^{++}/l

The regression coefficients of PO_2 on Time were -0.405

and -0.395 respectively for 5.0 and 0.5 mg Cu^{++}/l and an analysis has been carried out to determine if they differ significantly. The analysis of covariance is shown in Table 5.5.

TABLE 5.5 Comparison of Regression Coefficients of PO_2 on Time in 5.0 and 0.5 mg Cu^{++}/l

Treatment (mg Cu^{++}/l)	d.f.	Σx_2^2	$\Sigma x_2 y$	Σy^2	devs. from Regr. d.f.	s.s.	m.s.	F
5.0	5	3937.5	-1057.5	294.8	4	10.8	2.7	
0.5	8	13500	-5145	2074.9	7	114.1	16.3	0.166
					11	124.9	11.35	
Pooled	13	17437.5	-6202.5	2369.7	12	163.5	13.63	
Difference between slopes					1	38.6	38.6	3.40

The residual variances were homogeneous as shown by a value for F of 0.166 while at the probability level of 0.05 the value of F for 7 and 4 d.f. is 4.12. The difference between the slopes (i.e. the regression coefficients) with F = 3.40 was not significant; the theoretical value of F for 1 and 11 d.f. = 4.84. This result is of interest since the analysis shows that the rate of oxygen uptake did not differ significantly in the time intervals compared despite a ten-fold difference in the concentration of copper.

A comparison of the oxygen uptake by 50% of the daphnia population is shown in Table 5.6 for 5.0 and 0.5 mg Cu^{++}/l.

TABLE 5.6 Oxygen Uptake by 50% of the Daphnia Population and Uptake by one Daphnia per minute

Treatment (mg Cu^{++}/l)	Oxygen Uptake as PO_2 by 50% Daphnia Pop.	Time (mins)	Oxygen Uptake (PO_2) by 1 Daphnia/min
5.0	12.3	37.5	0.0219
0.5	23.0	60.75	0.0252

The similarity in oxygen uptake between these differing concentrations of copper is an unexpected result since an uncomplicated toxicological effect would show a dose response unless a threshold level was reached at the lower concentration.

5.2.3 To Determine the Influence of 0.25 mg Cu^{++}/l on the Rate of Oxygen Uptake by Daphnia

The same procedure was used as in the previous experiments and the results are shown in Table 5.7.

TABLE 5.7 Daphnia Population (X_1) in 0.25 mg Cu^{++}/l in Respirometer and PO_2 values (Y) recorded at 15 minute intervals (X_2)

Daphnia Population X_1	Time (mins) X_2	Radiometer Reading	Corrected PO_2 (mm Hg) Y	$\hat{Y}$	$Y - \hat{Y}$
30	0	160	157	149.2	+7.8
30	15	146	143	144.1	-1.1
30	30	140	137	139.1	-2.1
30	45	135	132	134.0	-2.0
30	60	132	129	129.0	0.0
30	75	129	126	123.9	+2.1
23	90	120	118	120.2	-2.2
16	105	115	113	116.5	-3.5
12	120	111*	109	112.2	-3.2
5	135	106	104	108.5	-4.5
4	150	105	103	103.6	-0.6
4	165	101	99	98.5	+0.5
4	180	97	95	93.5	+1.5
0	195	97	95	89.2	+5.8
Total 248	1365		2660		-1.5
Mean 17.7	97.5		118.6		

$\Sigma X_1^2 = 6402$ $\Sigma X_2^2 = 184275$ $\Sigma Y^2 = 201658$

C = 4393.1 C = 133087.5 C = 196828

$\Sigma x_1^2 = 2088.9$ $\Sigma x_2^2 = 51187.5$ $\Sigma y^2 = 4830$

$\Sigma X_1 X_2 = 14595$ $\Sigma X_1 Y = 32258$ $\Sigma X_2 Y = 146400$

C = 24180 C = 29405.7 C = 161850

$\Sigma x_1 x_2 = -9585$ $\Sigma x_1 y = 2852.3$ $\Sigma x_2 y = -15450$

*Estimated

$$b_1 = \frac{51187.5 \times 2852.3 - (-9585 \times -1540)}{10958343} = -0.19$$

$$b_2 = \frac{2008.9 \times -15450 - (-9585 \times 2852.3)}{10958343} = -0.337$$

$$Y = 154.9 - 0.19X_1 - 0.337X_2$$

This analysis shows that a decrease of one daphnia was accompanied by an average reduction of 0.19 units of PO_2 and there was an average reduction of 0.337 units of PO_2 per minute. The values plotted in Figure 5.3 indicate a trend towards a partly sigmoid curve which has been caused by six initial readings when the daphnia population remained static, and similarly by three points near the end of the curve, while the intervening points formed a straight line. Transformation of the data would not modify the slope and it was decided to apply multiple regression analysis to the data with the knowledge that this technique was being applied to include some outlying points notably those recorded at 45, 60 and 75 minutes. The oxygen uptake by 50% of the daphnia population is seen from Figure 5.3 to be 29.5 units of PO_2 in 97.5 minutes.

The regression coefficients of PO_2 on Time for 0.5 mg Cu^{++}/l at 0.25 mg Cu^{++}/l were -0.395 and -0.337 respectively and the significance of the difference between them is considered below in Table 5.8.

TABLE 5.8 Covariance Analysis of the Regression Coefficients of PO_2 on Time in 0.5 and 0.25 mg Cu^{++}/l

Treatment (mg Cu^{++}/l)	d.f.	Σx_2^2	$\Sigma x_2 y$	Σy_2	Devs. from Regr. d.f.	s.s.	m.s.	F
0.5	8	13500	-5145	2074.9	7	114.1	16.30	1.173
0.25	13	51877.5	-15450	4830	12	166.7	13.89	
					19	280.8	14.79	
Pooled	21	64687.5	-20595	6904.9	20	347.9	17.40	
	Difference between slopes				1	67.1	67.1	4.537*

The 5% value for F with 7 and 12 d.f. is 2.92 which exceeds the observed value 1.173 thus demonstrating that the residual variances do not differ significantly. The difference between the slopes is significant at the 5% level with an observed value of 4.537 against the theoretical value for F with 1 and 19 d.f. = 4.38. This means that the regression coefficient -0.337 units of PO_2 was significantly lower than -0.395 indicating a lower rate of oxygen uptake by daphnia exposed to 0.25 mg Cu^{++}/l compared with the rate of uptake in 0.5 mg Cu^{++}/l.

The oxygen uptake by 50% of the daphnia population in concentrations of 0.5 and 0.25 mg Cu^{++}/l is shown in Table 5.9.

TABLE 5.9 Oxygen Uptake as PO_2 (mm Hg) by 50% of the Daphnia Population and Uptake by 1 daphnia/min in 0.5 and 0.25 mg Cu^{++}/l

Treatment (mg Cu^{++}/l)	Oxygen Uptake by 50% Daphnia Population	Time (mins)	Oxygen Uptake by 1 Daphnia/min
0.5	23.0	60.75	0.0252
0.25	29.5	97.50	0.0201

The oxygen uptake expressed in terms of uptake by one daphnid per minute appeared to be very similar but since the regression coefficients differed significantly it is concluded that the daphnia in 0.25 mg Cu^{++}/l had a lower rate of respiration than those in 0.5 mg Cu^{++}/l.

The mortality of the daphnia in the respirometer is compared with that in the reference controls in Table 5.10.

TABLE 5.10 Daphnia Mortality in Respirometer and in Reference Controls at a concentration of 0.25 mg Cu^{++}/l

Time (mins)	Respirometer (0.25 mg Cu^{++}/l)	Control 3 x 10 Daphnia (0.25 mg Cu^{++}/l)				Control 1 x 10 (0.0 mg Cu^{++}/l)
		a	b	c	Total	
90	7	3	4	5	12	0
105	14	7	4	5	16	0
135	25	8	8	7	23	0
150	26	8	8	7	23	0
165	26	8	9	9	26	0
180	26	9	9	9	27	0
195	30	10	10	10	30	0

It is obvious from the above observations that there were only small, and as assessed from the previous mortality analysis, insignificant differences between the mortality in the respirometer and the appropriate control.

5.2.4 To Determine the Rate of Oxygen Uptake by Daphnia exposed to 0.125 mg Cu^{++}/l

The same procedure was followed as in the previous experiments with the exception that the observations on PO_2 and mortality were taken at intervals of 1 hour since it was anticipated

that this experiment would extend over a much longer time scale and there would be no advantage in recording a successive number of identical population values or small changes in PO_2 readings during the extended course of the experiment.

TABLE 5.11 Daphnia Population and PO_2 Readings at Hourly Intervals at a concentration of 0.125 mg Cu^{++}/l

Daphnia Population	Time (Hours)	Radiometer Reading	PO_2 (mm Hg)
30	0	100	96
30	1	96	92
30	2	105	101
30	3	85	82
30	4	60	58
29	5	51	49
29	6	47	45
29	7	40	38
28	8	31	30
25	9	23	22
24	10	11	11
18	11	0	0

During preliminary experiments it was observed that daphnia in a culture solution containing no added copper survived for about 1 hour in the apparent absence of ambient oxygen. This is not unusual among Invertebrates and several hypotheses have been advanced on the subject of tissue function in the absence of ambient oxygen (Jones, 1975). This characteristic is demonstrated in the above result when there were 18 surviving daphnia in the absence of ambient oxygen. It is assumed that the 18 daphnia utilized a storage capacity for oxygen before the PO_2 level diminished to zero thus allowing

survival to continue for a limited time.

The PO_2 readings plotted against Time produced a curvilinear response and for this type of regression analysis transformation of the data is required to give a linear arrangement. In order to avoid the uncertainties of transformation an appropriate method of obtaining a linear response is to add together the actual units of oxygen taken up at each successive interval expressed in terms of a single daphnid per hour. This is referred to as the cumulative PO_2 value per 1 daphnid in the table of results. A linear regression of Time on cumulative PO_2/1 daphnid has been carried out on the data in Table 5.12 and this is subsequently compared with a copper-free control over the same time scale. It will be seen from Table 5.11 that the PO_2 value showed an increase at the recording taken at 2 hours and since this was probably caused by an electrical fault in the instrument or temperature fluctuation the readings at 0 and 1 hours have been disregarded and the calculated PO_2 per 1 daphnid begins at 2 hours.

TABLE 5.12 Cumulative Units of PO_2 taken up by 1 Daphnid at Hourly Intervals in Solution of 0.125 mg Cu^{++}/l

Time (Hours) X	Cumulative PO_2 per 1 daphnid Y	$\hat{Y}$	$Y - \hat{Y}$
1	0.63	0.66	-0.03
2	1.43	1.05	+0.38
3	1.73	1.43	+0.30
4	1.87	1.82	+0.05
5	2.11	2.21	-0.10
6	2.40	2.60	-0.20
7	2.72	2.99	-0.27
8	3.18	3.37	-0.19
9	3.79	3.76	+0.03
Total 45	19.86		-0.03
Mean 5.0	2.21		

$\Sigma X^2 = 285$ $\Sigma Y^2 = 51.0$ $\Sigma XY = 119.7$

$C = 225$ $C = 43.8$ $C = 99.3$

$\Sigma x^2 = 60$ $\Sigma y^2 = 7.2$ $\Sigma xy = 20.4$

Regression coefficient $b = \frac{\Sigma xy}{\Sigma x^2} = 0.340$ cumulative PO_2 units per 1 daphnid/hour

$\hat{Y} = \bar{Y} + b(X - \bar{X}) = 0.27 + 0.338X$

S.S. of deviation $= \Sigma y^2 - \frac{\Sigma xy^2}{\Sigma x^2} = 0.26$

M.S. deviation from regression $= \frac{0.26}{n-2} = 0.037$

Std. deviation from regression $= \sqrt{0.037} = 0.19$ cumulative PO_2 units

Std. deviation of regression coefficient $= \frac{0.19}{\sqrt{60}} = 0.025$

$t = \frac{0.340}{0.025} = 13.6$

t (7 d.f.) = 5.405 (0.001)

The regression of cumulative PO_2/l daphnid on Time is seen to be highly significant.

The fitted regression line in Figure 5.4(a) is compared against the plot of the observed values, the linearity of which validates expressing the results in terms of cumulative PO_2/l Daphnid. The uptake of cumulative PO_2/l daphnid over 4.5 hours amounted to 2.0 units.

A comparison of the daphnia mortality in the 0.125 mg Cu^{++}/l solution in the respirometer and in the open specimen tube controls is shown in Table 5.13.

TABLE 5.13 Mortality of Daphnia in 0.125 mg Cu^{++}/l in the Respirometer and in reference Controls.

Exposure Time (Hours)	Mortality in Respirometer (0.125 mg Cu^{++}/l)	Mortality in Reference Control (0.125 mg Cu^{++}/l)				Control 1 x 10 Daphnia (0.0 mg Cu^{++}/l)
		a	b	c	Total	
5	1	0	1	0	1	0
6	1	1	2	2	5	0
7	1	3	3	4	10	0
8	2	5	4	4	13	0
9	5	7	5	6	18	0
10	6	7	5	6	18	0
11	12	8	7	8	23	0

There was a significantly higher mortality in the reference control than in the respirometer, the observed 't' value being 2.58 against a theoretical value of 2.179.

In order to compare the regression coefficients of 0.125 and 0.25 mg Cu^{++}/l, the PO_2 values for the daphnia population in 0.25 mg Cu^{++}/l (Table 5.7) have been converted into cumulative

units of PO_2 for a single daphnid as expressed for 0.125 mg Cu^{++}/l. They are shown in Table 5.14.

TABLE 5.14 Cumulative Units of PO_2 taken up by 1 Daphnid at Intervals Designated below in a Solution of 0.25 mg Cu^{++}/l

Time (Hours) X	Cumulative PO_2/l Daphnid Y
0.25	0.47
0.50	0.67
0.75	0.84
1.0	0.94
1.25	1.04
1.50	1.34
1.75	1.60
2.0	1.89
2.25	2.48
2.50	3.15
2.75	4.15
3.0	5.15
Total 19.50	23.72

$\Sigma X^2 = 40.6$ $\Sigma Y^2 = 71.1$ $\Sigma XY = 52.2$

C = 31.7 C = 46.9 C = 38.5

$\Sigma x^2 = 8.9$ $\Sigma y^2 = 24.2$ $\Sigma xy = 13.7$

$$\text{Regression coefficient} = \frac{13.7}{8.9} = 1.539$$

TABLE 5.15 Comparison of Residual Variances and Slopes of Regression Lines Relating Cumulative PO_2/Single Daphnid at Time (Hours) in 0.25 and 0.125 mg Cu^{++}/l

Treatment (mg Cu^{++}/l)	d.f.	Σx^2	Σxy	Σy^2	Devs. from Regr. d.f.	s.s.	m.s.	F
0.25	11	8.9	13.7	24.2	10	3.11	.31	7.75
0.125	8	60	20.4	7.2	7	.26	.04	
					17	3.37	.20	
Pooled	19	68.9	34.1	31.4	18	14.52	.81	
Difference between slopes					1	11.15	11.15	55.75

The F values for 10 and 7 d.f. and 1 and 17 d.f. are 3.63 and 4.45 at P = 0.05. Thus the residual variances were significantly different. The regression coefficients 1.539 for 0.25 mg Cu^{++}/l, and 0.340 for 0.125 mg Cu^{++}/l were also significantly different.

5.2.5 To Determine the Uptake of Oxygen by Daphnia in Culture Solution Containing No Added Copper

In this experiment the uptake of oxygen was recorded from daphnia in the respirometer containing only the culture solution for comparison against the 0.125 mg Cu^{++}/l concentration in the previous experiment. The results are shown in Table 5.16. The uptake at 4 and 5 hours was not recorded and the values shown are estimated from 3 and 6 hours and the value for 8 hours has been calculated from the observed reading which was taken at 8.5 hours.

TABLE 5.16 Daphnia Population and Radiometer Readings and PO_2 (mm Hg) Uptake by Daphnia in Culture Medium Control

Daphnia Population	Time (Hours)	Radiometer Reading	PO_2 (mm Hg)
30	0	145	139
30	1	130	125
30	2	122	117
30	3	117	113
30	4	113	109
30	5	109	105
30	6	100	96
28	7	87	84
28	8	69	66
28	9	55	53

As in the previous experiment the uptake of oxygen has been calculated as cumulative PO_2 units per single daphnid and this is shown in Table 5.17.

TABLE 5.17 Oxygen Uptake in Daphnia in Culture Solution Expressed as Cumulative Units of PO_2/1 Daphnid

Time (Hours) X	Cumulative PO_2 /1 daphnid Y	$\hat{Y}$	$Y - \hat{Y}$
1	0.47	0.26	+0.21
2	0.74	0.56	+0.18
3	0.87	0.85	+0.02
4	1.00	1.15	-0.15
5	1.13	1.44	-0.31
6	1.43	1.73	-0.30
7	1.86	2.03	-0.17
8	2.50	2.32	+0.18
9	2.96	2.62	+0.34
Total 45	12.96		0.0
Mean 5.0	1.44		

$\Sigma X^2 = 285$ $\Sigma Y^2 = 24.30$ $\Sigma XY = 82.45$

$C = 225$ $C = 18.66$ $C = 64.80$

$\Sigma x^2 = 60$ $\Sigma y^2 = 5.64$ $\Sigma xy = 17.65$

Regression coefficient $b_1 = \frac{17.65}{60} = 0.294$

$\hat{Y} = \bar{Y} + b(X - \bar{X}) = -0.03 + 0.294X$

The fitted regression line is shown in Figure 5.4(b). It is of interest to note that in 4.5 hours, the cumulative PO_2 value for 1 daphnid was 1.25 units compared with 2.0 units for 1 daphnid in 0.125 mg Cu^{++}/1. To test the statistical significance of the difference in uptake the covariance analysis in Table 5.18 has been carried out.

TABLE 5.18 Comparison of residual Variances and Slopes of Regression Lines Relating Cumulative PO_2/single Daphnid and time (Hours) in 0.125 mg Cu^{++}/l and Control

Treatment (mg Cu^{++}/l)	d.f.	Σx^2	Σxy	Σy^2	Devs. from Regr. d.f.	s.s.	m.s.	F
0.125	8	60.0	20.4	7.2	7	0.26	0.04	
0.0	8	60.0	17.65	5.64	7	0.45	0.06	0.67
					14	0.71	0.05	
Pooled	16	120	38.05	12.84	15	0.78	0.05	
Difference between slopes					1	0.07	0.07	1.4

The differences between the residual variances or the slopes of the regression lines were not statistically significant.

The experiments reported have all been carried out with non-pigmented daphnia which had been cultured in well-aerated water. In the 0.125 mg Cu^{++}/l experiment it was observed that as the PO_2 reading dropped to near zero this was accompanied by a gradual synthesis of haemoglobin and in the final hour of the experiment the daphnia were red in colour. This also occurred in the control experiment in culture medium, the haemoglobin synthesis becoming apparent a few units above zero. It was considered likely that haemoglobin would generate a more rapid uptake of oxygen and yet it was observed that the pigmented daphnia were capable of surviving for 1 hour in zero PO_2. The experiment reported below was carried out to obtain data on the rate of oxygen uptake by pigmented daphnia.

5.2.6 To Determine the Rate of Oxygen Uptake in Pigmented Daphnia

Thirty pigmented daphnia obtained by maintaining a small culture at low PO_2 were transferred to the culture medium in the respirometer and the radiometer readings are shown in Table 5.19.

TABLE 5.19 Oxygen Uptake as PO_2 (mm Hg) in Pigmented Daphnia

Daphnia Population	Time (Hours)	Radiometer Reading	PO_2 (mm Hg)
30	1	100	100
30	2	60	59
30	3	34	34
30	3.3	12	12
30	4	3	3
18	4.5	0	0
12	4.7	0	0
5	4.8	0	0
0	4.9	0	0

It is obvious without analysis that the pigmented daphnia exhibited a higher oxygen uptake than the non-pigmented daphnia reported in the previous experiment. In terms of uptake per minute one pigmented daphnia consumed 0.016 units of PO_2 per minute compared with 0.0046 units in the case of one non-pigmented daphnia. This does not necessarily mean that the pigmented daphnia had a greater metabolic activity but it does suggest an accelerated rate of oxygen transport and possibly a capacity for storing oxygen to permit survival for about 1 hour in the absence of ambient oxygen.

5.3 Discussion of Results

Since the difference between 0.5 and 0.25 mg Cu^{++}/l was just significant and the value from 0.5 was higher but not significantly

so than that from 5.0, it seemed appropriate to test the significance of the difference between the slopes of 5.0 and 0.25 mg Cu^{++}/l. The analysis of covariance is in Table 5.20.

TABLE 5.20 Covariance Analysis of the Regression Coefficients of PO_2 on Time in 5.0 and 0.25 mg Cu^{++}/l

Treatment (mg Cu^{++}/l)	d.f.	Σx^2	$\Sigma x_2 y$	Σy^2	Devs. from Regr. d.f.	s.s.	m.s.	F
5.0	5	3937.5	-1057.5	294.8	4	10.8	2.7	
0.25	13	51877.5	-15450	4830	12	166.7	13.89	0.19
					16	177.5	16.59	
Pooled	18	55815	-16507.5	5124.8	17	242.6	14.27	
Difference between slopes					1	65.1	65.1	3.92

The value of F for 1 and 16 d.f. is 4.49 (P = 0.05) which is greater than the observed value 3.92. Thus there is no significant difference between the regression coefficients from 5.0 and 0.25 mg Cu^{++}/l. This result is of considerable interest since it suggests that no real difference in respiratory rate occurred between these widely differing concentrations of copper.

The rate of oxygen uptake expressed as PO_2 units taken up by 1 daphnid per minute is shown in Table 5.21 for the various concentrations of copper and the control. The significance of the difference in the mortality of daphnia in the respirometer and in the open specimen tubes is also shown in the table.

SUMMARY TABLE 5.21 Uptake of Oxygen as PO_2/single daphnid/min and statistical significance of differences between the various concentrations of Cu^{++} Significance of difference in mortality of daphnia in respirometer and open specimen tubes

Treatment (mg Cu^{++}/l)	Oxygen Uptake by 1 daphnid/min	Sig. Diff.	Mortality of Daphnia Respirometer	Mortality of Daphnia Open Tubes
5.0	+0.0219			N.S.
		N.S.		
0.5	0.0252			N.S.
		S.D.		
0.25	+0.0201			N.S.
		S.D.		
0.125	0.0074			S.D.*
		N.S.		
0.0 (Control)	0.0046			--

N.S. - not significantly different
S.D. - significantly different
\+ - N.S.

*Higher mortality in open tubes

There was remarkably close agreement in the rate of oxygen uptake at 5.0, 0.5 and 0.25 mg Cu^{++}/l. Although analysis showed that the regression coefficient of 0.25 mg Cu^{++}/l was significantly lower than that of 0.5 mg Cu^{++}/l analysis also demonstrated that there was no significant difference between 0.25 and 5.0 mg Cu^{++}/l. There was a marked dose response at 0.125 mg Cu^{++}/l which showed a large and significant reduction in uptake compared with 0.25 mg Cu^{++}/l. There was no significant difference between oxygen consumption in 0.125 mg Cu^{++}/l and the control. It is of interest to observe that there was no significant difference in the mortality of daphnia in the respirometer and in the open specimen tubes when compared

within each concentration of 5.0, 0.5 or 0.25 mg Cu^{++}/l. There was significantly higher mortality in the open specimen tubes at 0.125 mg Cu^{++}/l compared with the respirometer suggesting no increase in copper toxicity was associated with diminishing levels of oxygen in the respirometer.

A consideration of the oxygen consumption by a single daphnid per minute at 5.0, 0.5 and 0.25 mg Cu^{++}/l shows it was 4 to 5 times greater than in the control and this leads to the tentative conclusion that such an accelerated rate of respiration would rapidly deplete the available energy and cause death of the organism. That this is not necessarily the case is seen from the statistically similar uptake rates of 5.0 and 0.5 mg Cu^{++}/l which had survival rates to the extinction of the total population of daphnia of 75 and 120 minutes. However, it is likely that the accelerated rate of respiration associated with the concentration of copper was a contributory factor in mortality. There is some support for this view in the results of an energetics study with ^{14}C labelled Chlorella vulgaris fed to D. magna (Gulati, 1973). Daphnia consumed 30 - 50 ug organic matter m.g. D.W./hr, and of this amount 18 - 30 ug mg D.W./hr was absorbed into the digestive tract and the remainder was ejected as unutilised or partly digested material. A metabolic study demonstrated that respiration accounted for 7 - 10 ug mg D.W./hr of the absorbed organic matter. The respiratory rate was not stated but assuming it was normal, a respiration rate elevated 2 or 3 times above normal, while generating an increased food intake, would drastically restrict the amount of energy available for other vital functions.

The respiratory movements in daphnia consist of synchronous sweeping movements of the appendages which beat fairly regularly

in approximately 140 to 200 beats per minute with occasional lapses lasting a few seconds. This rhythm must be controlled by the nervous centres and the peripheral muscular mechanism, and it is of interest to speculate on the action of copper as a toxin of the general ganglionic centres or as a specific neuromuscular poison. Sollman and Webb (1940) demonstrated that drugs which depress vertebrate respiration also depressed the respiration of D. magna but drugs which stimulated vertebrate respiration, e.g. nicotine, carbon dioxide, and strychnine depressed the respiration of daphnia. The respiratory action of daphnia appears to be a sensitive indicator of toxins and the observed responses to copper may be typical of heavy metals.

In experiments with the anaesthetics Procaine HCl and MS22 which were evaluated in daphnia as a prelude to facilitate the introduction of the nymphs into a Cartesian diver which was initially considered for the experiments reported in this chapter it was found that high doses caused convulsions characterized by continuous beating of the antennae which resulted in rapid circling and looping movements. These responses may be regarded as typical of toxins acting on the ganglionic centres. Such movements were not observed from copper intoxication and it is more likely that the action of copper is neuro-muscular and one of its effects is displayed on the musculature of the peripheral appendages which respond by beating more rapidly thereby promoting an increase in the rate of oxygen uptake.

Conclusion

It is concluded that 5.0, 0.5 and 0.25 mg Cu^{++}/l increased the rate of oxygen uptake by 4 to 5 times that observed in the control.

A dose response was observed between 0.125 mg Cu^{++}/l and 0.25 mg Cu^{++}/l and the maximum stimulus among the concentrations examined was probably at 0.25 mg Cu^{++}/l, this latter concentration representing the threshold level since no further increase in the rate of oxygen uptake was observed at 5.0 mg Cu^{++}/l. The rate of oxygen uptake at 0.125 mg Cu^{++}/l did not differ significantly from the non-copper control. A comparison of daphnia mortality within the different copper concentrations in the respirometer and in the open specimen tubes showed that there was no increase in copper toxicity associated with diminishing levels of oxygen in the respirometer.

Finally the increased respiratory rate as a measure of metabolic activity is regarded as an important factor contributing to the acute toxicity of copper in daphnia.

These conclusions must be qualified by the fact that the daphnia in the 0.25 mg Cu^{++}/l concentration weighed about half that in 0.5 and 0.125 mg Cu^{++}/l and there is a regrettable omission of the weights for the 5.0 mg Cu^{++}/l concentration and the untreated control. The lack of certainty about the species is not considered so important since it is unlikely that a change in species would materially influence the trend of the results.

Summary

1. The object of the study was to determine the influence of copper on the rate of oxygen uptake by daphnia.
2. A population of 30 daphnia was enclosed in a respirometer containing the test solution and oxygen uptake as PO_2(mm Hg) was recorded by a PO_2 electrode which measured changes in the oxygen tension in the liquid.
3. The test solutions were 5.0, 0.5, 0.25 and 0.125 mg Cu^{++}/l as $CuSO_4$, and a control consisting of the culture medium in which the test solutions were made up.
4. In order to determine if interaction of copper and diminishing oxygen tension occurred in the respirometer, control daphnia were placed in open specimen tubes containing the same solution and at the same ratio of daphnia to volume of liquid as in the respirometer. Observations on mortality in both systems were analysed in 't' tests for statistical significance.
5. The relationship between PO_2 values, exposure time and mortality of daphnia was determined by multiple regression or linear regression analysis. Covariance analysis was used to determine the significance of the difference between the regression coefficients.
6. There was no interaction of copper and diminishing oxygen tension in 5.0, 0.5 or 0.25 mg Cu^{++}/l in the respirometer suggesting no increase in copper toxicity associated with reduced PO_2 values. At 0.125 mg cu^{++}/l the mortality rate was significantly higher in the controls exposed to the air than in the respirometer, which validates the previous observation.

7. The rate of oxygen uptake in copper concentrations of 5.0, 0.5 and 0.25 mg Cu^{++}/l was 4 to 5 times higher than in the control and statistically significant. At a concentration of 0.125 mg Cu^{++}/l the rate of oxygen uptake did not differ significantly from the control. The plateau effect of copper on oxygen consumption occurs at 0.25 mg Cu^{+} /l.
8. It is probable that the increased metabolic rate caused by the accelerated respiratory rate contributed to the acute toxicity of copper in daphnia.

Fig. 8.1. Conc. of Cu^{++} = 5.0 mg/l.

Multiple regression of PO_2 (Y) on daphnia population (X_1) and Time (X_2)

$\hat{Y} = 115.8 - 0.344\ X_1 - 0.405\ X_2$

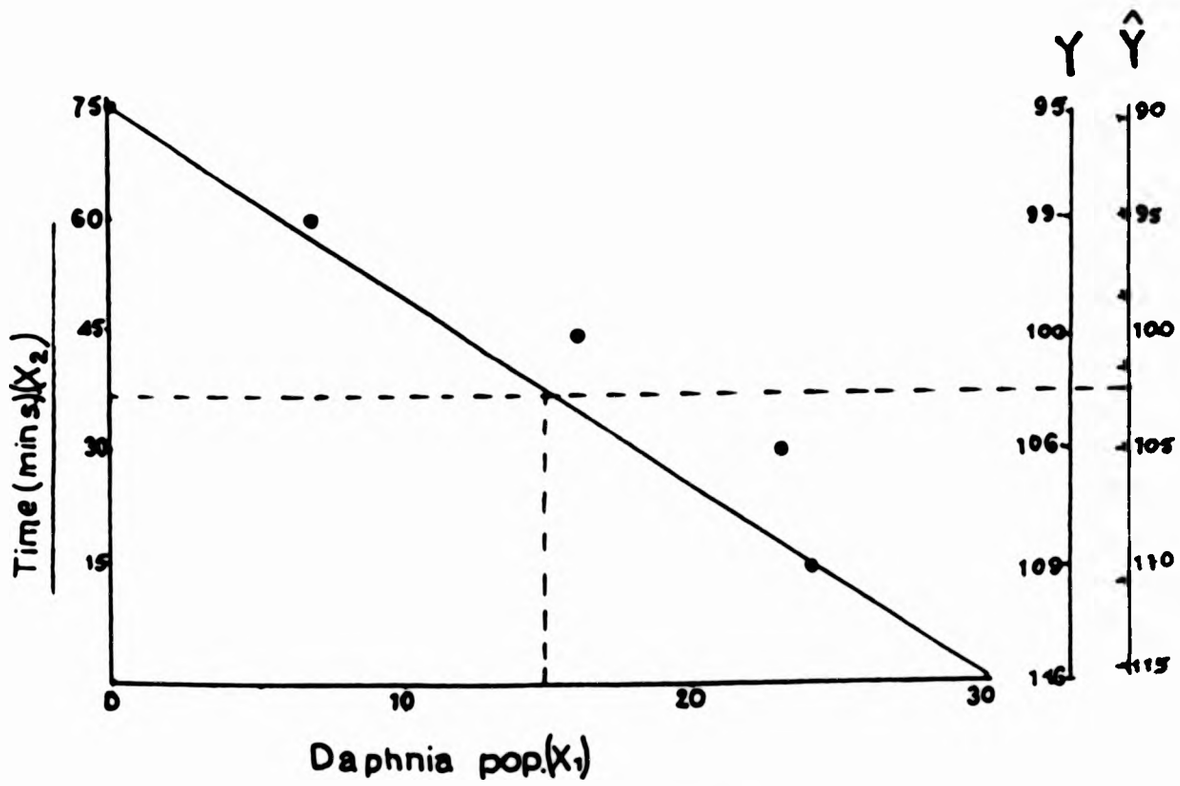


Fig. 5.2. Conc. of Cu^{++} = 0·5 mg/l.
Multiple regression of PO_2 (Y)
on Daphnia population (X_1)
and Time (X_2). Predicted value = $\hat{Y}$

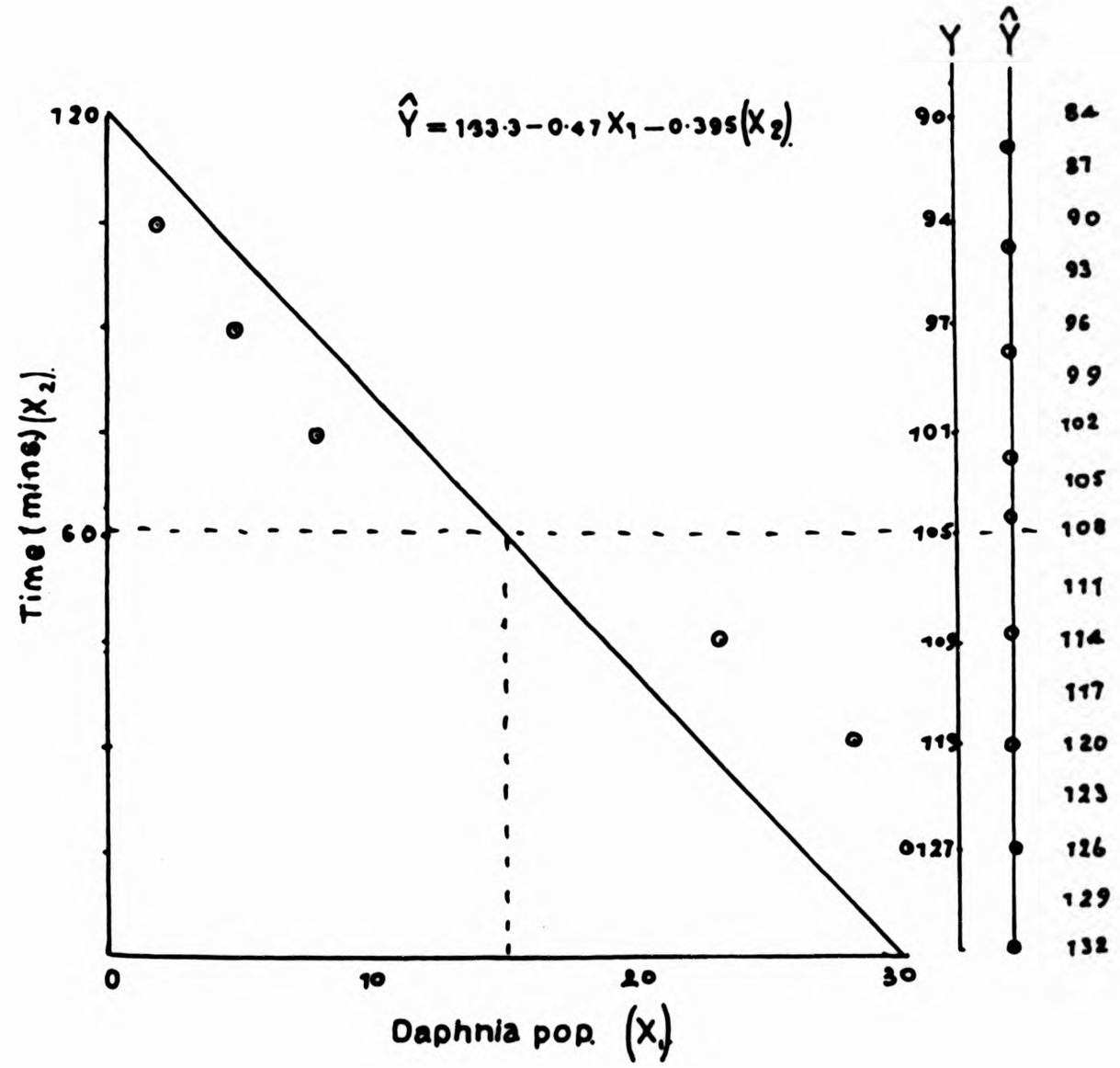


Fig. 5.3. Conc. of Cu^{++} - 0·25 mg./l.
Multiple regression of PO_2 (Y) on Daphnia population (X_1) and Time (X_2).

$$\hat{Y} = 154·9 - 0·19\ X_1 - 0·337\ X_2$$

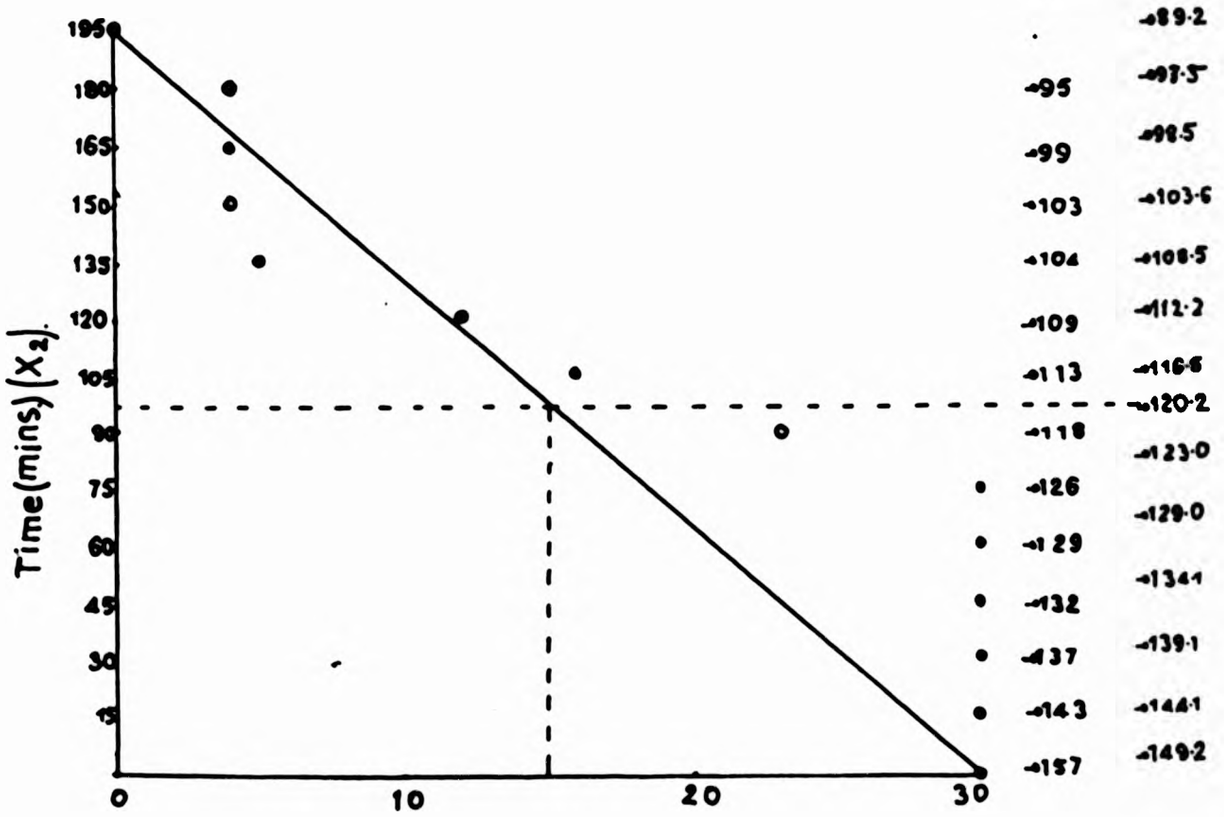
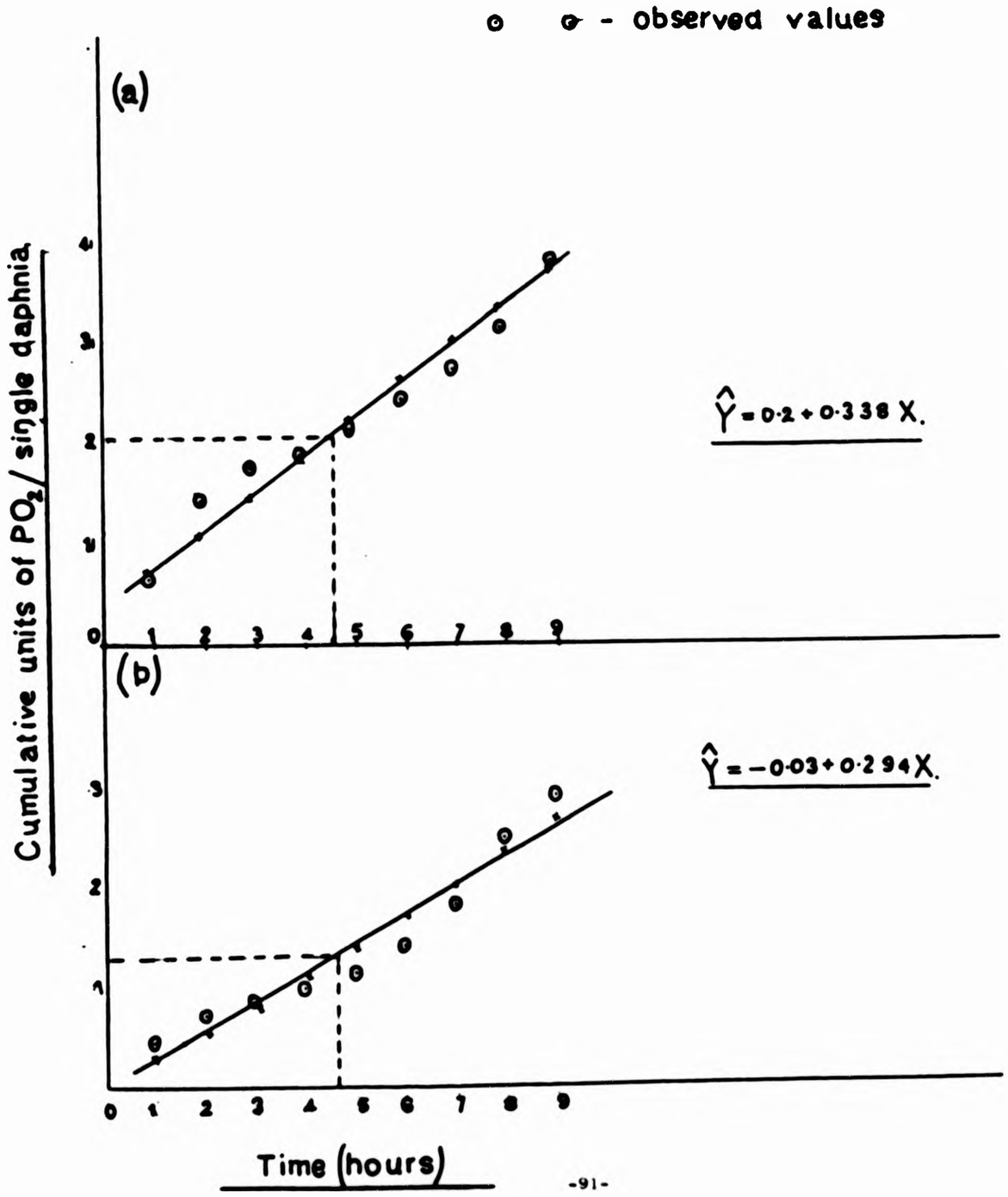


Fig. 54. Regression of Time (hours) on Cumulative units of PO_2/1 daphnia in (a) 0·125 mg. Cu"/l and (b) control.

x——x - fitted regression line

⊙ ⊙ - observed values



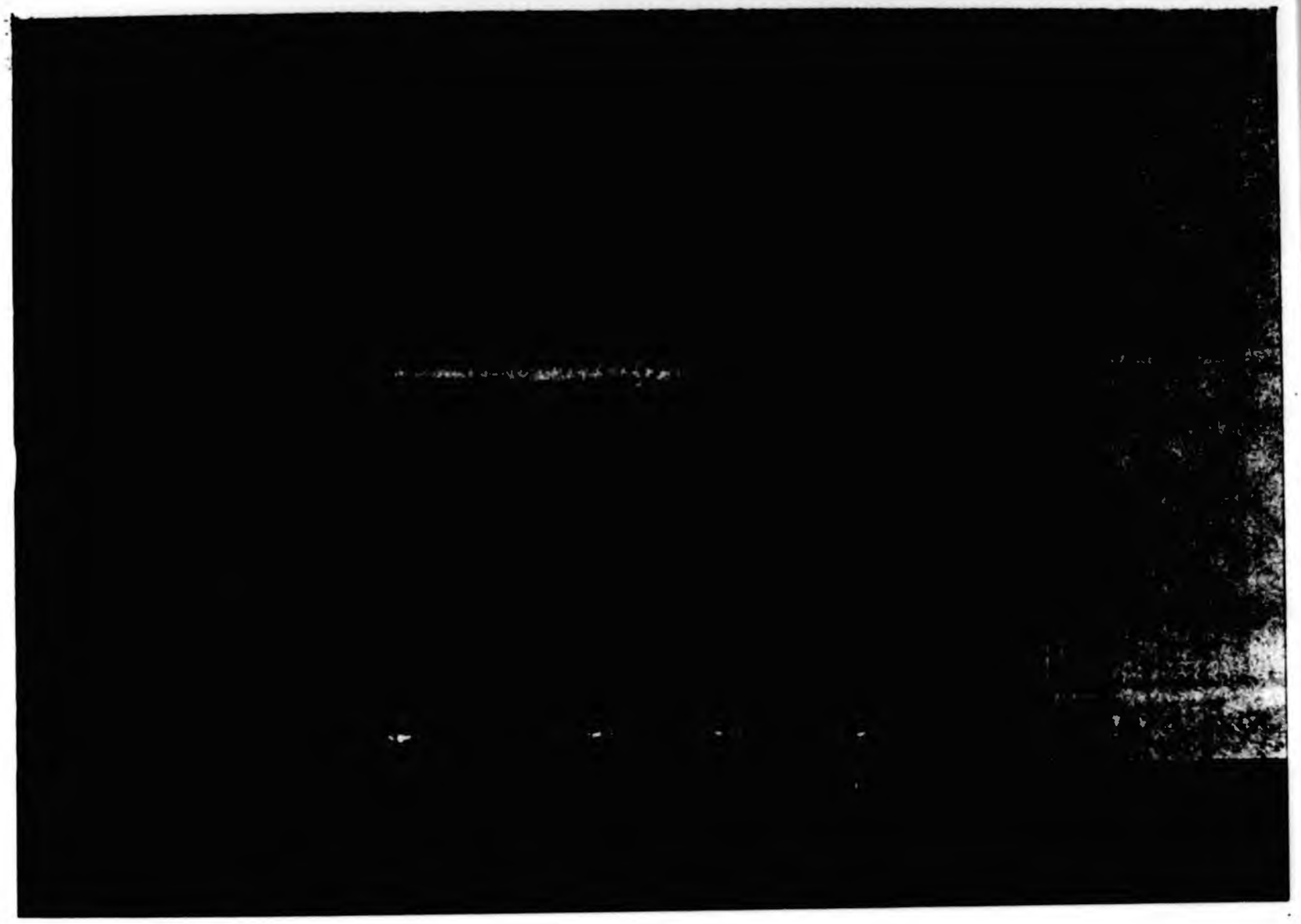


FIGURE 5.P.1

1. PO_2 electrode
2. Respirometer
3. Radiometer

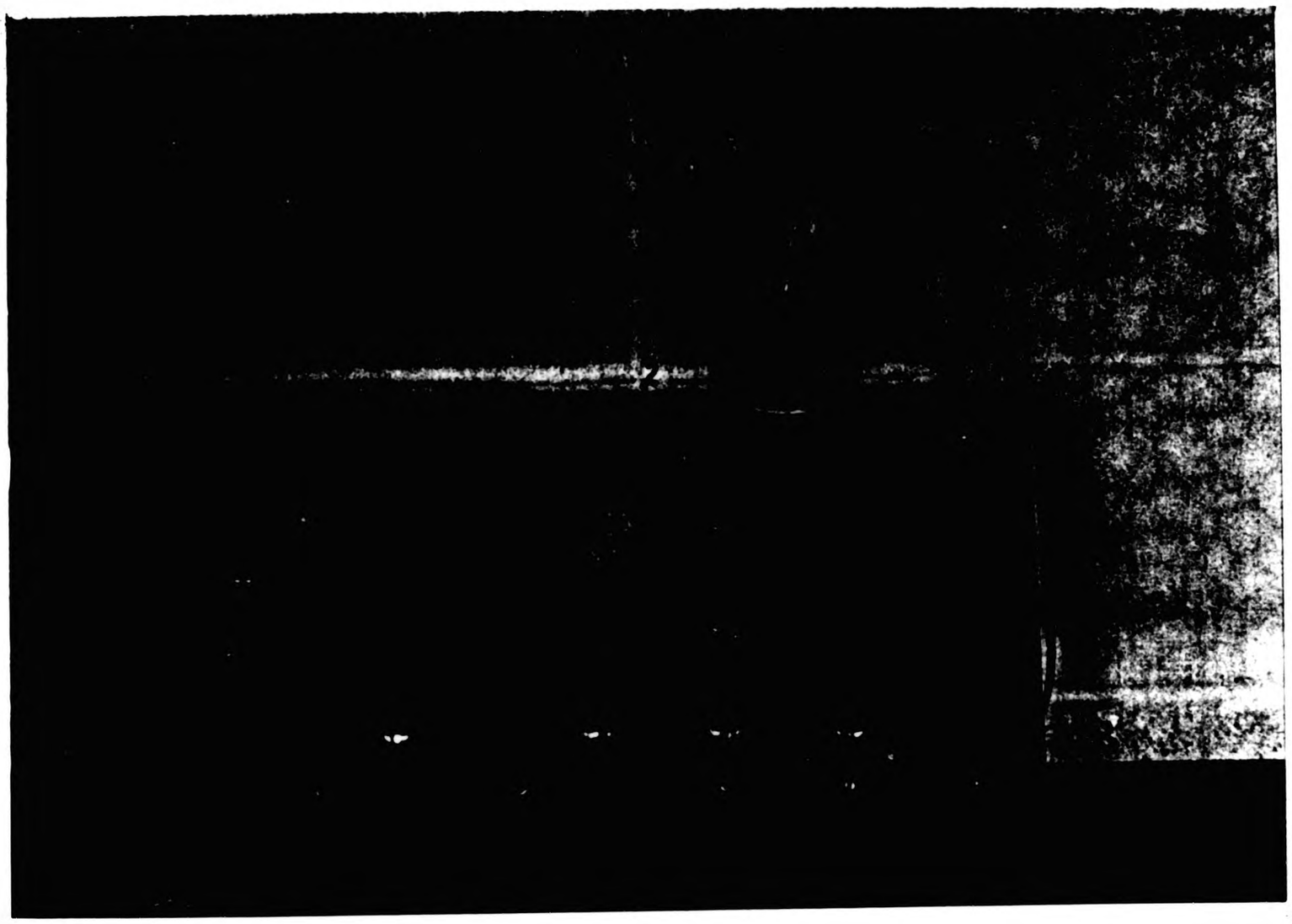


FIGURE 5.P.1

1. PO_2 electrode
2. Respirometer
3. Radiometer

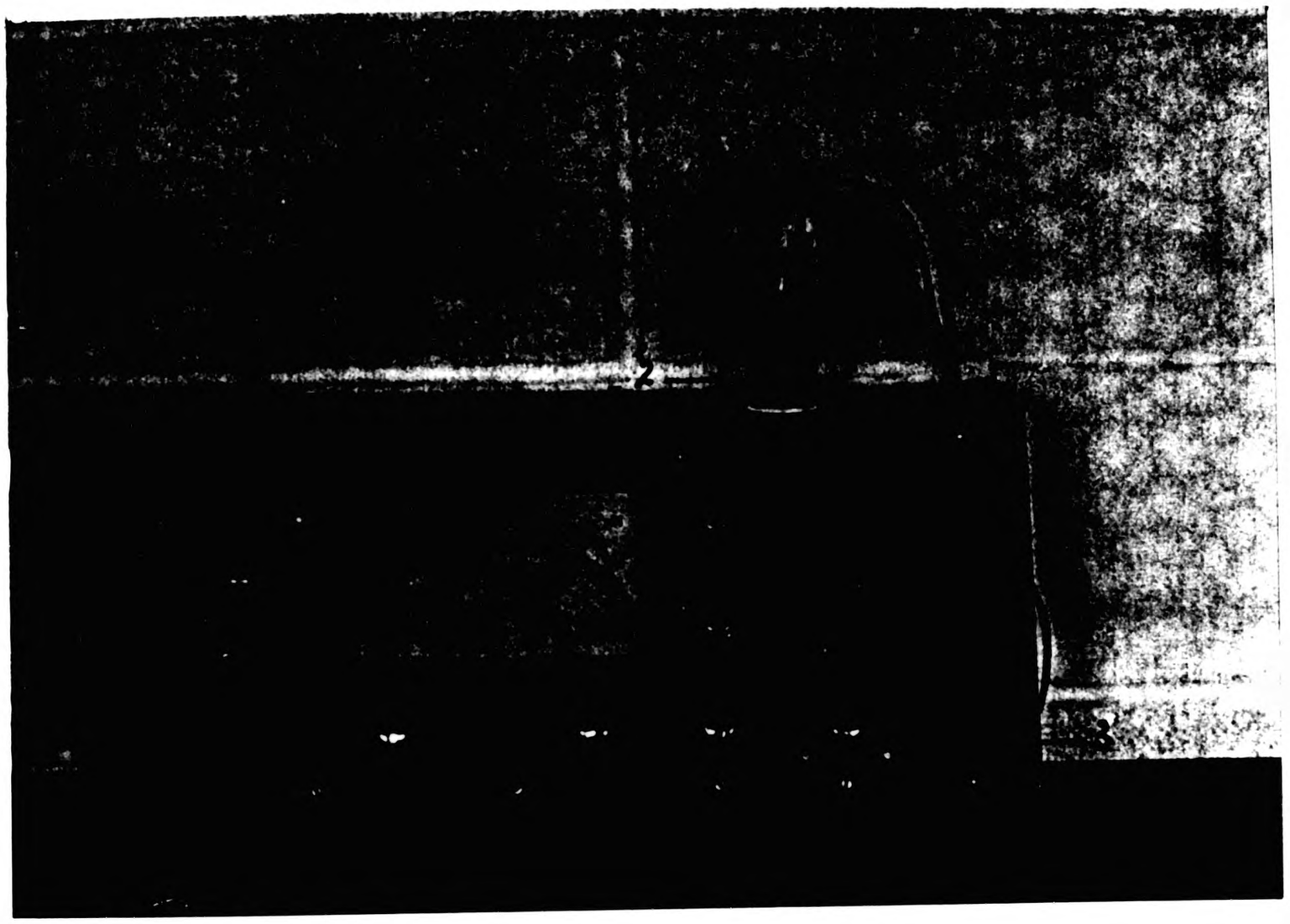


FIGURE 5.P.1

1. PO_2 electrode
2. Respirometer
3. Radiometer

CHAPTER 6

RADIOTRACER STUDIES TO DETERMINE UPTAKE OF $^{64}Cu^{++}$ IN *D. magna*

6.1 Introduction

Notable advances in instrumentation and techniques have been made over the last 20 years in the development of radioassay in a variety of disciplines including studies on the genesis or rock formation, medical physics, metabolism of natural and foreign substances in the body and in a large number of biological organisms. The universal appeal of radioassay continues to be the ultra high sensitivity of detection from electron probe analysis which combined with autoradiography permits a precise conclusion on metal pathways and metabolism.

6.1.1 Experimental Objectives

In view of the unresolved difficulties in the determination of trace amounts of copper in water and daphnia by the conventional methods of analysis reported in 3.1.3 it was decided to apply the more sensitive technique of radioassay in attempts to solve this problem and to identify the rate of uptake of labelled copper as a preliminary experiment in daphnia. The second objective was to determine the influence of NaCl or EDTA on the rate of uptake of labelled copper in daphnia. The third objective was to identify the location of labelled copper in various organs in daphnia in order to postulate hypotheses on pathways and sites of accumulation of the metal. Since the length of daphnia is about 2.5 mm and the organs are correspondingly small it was considered impractical to carry out radioactivity readings on individual organs. The alternative was to compare readings on entire animals with those in which specific organs had been dissected and removed and attribute the difference in the readings to the abstracted organs. The location of the radioactive metal would be demonstrated by serial sections

of whole body autoradiography.

6.1.2 Literature Survey

The literature search revealed an almost total lack of critical studies on the uptake of labelled metals by daphnia in the last 30 years. Potts and Fryer (1979) published an account on the effects of pH and salt content on sodium balance in *D. magna* They measured sodium influxes by placing daphnia in 5 or 10 ml of medium of known composition labelled with ^{22}Na. After intervals of ½ hour or 1 hour the daphnia were removed and washed by transfer through three beakers each containing about 200 ml of water of the same compcsition as the loading solution. The washing time was brief averaging abo..t 30 secs in each beaker. Radioactivity was measured in a scintillation counter with a sodium iodide well crystal. The authors showed that the effect of the concentration of external sodium on the rate of sodium uptake was similar to that between the rate of an enzymatic reaction and the concentration of the substrate and could be formalised by an equation similar to the Michal's-Menton equation:

$$f = f_{max} \left(\frac{c}{c + km}\right)$$

where:

f is the rate of uptake at concentration c,

f_{max} is the maximum rate of uptake when the transport system is saturated at high concentrations

k_m is a constant, a measure of the affinity of the carrier for sodium ions and equivalent to the concentration at which uptake was half the maximum.

By using the radioisotope ^{22}Na which emits gamma photons the radioactivity was measured directly in the entire daphnia thus eliminating the need for conversion of the sample to a form suitable for liquid scintillation counting of beta particles and gamma photons as in ^{24}Na.

6.1.3 Route of Entry of Copper in Daphnia

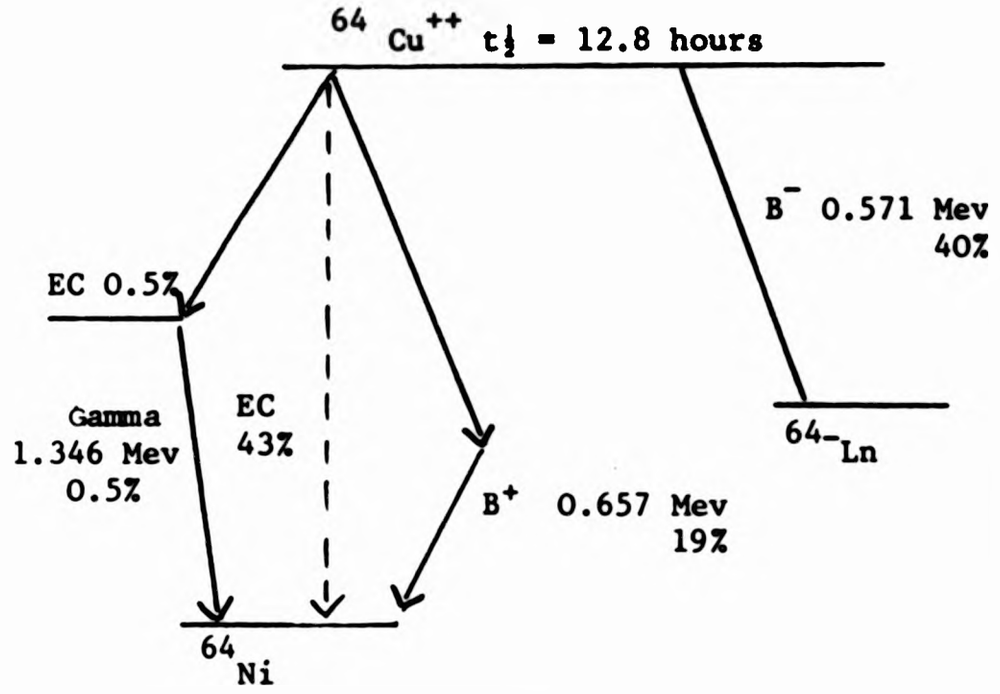
The radiotracer dissolved in the aqueous medium would be taken up into the body of daphnia principally through the anus (Potts and Fryer, 1979). In the lower part of the gut, the sphincter muscle performs an anti-peristaltic movement causing two main effects (1) the ejection of excess food and waste products through the anus, and (2) the intake of water through the anus. A proportion of the incoming water will be expelled along with the waste products but the major portion will travel upwards along the gut to the oesophagus and mandibles and en route will be absorbed through the gut to the haemocoel and thence to the various organs of the body including the ovary, egg sac, appendages, carapace and heart.

6.1.4 Radioisotopes of Copper

There are two principal radioisotopes of copper namely $^{64}Cu^{++}$ and $^{67}Cu^{++}$, the former having a half-life of 12.8 hours and the latter with a half-life of 68 hours. The preparation of $^{67}Cu^{++}$ is complex consisting of a two-stage reaction process and this isotope is not available from reactor centres in the U.K. Despite the disadvantages of a short life, the studies reported had to be performed with $^{64}Cu^{++}$.

6.1.5 Disintegration Scheme of $^{64}Cu^{++}$

The disintegration scheme of $^{64}Cu^{++}$ is illustrated below (after Faires, 1981).



The nuclear energy is expressed in terms of Milli electron volts. The electron volt is defined as the energy acquired by an electron in falling through a potential difference of one volt.

$$1ev = 1.602189 \times 10^{-19} \text{ joules (about } 3.83 \times 10^{-20} \text{ calories)}$$

In about 0.5% of the disintegrations a gamma photon of energy 1.346 Me v is emitted, and in 19% of the disintegrations a positron termed a beta^{+} particle is discharged yielding ^{64}Ni. In 40% of the disintegrations a fast negative electron called a beta^{-} particle of energy 0.571 Me v is emitted and the decay product is ^{64}Zn. Radioactive decay to the extent of 43.5% is caused by electron capture (EC) which occurs when one of the inner orbital electrons combines with a nuclear proton to form a neutron and in this event there is no emission of beta radiation.

The disintegration scheme can be used in a determination of the counting efficient of the scintillation counter - which only

records a proportion of the total number of disintegrations which occur. Using the micro-curie (u Ci) as the unit of activity, which is a disintegration rate of 3.7×10^4 disintegrations per second a calculation from the above scheme shows that:

> 0.027 uc Ci $^{64}Cu^{++}$ emits 590 beta particles (190 beta$^+$ and 400 beta$^-$ particles) and 5 gamma photons per second

The count from the same activity of $^{64}Cu^{++}$ in a scintillation counter can be calculated as a percentage of disintegrations per second to obtain a measure of the efficiency of the instrument.

6.1.6 Rate of Radioactive Decay

During an experiment with a radioactive isotope the atoms decay at a constant rate and in order to compare, for example, the rate of uptake through a series of time exposures it is necessary to equate the radioactivity readings to a fixed point in time which may be chosen arbitrarily although it is customary to correct the readings back to zero time, that is the time the isotope was withdrawn from the reactor. The extent of decay which has occurred at a given time can be calculated by applying the law of radioactive decay.

$$\lambda = \frac{-dN/dt}{N} \quad \text{or} \quad \frac{-dN/N}{dt}$$

where λ = decay constant ($-\frac{\text{No. of atoms decaying in unit time}}{\text{No. of atoms originally present}}$)

N = the number of atoms at time t

dN = the number of atoms which disintegrate in interval dt

By rearranging the above equation to:

$dN/N = -\lambda dt$ and integrating through the limit No N, yields

$\log_n (N/No) = -\lambda t$ or

$$N = N_o e^{-\lambda t}$$

For practical application the $\log_n (N/No) = -\lambda t$ is converted to a form using logarithms to the base 10:

$$2.303 \log_{10} N/No = -\lambda t$$

A plot of radioactivity against time reveals the exponential nature of radioactive decay and if plotted on semi-logarithmic paper the decay curve becomes a straight line with a slope equal to the value of $-(\lambda/2.303)$.

In equation $N = No e^{-\lambda t}$, by putting

$N = No/2$, it follows that $e^{-\lambda t} = \frac{1}{2}$ and

therefore $t = \log e\ 2/\lambda = 0.693/\lambda$

This value is the half life since it is the time taken for the activity to be reduced to half its original value.

The above equations have been taken from Faires (1981) and Wang and Willis (1965) and they are presented in order to show the methods used to determine the extent of radioactivity remaining in a radioisotope after a given period of time. The following example shows the percentage activity remaining in a sample of $^{64}Cu^{++}$, 7.5 hours after irradiation in the reactor. $^{64}Cu^{++}$ has a half life of 12.8 hours.

$$\text{Decay constant } \lambda = \frac{0.693}{12.8} = 0.054/\text{hr}$$

$$2.303 \text{ lot } \frac{No}{N} = \lambda t$$

$$\text{let } N = 100\%$$

$$2.303 \log \frac{100}{N} = 0.054 \times 7.5 = 0.41$$

$$\log \frac{100}{N} = \frac{0.41}{2.303} = 0.18$$

$$\log 100 - \log N = 0.18$$

$$\log N = \log 100 - 0.18$$

$$N = 66.0\%$$

At 7.5 hours 66.0% of the initial activity remains in the sample. This procedure has been used for decay correction in all the subsequent experiments.

From the exponential nature of the decay, after one half life the activity is reduced by one half, after two half lives the activity will be reduced by N/4 and after m half lives by $N/2^m$. In a nucleide with a half life of 12 hours the activity after one week will be $N/2^{14}$, that is it will be reduced by a factor of 16384. The exposure times in the experiments with $^{64}Cu^{++}$ were therefore restricted by the short half life of the radioisotope to a few hours.

6.1.7 Quenching

Having considered the counting efficiency of a scintillation counter (Section 6.1.6) it is now necessary to consider how scintillating fluids which are used in the transfer of energy from the sample to the recording mechanism of the instrument can influence the radioactivity count. The function of a scintillating

fluid is to provide a source of interacting atoms and to convert the energy arising from the interaction of emitted nuclear particles and these atoms into the ultimate production of photons with a wavelength in the visible or near u.v. region. The pathway of these photons may be partially impeded by the primary solvent of some scintillating fluids or by solvents used in the preparation of the radioactive sample forming a coloured complex with the scintillation fluid. This complex would absorb a proportion of the light energy, and there would be a consequent reduction in the activity count recorded, together with a compression of the spectrum to lower pulse heights thus changing the ratio of the counts in the recording channels of the instrument. These effects are described by the inelegant word 'quenching' - the verb is derived from the anglo-saxon 'cwencan' meaning to extinguish or dwindle. In order to reduce the amount of quenching which may occur it is obviously desirable to aim for clear, homogeneous, solution in the scintillating vial but in any event it is necessary to calculate the degree of quenching which occurred in order to obtain comparable counts of emitted radioactivity.

Correction factors for quenching can be obtained by counting variously quenched samples containing known amounts of radioactivity in two recording channels of the counter and constructing a standard curve relating counting efficiency to the ratio of the net counts in the two channels. Unknown samples can then be counted in these two channels and the count adjusted by reference to the standard curve (Packard Tech. Bull., 1965). An alternative method consists of using the External Standard radioisotope of the counter. The gamma rays from this source penetrate the scintillation vial containing the sample and produce free electrons in the scintillator by Compton

scattering. The energy spectrum of the Compton electrons does not exactly match that of the beta radiation but the gamma source is chosen to give the best possible match. This was the method used to determine the degree of quenching which occurred in the first experiment. The gamma source was ^{226}Ra and the counts from this radioisotope were recorded directly in two channels of the counter and compared with the counts obtained when ^{226}Ra was positioned immediately behind samples of daphnia which had been exposed to three concentrations of $^{64}Cu^{++}$.

6.1.8 Calculation of Quantity of $^{64}Cu^{++}$ Required

The amount of radioactivity initially present in a radionuclide is referred to as the specific activity which is a measure of the proportion of radioactive atoms present in the total number of atoms of an irradiated sample. For example, if the specific activity of $^{64}Cu^{++}$ is 1 uCi/100 ug Cu^{++} this means that a solution of 0.1 mg/l of copper will contain 1 u Ci $^{64}Cu^{++}$. The quantity of radioactive material required is calculated on the initial specific activity and several other factors as indicated in the following example (after Wang and Willis, 1965). If the background counting rate is 100 counts per minute and if the final 5 ml water samples and 5 ml daphnia samples are five times background, then a final sample activity of 500 c.p.m. is required. If it is assumed that the uptake of $^{64}Cu^{++}$ by daphnia and the loss of the isotope by adsorption on the glass of the bioassay vessel will deplete the solution by a factor of 10, an initial activity of 5000 c.p.m./5 ml will be required. If the detection efficiency of the counter is 25% the counting rate to aim for is 20,000 c.p.m. per 5 ml solution. The activity to be introduced per litre is

$$\frac{20{,}000}{5 \text{ ml sample}} \times 1000 \text{ ml} = 4 \times 10^6 \text{ c.p.m.}$$

The decay rate of 1 u Ci of radium is 2.220×10^6 d.p.m.

Thus 4×10^6 c.p.m. = 1.8 u Ci of $^{64}Cu^{++}$ per litre

If the specific activity of the isotope is 10 mc/g Cu^{++} the weight of irradiated sample required is

$$(\frac{1.8}{10^4} \times 10^3) = 0.18 \text{ mg } ^{64}Cu^{++} + Cu$$

6.1.9 Statistics of Radioactive Counts

Radioactive decay occurs at random, that is the decay of a given atom is an entirely random event which can be demonstrated by making repeated measurements of activity on a nuclide with a long half life. The results will be distributed over a range with the majority of observations near the centre of the range and if plotted they form a normal distribution curve. With a large number of observations an accurate error can be determined but since in radioassay it is common to make only one or two activity determinations per sample the accuracy of the variance is accordingly reduced, and the standard deviation of a single count of magnitude n is customarily taken as $\sqrt{n}$ and the relative standard deviation as $100/\sqrt{n}$. Thus the larger the count, the smaller the standard deviation becomes.

Since the radioactivity count is made up of the activity from the sample and the background the standard deviation is a combination of the error of each source. Inclusive of the background error the standard deviation of a sample is:

$$S.D. = \sqrt{\frac{rg}{tg} + \frac{rb}{tb}}$$

where rg = gross count of sample

rb = background count

tg = time interval of gross count

tb = time interval of background count

Wang and Willis (1965)

6.1.10 Experimental Methods

Conversion of daphnia to a form suitable for counting the activity of $^{64}Cu^{++}$ in a liquid scintillation counter

Since daphnia consists of a hard chitinous carapace and soft body tissue, solvents which were found satisfactory by earlier workers Badman and Brown (1961) and Schwebel et al. (1951) in the disintegration of similar structures were tested, namely 1M hyamine hydroxide and N - N - dimethyl formamide. The results of the solvents at various exposure times are shown in Table 6.1.

TABLE 6.1 Effect of hyamine hydroxide and N - N - dimethyl formamide on the disintegration of daphnia

Compound	Exposure Time	Temperature (^{o}C)	Effect on Daphnia	
1 M hyamine hydroxide	1 hr	20	Soft tissue dissolved	No effect on carapace
	2 hrs	"	" "	" " "
	3 hrs	"	" "	" " "
	4 hrs	"	" "	" " "
	1 hr	100	" "	Slight "
	2 hrs	"	" "	" " "
	3 hrs	"	" "	" " "
	4 hrs	"	" "	" " "
N-N-dimethyl formamide	1 hr	100	Soft tissue dissolved	Carapace broken up
	2 hrs	"	" "	" "
	3 hrs	"	" "	" "
	4 hrs	"	" "	" "
	5 hrs	"	" "	" "

Hyamine hydroxide (methyl bensethonium hydroxide)at a concentration of 2 ml/5 daphnia and at a temperature of 20^{o}C dissolved the body tissue of daphnia but had no effect on the carapace irrespective of the exposure time tested. At 100^{o}C there was a slight effect on the carapace, that is, one or two large pieces had become detached from the main shell. This temperature caused a colour change in the hyamine hydroxide from transparent to brown and finally black which would in any event render the compound unsuitable for use in a scintillation counter.

At a concentration of 2 ml/5 daphnia, N - N - dimethyl formamide at 100^{o}C dissolved the soft tissue and fractured the shells of the carapace into small pieces but did not cause total disintegration even at the longer exposure intervals. It was observed that the

main effect on the carapace occurred within the first few minutes and to reduce the possible quenching effect from the solvent a reduced concentration of 0.5 ml/5 daphnia was tested at 100°C. This dissolved the soft tissue and broke up the carapace into small pieces which were further reduced by stirring manually with a glass rod. Some pieces of the broken fragments still remained and in an attempt to cause further disintegration the samples were exposed to sonication in the water bath of an ultrasonics generator. After sonication for 3 mins the samples had totally disintegrated and the suspension was visually clear.

The choice of scintillating fluid to use was made by eliminating those with a known high quenching factor or rejecting others which were not compatible with N - N - dimethyl formamide. The most suitable scintillating fluid was a proprietory compcund, Insta-gel which was developed by the Packard Instrument Co. for the counting of radionuclides in aqueous or biological samples. The manufacturers claimed a high counting efficiency with several inorganic nuclides in Insta-gel. For example ^{60}Co which has a similar mode of decay to $^{64}Cu^{++}$ was counted to an efficiency of 80% and ^{36}Cl to 100%.

The addition of a sonicated sample of 5 daphnia/0.5 ml N - N - dimethyl formamide to the recommended volume of 10 ml Instagel did not cause precipitation or a change in the colour of Insta-gel. In order to obtain uniform distribution of the samples in Insta-gel, the vials were also sonicated for 3 mins.

To permit a comparison of the radioactivity counts in the loading solutions with those from daphnia, each 1 ml sample of loading solution was introduced into 10 ml Insta-gel + 0.5 ml

N - N - dimethyl formamide.

6.1.11 Expression of Activity in Loading Solutions

Since the specific activity of the irradiated copper samples varied from one experiment to another, the variation arising from the length of time the metal was in the reactor, the loading solutions if expressed in terms of mg/l would not convey the amount of activity initially present in each solution. Hence the loading solutions are expressed in the tables of results as microcuries (u Ci) of $^{64}Cu^{++}$ per millilitre.

6.1.12 Liquid Scintillation Counter

The first two experiments were carried out on a Tricarb 2000 spectrometer at the Scottish Universities Research and Reactor Centre, East Kilbride, Glasgow. The instrument was a 2-channel model adapted for manual or automatic operation and included ^{226}Ra as the external Standard isotope in the automatic system. Two further experiments were carried out in a Tricarb Spectrometer in the Biology Department, University of Stirling.

6.2 To Determine the Rate of Uptake of $^{64}Cu^{++}$ in Daphnia

The objective of this preliminary experiment was to determine the rate of uptake of radioactive copper in daphnia exposed to three concentrations of the isotope in deionized water. Two exposure intervals were proposed namely 1 and 3 hours.

6.2.1 Experimental Methods

D. magna

Adults of daphnia were obtained by screening a mass culture through a 850 micron mesh sieve. In order to reduce the

presence of food in the gut as a source for accumulation of copper during the subsequent exposures to the radioactive solutions, the daphnia were unfed during a 24-hour period prior to the experiment.

6.2.2 Preparation of $^{64}Cu^{++}$

The radioactive copper was prepared by neutron bombardment of 1.0168 mg of copper foil which was placed for six hours in the reactor at the Research and Reactor Centre, East Kilbride, Glasgow. This generated a specific activity of 380 u Ci $^{64}Cu^{++}$/1.0168 mg copper. The radioactive copper was dissolved by the addition of a few drops of concentrated HNO_3 and after a few minutes diluting with 100 ml double deionized water and heating to boiling point for 10 minutes to complete the solution and to drive off fumes of NO_3. The solution was filtered through a Whatman No. 2 filter paper and made up to 1 litre in double deionized water. Dilutions were prepared to give initial concentrations of 3.80^{-1} u Ci $^{64}Cu^{++}$/ml, 3.80^{-2} u Ci $^{64}Cu^{++}$/ml and 3.80^{-3} u Ci $^{64}Cu^{++}$/ml, which were then dispensed each at a volume of 20 ml into 40-ml capacity glass specimen tubes. Five daphnia were added to each specimen tube for exposure periods of 1 and 3 hours. In the lowest concentration of the 1 hour exposure interval two of the five daphnia died, although none died in the higher concentrations. None of the daphnia in the projected 3 hour exposure survived beyond 1½ hours.

6.2.3 Preparation of Samples for Radiation Counting

After exposure for one hour the daphnia were transferred from the loading solutions to watch glasses containing double deionized water. The daphnia were rinsed for 10 seconds in three changes of water, using separate watch glasses for each concentration.

The superficial water on the daphnia was removed by rotating the individuals in blotting paper. To remove the water and traces of superficial copper held within the carapace, the shells of each animal were opened with a pair of fine tweezers and the individuals set briskly on filter paper to release a few drops of solution. Each sample of daphnia was weighed and prepared for scintillation counting as described earlier (6.1.11).

The pulse spectrum of $^{64}Cu^{++}$ had earlier been determined and based on this, Channel A - B was set at 10 - 500 and C - D at 10 - 250 and gain at 2%.

6.2.4 Results

In order to obtain data on quenching, radioactivity counts were taken of the external standard ^{226}Ra alone, and in the presence of the stock solution, and the suspensions of daphnia which had been exposed for one hour to three concentrations of labelled copper. Each count was taken for 1 minute and the counts for both Channels, and the Channels Ratio are shown in Table 6.2.

TABLE 6.2 Radioactivity in counts per minute (c.p.m.) of External Standard ^{226}Ra, ^{226}Ra + Stock solution, ^{226}Ra + Daphnia exposed to three concentrations of $^{64}Cu^{++}$, to determine degree of quenching

Sample	Channel A - B	Channel C - D	Channels Ratio $\frac{A - B}{C - D}$
^{226}Ra	948006	672212	1.410
^{226}Ra	956252	672212	1.410
^{226}Ra + 0.1 ml 3.80^{-1} u Ci Cu^{++}/ml	913292	638392	1.430
" " " "	926427	649006	1.427
^{226}Ra + daphnia exposed to:			
3.80^{-1} u Ci $^{64}Cu^{++}$/l	659483	480030	1.373
3.80^{-2} u Ci $^{64}Cu^{++}$/l	870626	605332	1.438
3.80^{-3} u Ci $^{64}Cu^{++}$/l	889050	618260	1.437

The duplicate counts of ^{226}Ra were of the same order and the Channels Ratios were identical. Compared with ^{226}Ra, there was a slight reduction in the counts from ^{226}Ra + 0.1 ml of 3.80^{-1} u Ci $^{64}Cu^{++}$/ml. There was a marked reduction in the count from ^{226}Ra + Daphnia exposed to 3.80^{-1} u Ci $^{64}Cu^{++}$ compared with the count from ^{226}Ra, and this corresponded with a reduced Channels Ratio value, both results indicating that quenching had occurred in this sample. There were smaller reductions in the counts from $^{2}{}^{26}Ra$ + 3.80^{-2} or 3.80^{-3} u Ci $^{64}Cu^{++}$/ml but no decrease in the Channels Ratio values were observed compared with ^{226}Ra alone which indicated that only a small degree of quenching occurred.

In order to construct a quench correction curve for application to the subsequent results in Table 6.4, the counts from the ^{226}Ra External Standard have been taken to represent 100% efficiency and the counts from ^{226}Ra + the stock solution, and those from concentrations of $^{64}Cu^{++}$ have been expressed as a percentage of the ^{226}Ra External Standard. The percentage efficiency of each treatment is shown in Table 6.3 and illustrated with respect to the Daphnia samples, in Figure 6.1.

TABLE 6.3 Degree of Quenching in the $^{64}Cu^{++}$ Stock Solution and in the Daphnia samples exposed to three concentrations of $^{64}Cu^{++}$, expressed as a percentage of ^{226}Ra External Standard

Sample	Mean c.p.m. Channel A - B	% Efficiency
^{226}Ra	952129	100
^{226}Ra + 0.1 ml of 3.8^{-1} u Ci $^{64}Cu^{++}$/ml	919860	96.6
^{226}Ra + daphnia exposed to:		
3.80^{-1} u Ci $^{64}Cu^{++}$/ml	659483	69.3
3.80^{-2} u Ci $^{64}Cu^{++}$/ml	870626	91.4
3.80^{-3} u Ci $^{64}Cu^{++}$/ml	889050	93.3

Taking ^{226}Ra as showing 100% efficiency, there was a high counting efficiency in the sample of stock solution and in the daphnia samples exposed to the lower levels of radioactive copper while there was a marked reduction in the highest daphnia concentration. Since there was an indication of a dose response in the two lower daphnia levels and since the stock solution had a high efficiency

TABLE 6.4 Radioactivity Counts of Stock Solution and *D. magna* exposed for 1 hour to three concentrations of $^{64}Cu^{++}$

Sample	Time in Counter (mins)	Counts per Channel A - B	Counts per Channel C - D	Channels Ratio $\frac{A-B}{C-D}$	c.p.m. in Channel A - B minus background	c.p.m. after quenching adjustment	Decay correction to zero time: % Initial Activity remaining at time of count	Decay correction to zero time: c.p.m. at zero time
Stock Solution								
3.80^{-1} u Ci $^{64}Cu^{++}$/0.1ml	1.0	8381±92	5350±74	1.567	8314	8606	71%	12121
Daphnia exposed to:								
3.80^{-1} u Ci ^{64}Cu/ml	20	11182±25	10117±24	1.105	492	807	68%	1187
3.80^{-2} u Ci ^{64}Cu/ml	20	6346±20	4477±17	1.417	250	305	70%	436
3.80^{-3} u Ci ^{64}Cu/ml	50	9739±16	6786±14	1.435	128	132	60%	220
Background	20	1345						

the marked reduction in the daphnia sample 3.80^{-1} u Ci $^{64}Cu^{++}$/ml may be associated with a secretion of reaction products which may have reduced the wavelength energy of the radium photons.

6.2.5 Radioactivity Counts of Samples

The radioactivity counts of the stock solution and in the daphnia exposed to the three concentrations of loading solution for 1 hour are shown in Table 6.4 together with the adjustments for quenching and decay correction to zero time.

The quenching already demonstrated in daphnia exposed to 3.80^{-1} u Ci $^{64}Cu^{++}$/ml is readily apparent by the lower Channels Ratio value 1.105 compared with values of 1.417 and 1.435 for the lower exposure rates. By application of the quench correction curve in Figure 6.1 the counts were adjusted for quenching, and by using the decay correction equations the counts were converted to zero time. The uptake per single daphnia is shown in Table 6.5.

TABLE 6.5 Final calculated uptake based on data in Tables 1 - 4

Sample	No. of Daphnia per treatment	c.p.m. per single daphnia	Uptake (ug) of $^{64}Cu^{++}$/hour/ single daphnia
Daphnia exposed to:			
3.80^{-1} u Ci $^{64}Cu^{++}$/ml	5	237	0.72^{-3}
3.80^{-2} u Ci $^{64}Cu^{++}$/ml	5	87	0.27^{-3}
3.80^{-3} u Ci $^{64}Cu^{++}$/ml	3	73	0.23^{-3}

The calculation of uptake has been made on a single daphnia in preference to uptake per unit weight of daphnia. The reason for this is that uptake was considered to occur in the gut and in

the soft body tissues and not in the appendages or carapace and since the latter organs constitute the major proportion of the weight of daphnia and since weight variations (from 0.60 mg to 1.14 mg in this experiment) could unduly distort uptake if expressed in terms of body weight, uptake per individual seemed more appropriate, a form of expression also used by other workers (Potts and Fryer, ref. cit.).

The calculation of the uptake of $^{64}Cu^{++}$ at 1 hour is shown below:

3.80^{-1} u Ci $^{64}Cu^{++}$/0.1 ml = 12121 counts per minute

12121 = 1.0168×10^{7} g or 0.10168 ug $^{64}Cu^{++}$ + Cu

Specific activity of sample = 38%

i.e. % of labelled copper in total sample = 38%

38% of 0.01068 = 0.039 ug $^{64}Cu^{++}$

12121 = 0.039 ug $^{64}Cu^{++}$

c.p.m. from single daphnia for highest exposure rate = 237

$$237 = \frac{0.039}{1} \times \frac{237}{12121} = 0.72^{-3}\ ^{64}Cu^{++}$$

The trace of radioactive copper which accumulated after 1 hour's exposure to a solution of 3.8^{-1} u Ci $^{64}Cu^{++}$/ml must limit the validity of a single figure in isolation. The fact that this value is about three times the level of radioactive copper accumulated from a loading solution of 3.8^{-2} u Ci $^{64}Cu^{++}$/ml is not unexpected, in contrast to the result with the lowest loading solution which is very close to that from the middle concentration. This similarity suggests that the technique to remove superficial radioactive copper was inadequate. The absence of data from the projected 3 hour exposure due to premature mortality of the daphnia greatly limits

the interpretation of the results from the 1 hour exposure. Several possible reasons for the mortality may be considered including metal contamination, nitrate from the original use of HNO_3 or the action of the decay products, zinc and nickel. These reasons were discarded since if involved the daphnia in the highest concentration would show the greatest mortality which was not the case. In earlier experiments with non-labelled copper it was found that daphnia survival was much less certain, even for brief periods of a few hours, in deionized water than in the standard culture medium, although it was unexpected that mortality would occur within 1½ hours.

When it was observed that mortality occurred in the radioactive solutions in deionized water, samples of daphnia were exposed to the same level of radioactivity in the culture medium. It was found that swimming behaviour was normal and there was no mortality for at least 2 hours. Since the projected exposure times were to be increased to 6 hours in subsequent experiments it was decided to use the culture medium in preference to deionized water as the loading solution.

It is, however, of interest to speculate on the possible reason for the mortality of daphnia in deionized water. Determinations with D_2O (Krogh, 1939) revealed the remarkably high water exchange of 80% in less than 2 minutes in daphnia and since the haemolymph is hyperosmotic (Fritsche, 1916, quoted by Holm-Jensen, 1948) a considerable inflow of water must occur. This inflow must be balanced by a corresponding secretion of urine containing salts. The loss or deprivation of a minimum level of salts required for normal body functions possibly accounted for the observed mortality in daphnia in the deionized water.

In order to eliminate any possibility of metal contamination from the use of metallic copper, subsequent experiments were carried out with anydrous $CuSO_4$ using a sample from the laboratory grade salt which was used successfully in the toxicity and respiration studies already reported.

The third change in procedure concerned the need to use a more sophisticated method to remove the superficial traces of labelled copper held within the carapace which was possibly the source of contamination accounting for the absence of a doze response in the uptake of labelled copper from the lower concentrations examined. The modified procedure was based on centrifuging the daphnia in order to cause the shells of the carapace to open and thereby lose the traces of labelled copper and water droplets by centrifugal force (Holm-Jensen, 1948).

6.3 To Determine the Influence of NaCl or EDTA on the Uptake of $^{64}Cu^{++}$ in Daphnia

6.3.1 Introduction

Toxicity experiments by Holm-Jensen (ref. cit.) demonstrated that the addition of NaCl to the culture medium for daphnia led to a considerable reduction in the toxicity of copper. The chelating agent EDTA has frequently been used to reduce metal toxicity and its effects are well documented. The objective of this experiment was to determine the influence of 2 mM NaCl or 0.009 mM EDTA on the uptake of labelled copper in daphnia.

If labelled copper accumulated to the same extent irrespective of the presence of 2 mM NaCl in the loading solution the protective action of NaCl might be attributed to an increase in the membrane transport of ions, the inhibition of which was postulated as the

In order to eliminate any possibility of metal contamination from the use of metallic copper, subsequent experiments were carried out with anydrous $CuSO_4$ using a sample from the laboratory grade salt which was used successfully in the toxicity and respiration studies already reported.

The third change in procedure concerned the need to use a more sophisticated method to remove the superficial traces of labelled copper held within the carapace which was possibly the source of contamination accounting for the absence of a doze response in the uptake of labelled copper from the lower concentrations examined. The modified procedure was based on centrifuging the daphnia in order to cause the shells of the carapace to open and thereby lose the traces of labelled copper and water droplets by centrifugal force (Holm-Jensen, 1948).

6.3 To Determine the Influence of NaCl or EDTA on the Uptake of $^{64}Cu^{++}$ in Daphnia

6.3.1 Introduction

Toxicity experiments by Holm-Jensen (ref. cit.) demonstrated that the addition of NaCl to the culture medium for daphnia led to a considerable reduction in the toxicity of copper. The chelating agent EDTA has frequently been used to reduce metal toxicity and its effects are well documented. The objective of this experiment was to determine the influence of 2 mM NaCl or 0.009 mM EDTA on the uptake of labelled copper in daphnia.

If labelled copper accumulated to the same extent irrespective of the presence of 2 mM NaCl in the loading solution the protective action of NaCl might be attributed to an increase in the membrane transport of ions, the inhibition of which was postulated as the

reason for copper toxicity in pigeon brain (R. A. Peeters, 1968). If copper accumulated at a lower level in the presence of NaCl it might be postulated that an exchange of Na^{+} for Cu^{++} occurred in the membrane, or that the majority of sites for metal accumulation were preferentially saturated by sodium.

If EDTA did not influence the uptake of labelled copper it might be postulated that a tightly bound Cu - EDTA complex was held at a toxicologically insensitive site in the daphnia. If EDTA caused a reduced level of accumulation of radioactive copper, this might have been due to the trapping of the molecular complex in the filter-feeding structure of daphnia.

6.3.2 Experimental Design

The design of the experiment consisted of three concentrations of radioactive copper in each of theee exposure intervals of 1, 3 and 6 hours; each concentration was to be dispensed in the following solution:

(a) Culture medium (c.m.)

(b) " " + 2 mM NaCl

(c) " " + 0.009 mM EDTA

The loading solutions would be sampled at the start of the exposure period, and the daphnia removed at the end of each exposure interval, prepared as described earlier, and radioactivity counts recorded from the initial loading solutions and daphnia.

A considerable amount of preparatory work was required in this experiment and I acknowledge the assistance of a technician from the Biology Department, University of Stirling. He assisted in the preparation of the dilutions, weighed the daphnia and helped to prepare the samples for radioactivity counts.

6.3.3 Procedure

The radioisotope was prepared by neutron bombardment of 5.026 anydrous $CuSO_4$ at the Reactor centre. The sample had a specific activity of 385 u Ci/1 mg Cu^{++}. A stock solution of 20 ppm was made up from the 5.026 mg irradiated sample and dilutions added to the above media (a), (b) and (c), each of which had initial concentrations of 1.95^{-1} u Ci $^{64}Cu^{++}$/ml, 0.98^{-2} u Ci $^{64}Cu^{++}$/ml and 0.98^{-3} u Ci $^{64}Cu^{++}$/ml. These concentrations were dispensed into 40 ml capacity specimen tubes and five daphnia were added to each tube. A sample of 1 ml of each loading solution was taken for subsequent radioactivity counts.

After an exposure period of 1 hour the daphnia were removed from the dosing solutions and rinsed for about 5 seconds in two changes of deionized water. Each sample was then enclosed in a piece of fine muslin and centrifuged at 2000 r.p.m. for 2 minutes. This procedure removed all obvious traces of solution from the daphnia which, when withdrawn from the centrifuge, were brittle and dehydrated. The samples were weighed and prepared for scintillation counting as described. The same procedure was used for all samples which contained viable daphnia at the end of the exposure periods.

6.3.4 Results

After a period of 1½ hours, the five daphnia in the highest concentration of radioactive copper in the culture medium, 1.95^{-1} u Ci $^{64}Cu^{++}$/ml and in the culture medium + NaCl or EDTA were either dead or moribund and, therefore, no data are available for the 3 and 6 hour exposures of this treatment. No explanation is advanced for this unexpected mortality which was unaccountable in terms of copper toxicity or zinc or nickel toxicity. The daphnia in

the lower concentrations survived the 3 and 6 hour exposures, apart from some incidental mortality.

When all the samples of the holding solutions and daphnia were set in position in the scintillation counter it was found that the automatic system in the instrument failed to operate and the staff at the Centre were unable to correct the fault. The alternative system, manually operated, was fully functional but it was a time-consuming operation and by the time the lowest concentration of radioisotope in the loading solution of the culture medium was recorded the counts were scarcely above background. This meant that it was not possible to evaluate the data from the lowest concentrations of $^{64}Cu^{++}$ in the NaCl or EDTA treatments. This loss together with the restricted comparisons available at the highest concentration due to daphnia mortality invalidated the experiment and no results are presented. However, during the course of the experiments three main conclusions emerged to facilitate the technique and design of future experiments.

Firstly, the centrifuge speed of 2000 r.p.m. may have been too high and some $^{64}Cu^{++}$ may have been lost from within the body of daphnia. An improved system to remove the superficial traces of $^{64}Cu^{++}$ from daphnia would probably be achieved by rinsing the daphnia in a relatively high concentration, for example 10 mg Cu^{++}/l, of non-labelled $CuSO_4$. The ions of the non-labelled solution would first reach a state of equilibrium with the labelled ions and then displace them from the superficial sites on the body of daphnia and within the carapace. Secondly, there was an indication that the concentration of $^{64}Cu^{++}$ in daphnia decreased in time suggesting the presence of a detoxification system which led to

the excretion of $^{64}Cu^{++}$. Finally, instead of making comparisons between two compounds it would be more profitable to use an increased number of doses, and preferably replication, with a single compound. Thus the subsequent experiment was designed to study the influence of NaCl on the uptake of $^{64}Cu^{++}$ in daphnia.

6.4 To Determine the Influence of 2 mM NaCl on the Uptake of $^{64}Cu^{++}$ in Daphnia

This experiment consisted of various concentrations of $^{64}Cu^{++}$ in the culture medium containing no NaCl and in the culture medium plus 2 mM NaCl. The projected exposure times were 2, 4 and 8 hours. The labelled isotope was obtained from the Reactor Centre, East Kilbride, Glasgow and the experiment was carried out in the Liquid Scintillation Spectrometer, in the Department of Biology, University of Stirling.

At the end of the exposure periods 45 samples of the dosing solutions and daphnia were placed in the counter which was set to give 2 x 10 min readings of each sample. The sample included 1 ml of the culture medium and 1 ml of the culture medium + 2 mM NaCl for background counts. When the print-out was examined at the end of the experiment it was found that the background levels were abnormally high and about twice that found in the daphnia samples. This was rather unfortunate since it meant that all the results from the experiments had to be discarded. During previous tests with the instrument in calibration and quenching determinations, and in subsequent studies, background was always within the expected range. The culture solutions referred to above were identical to those used earlier at East Kilbride Reactor Centre and subsequently

in the Department of Biology, University of Stirling and in each case background was normal.

The day following the observation on background, it was found that background had declined by one-fifth of the original value but it was still well above normal. The only explanation that can be advanced for the high background is that it may have occurred when the high activity stock solution was adjacent to the sample vessels containing the culture solutions. In error both sets of vessels were placed together in the fume cupboard and the gamma rays from the stock solution may have penetrated through the glass of the vessels containing the culture solutions.

6.5 To Determine the Rate of Uptake and Rate of Loss of $^{64}Cu^{++}$ in Daphnia

This experiment was carried out in an attempt to acquire data on the uptake and excretion of radioactive copper in D. magna. In order to reduce the possibility of incidental mortality which occurred in daphnia in previous experiments, a fresh stock of D. magna was obtained from the Inveresk Research Centre, Mussleburgh, Edinburgh.

6.5.1 Procedure

Five daphnia were placed in solutions of radioactive $^{64}Cu^{++}$ as $CuSO_4$ and samples of the dosing solution were taken immediately before exposure started and at the end of the exposure interval of 6 and 9 hours. The samples of daphnia were removed at the end of the exposure periods, immersed for a few seconds in a solution of 10 mg/l $CuSO_4$, rinsed in deionized water, centrifuged at 500 r.p.m. for 2 minutes, and prepared for scintillation counting as described earlier.

Before the start of the experiment the liquid scintillation counter was checked and found to function normally. The background level was satisfactorily low (28 counts per minute). When all the samples were prepared for placing in the counter, it was found that another individual's sample had been earlier inserted in such a manner as to immobilize the reject mechanism of the instrument. Repeated attempts to correct the fault failed. It was unfortunate that this incident occurred at midnight on a Friday night. The The scintillation counter in the Department of Biochemistry was not available for use, nor was the instrument at the Scottish Universities Research and Reactor Centre, East Kilbride, which had closed down for the week-end. Since the radioisotope had been

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collected from the Reactor Centre at 9 a.m. on Friday the activity twelve hours later had diminished to one half life, and by the time the samples were ultimately counted the activity had declined to 1% of the initial value. Consequently only tentative conclusions can be reached from the results which are shown in Table 6.6.

TABLE 6.6 Radioactivity in Counts per Minute of $^{64}Cu^{++}$ in dosing solutions and in D. magna exposed for intervals of 6 and 9 hours

Conc. of $^{64}Cu^{++}$ (uCi $^{64}Cu^{++}$/ml) Dosing Solution	Exposure Interval (Hours)	c.p.m. minus background	% Initial Activity left	c.p.m. corrected to time zero	Mean Gain or Loss c.p.m.
0.80^{-1}	0	105	1.20	6432	
0.40^{-1}	0	72	1.19	4447	
0.20^{-1}	0	19	1.18	1184	
				Mean 4021	
0.80^{-1}	6	75	1.17	4712	
0.40^{-1}	6	42	1.15	2684	1233 loss
0.20^{-1}	6	15	1.14	967	
				Mean 2788	
0.80^{-1}	9	75	1.07	5152	
0.40^{-1}	9	36	1.05	2520	146 gain
0.20^{-1}	9	16	1.04	1130	
				Mean 2934	
Uptake in Daphnia from exposure in:					
0.80^{-1}	6	9	1.22	543	
0.40^{-1}	6	17	1.24	1007	
0.20^{-1}	6	0	--	--	
				Mean 517	
0.80^{-1}	9	7	1.10	467	181
0.40^{-1}	9	8	1.09	540	LOSS
0.20^{-1}	9	0	--	--	
				Mean 336	
Background		28			

After the exposure interval of 6 hours, the mean loss of radioactive copper in the dosing solutions compared with the mean pre-exposure count was 1233 counts per minute which is partly accounted for by a mean uptake in daphnia of 517 counts per minute. After 9 hours exposure, the mean count from the dosing solutions represented a gain of 146 counts per minute over the mean value from the 6-hour count. This suggests at least no further uptake of radioactive copper occurred in daphnia in the 3-hour interval between 6 and 9 hours.

In the 3-hour period between 6 and 9 hours there was a mean loss of 181 counts per minute in daphnia which indicated that daphnia had excreted the radioactive copper which was detectable in the dosing solution.

No radioactive copper was found in the daphnia exposed to the lowest concentration of dosing solution, 0.20^{-1} u Ci $^{64}Cu^{++}$/ml, but it will be observed that in the dosing solution a loss of 217 counts per minute occurred at 6 hours compared with the pre-exposure level, and a gain of 163 counts per minute at 9 hours and these changes may reflect the uptake and loss of radioactive copper in the daphnia.

Despite the low level of radioactivity in the samples, the results can be broadly interpreted to indicate a dynamic relationship between the levels of radioactive copper in the dosing solutions and in daphnia, and the possible operation of a detoxification system in daphnia which reduces the body concentration of the metal which is excreted.

6.5.2 Discussion and Conclusion

The absence of any recent studies on the uptake of radioactive copper in daphnia has meant that new techniques have had to be initially devised and evaluated throughout the course of the experiments. This applied with particular reference to the removal of superficial radioactive copper from the numerous surfaces in daphnia, including exterior cavities in the carapace shells, spines on the appendages and in the reservoir within the shells of the carapace. Rinsing the daphnia through several transfers of deionized water followed by spinning in a centrifuge removed a proportion of the superficial radioactive copper but the process had to be carefully controlled to minimise the loss of radioactive copper in accumulating organs and avoid the destruction of any vital body segments. In the final experiment reported, the technique of immersing the daphnia in a solution of non-labelled $CuSO_4$ in order to cause displacement of the superficial labelled copper which was then removed by rinsing appeared to be successful. This was followed by spinning in a centrifuge for 2 minutes at 500 r.p.m.

The techniques developed to convert daphnia into a form suitable for radioactivity counting were successful and yielded a homogeneous suspension which was compatible with the scintillating fluid, Insta-gel. The combined action of boiling the daphnia for 5 minutes in N - N - dimethyl formamide and sonication for 3 minutes caused complete disintegration of the daphnia.

Thus the entire methodology for radioactivity studies with daphnia is now available in a form which can be rapidly and effectively reproduced with a minimum of laboratory equipment. The methods are probably applicable to a number of organisms and

structures, and in view of the rapid operational speed they are particularly appropriate in experiments with short-lived radionuclides.

When the difficulties in the preparation of daphnia had been resolved, in theory the counting process should have proceeded routinely but mechanical faults in the scintillation counters, or in one experiment excessively high background limited the full exploitation of radioassay to generate conclusive results or to complete the projected programme of experiments. Time and finance inhibited the repetition of the faulted experiments.

The results indicated that the uptake of radioactive copper which occurred in daphnia during the initial exposures was followed by excretion of the radioisotope into the loading solution. This type of response is not uncommon and was reported over a quarter of a century ago by Borroughs et al. (1957) in several species of fish as exemplified in tuna fish which excreted 98% of ^{89}Sr in 24 hours. The excretion of radioactive copper suggests that the metal is not firmly bound to proteins in the first instance, and the daphnia possesses or develops a detoxification system to expel a proportion of the metal after a threshold level of intake is reached.

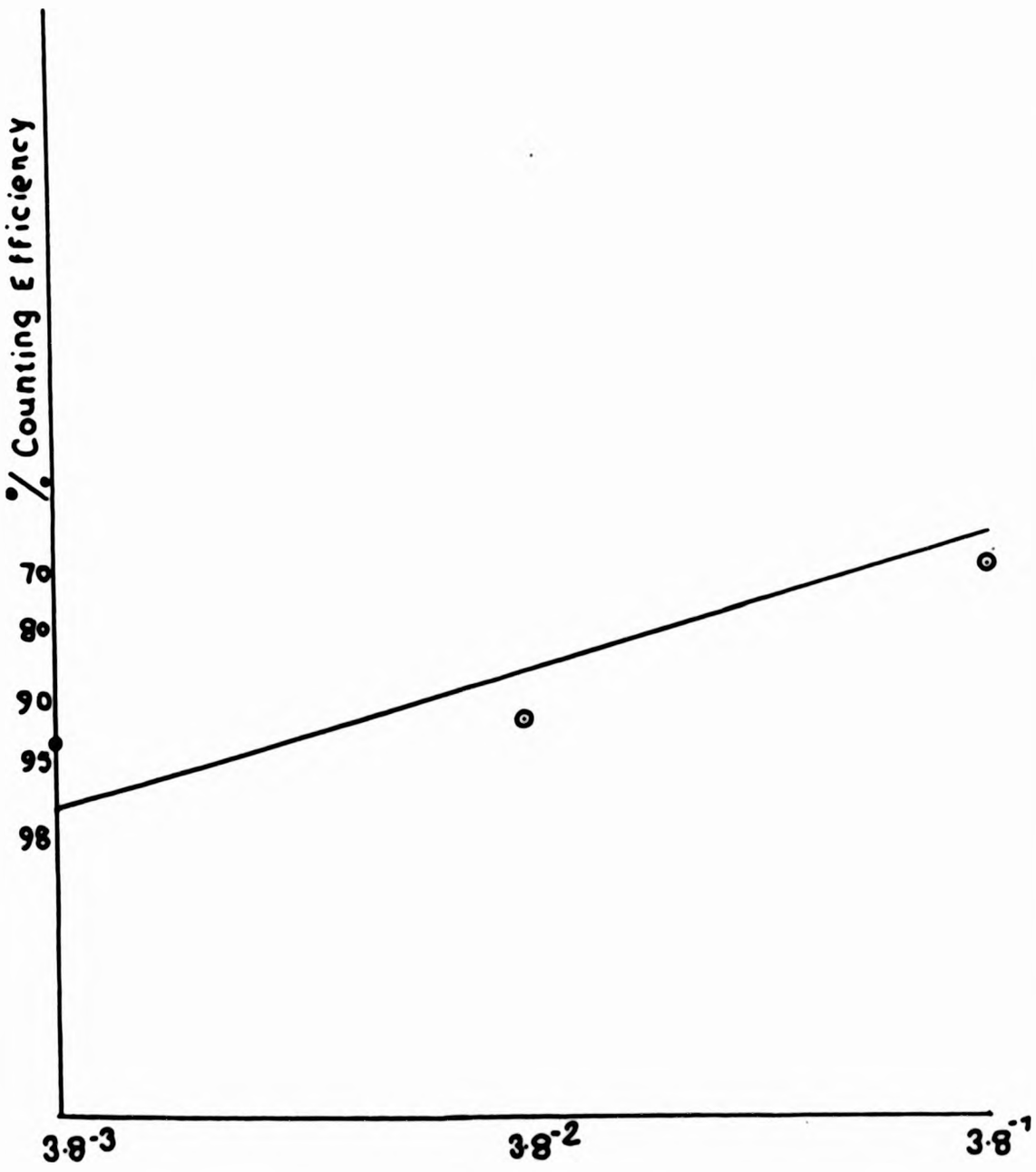
Summary

1. This chapter describes the techniques devised to determine the uptake of radioactive copper as $^{64}Cu^{++}$ by daphnia.
2. The disintegration scheme of $^{64}Cu^{++}$ is illustrated and described together with the equations for decay correction and the techniques to detect and correct quenching.
3. After exposure of daphnia to the dosing solutions, the removal of superficial radioactive copper was carried out by rinsing the samples of deionized water followed by spinning briefly in a centrifuge. This technique was improved by first immersing the daphnia in a solution of 10 mg/l non-labelled $CuSO_4$ followed by rinsing and spinning for 2 mins in a centrifuge at 500 r.p.m.
4. The conversion of daphnia into a form suitable for counting in a liquid scintillation counter was achieved by disintegration of the samples by boiling in N - N - dimethyl formamide followed by a 3-minute exposure to sonication in the water bath of an ultrasonic generator. This produced a homogeneous suspension which was compatible with the scintillating agent, Insta-gel.
5. After 1 hour's exposure to a concentration of 3.80^{-1} u Ci $^{64}Cu^{++}$/ml in a medium of double deionized water, radioactive copper accumulated to 0.72^{-3} ug $^{64}Cu^{++}$ per single daphnid, while dose concentrations of 3.80^{-2} u Ci $^{64}Cu^{++}$/ml or 3.80^{-3} u Ci $^{64}Cu^{++}$/ml showed accumulations of 0.27^{-3} and 0.23^{-3} ug $^{64}Cu^{++}$ respectively per single daphnid.
6. There was an indication of a dynamic relationship between the levels of radioactive copper in the dosing medium and in the uptake and loss of radioactive copper in daphnia.

However this could not be adequately validated since instrumental failure delayed the recording of activity counts to a stage where only 1% of the initial activity remained.

7. The results of one experiment had to be discarded due to excessively high background counts, and another experiment was lost by failure of the automatic counting system. Instrumental failure also reduced the validity of the results in the final experiment. These difficulties have meant a great reduction in the amount of data acquired and an awareness that interpretation of the studies must be necessarily limited by the absence of data which otherwise could be used in the presentation of influx and efflux curves to illustrate the presumed uptake and loss of radioactive copper in daphnia.

Fig. 6.1 Quench Correction Curve.



6.5. (a) Uptake of ^{64}Cu in D. Magna

6.5.1. (a) Introduction

A further experiment has been carried out to determine the rate of uptake of ^{64}Cu in D. magna which was exposed to 5 concentrations of irradiated copper for intervals up to 49.5 hours. The levels of radioactivity were recorded as direct emissions of gamma rays. The Daphnia for the experiment were provided by the Environmental Science Department Of The Huntingdon Research Centre, Huntingdon, Cambs., and the irradiated copper was prepared by The Liverpool and Manchester Universities Nuclear Reactor Centre, Risley, Warrington, which also made available the laboratory facilities for the experiment.

6.5.2. (a) Procedure

Mature specimens of Daphnia weighing on average 1.1mg per individual were disinfected by immersion for 90 minutes in a culture solution containing 100 units/ml. penicillin and 100 µg./ml. streptomycin, a technique reffered to by Hall (1982). The culture solution was identical to that used in the experiments on respiration (5.1.2.).
The irradiated copper was prepared by neutron bombardment of $CuSO_4$ to give a specific activity of 225.7 µ Ci/mg Cu^{++}. It was calculated that this level of activity would be adequate for observations throughout the exposure periods which were 5.5, 16.5 and 49.5 hours.
Dilutions were prepared to give concentrations of total copper of 66, 33, 16.5, 8.25 and 4.125 ppb, and were dispensed into specimen tubes of 20ml capacity. Each tube contained 18ml solution. Five Daphnia were transferred to each tube by Pasteur pipette. There were 5 tubes, one for each concentration, deployed for each of the 3 exposure periods and as precaution against loss of any treatment a duplicate series of 5 tubes was included. The pH of the culture medium was 7.3.

6.5.2. (a) Procedure Cont'd

At the end of the exposure period each specimen tube plus Daphnia was inverted into a plastic ring 1.5cm deep and 2.5cm in diameter with a muslin sieve at its base. The neck of the specimen tube fitted precisely into the plastic holder so that the tube could be inverted rapidly to discharge the Daphnia onto the sieve. A rapid inversion was essential to ensure that all the Daphnia were transferred simultaneously, otherwise some specimens adhered to sides of the tubes. In order to remove the superficial radioactive copper from the Daphnia, a spray of water was generously applied to the specimens on the sieve and they were then transferred to a stable solution of 10 ppm $CuSO_4$ in order to remove the irradiated copper held within the carapace (6.3.4.).

The activity in samples of the dosing solution and in Daphnia was measured in a sodium azide well linked to a Canberra Series 40 Multi-Channel Analyser for Nuclear Analysis, and the samples were exposed for either 500 or 1000 seconds. Background readings were taken at six intervals during the experiment and applied as appropriate to the radioactivity recorded in the samples. The readings were corrected for radioactivity decay, and the levels of radioactive copper in Daphnia and in the solutions were calculated from the specific activity of the isotope and the activity recorded in the samples. The radioactivity at 5.5 and 16.5 hours was recorded on 5 Daphnia at each concentration. At 49.5 hours some mortality occured, including 3 dead at 66 ppb and at 8.25 ppb.

The results are expressed in terms of radioactive copper accumulated per 1mg Daphnia and they are shown in Table 6.7. (a).

6.5.3. (a) Results

Concentration of ^{64}Cu in Daphnia (Ng. ^{64}Cu / 1 mg. Daphnia) at exposure intervals of 5.5, 16.5 and 49.5 hours.

Table 6.7. (a)

Conc. of ^{64}Cu in dosing sol. (Ng. ^{64}Cu /ml.)	Exposure Intervals (Hours) 5.5	16.5	49.5
14.9	0.99	3.75	10.10
8.0	0.67	1.73	6.59
4.0	0.38	0.86	4.55
1.5	0.23	0.83	1.29
0.96	0.19	0.48	0.98
Mean 5.87	0.49	1.53	4.70

Within each concentration of dosing solution there was a fairly uniform accumulation of ^{64}Cu as the exposure time increased from 5.5 hours to 16.5 hours and from 16.5 to 49.5 hours. The mean accumulation values showed a three-fold increase occured between the two intervals. There was also a reasonably regular dose response at each interval.

An analysis of covariance and a determination of the significance of the regression lines for the concentration of ^{64}Cu at 5.5 and 16.5 hours is shown in Table 6.8. (a).

Table 6.8. (a) **Comparison of Residual Variances and Slopes of Regression Lines relating concentration of ^{64}Cu in Dosing Solutions and uptake in Daphnia at 5.5 and 16.5 hours.**

Treatment Time of exposure (hours)	d.f.	$\sum x^2$	$\sum xy$	$\sum y^2$	Devs. from Regr. d.f.	Devs. from Regr. s.s.	Devs. from Regr. m.s.	F
5.5	4	132.68	7.71	0.45	3	0.01	0.0003	
16.5	4	132.68	29.94	7.01	3	0.25	0.08	
					6	0.26	0.04	
Pooled	8	265.36	37.65	7.46	7	2.12	0.30	
			Difference between slopes		1	1.86	1.86	xx 46.5

The value of F for 1 and 6 d.f. is 13.74 at the 0.01 probability level and since the observed value was 46.5 the slopes of the regression lines are highly significantly different. Thus uptake of ^{64}Cu at 16.5 hours was highly significantly greater than at 5.5 hours. A similar analysis at 49.5 hours showed that uptake at this interval was highly significantly greater than at 16.5 hours. The values suggest that uptake was approximately linear and this is illustrated in Figure 6.2. (a) in which the concentration factors of ^{64}Cu in Daphnia have been plotted against time of exposure. The concentration factors represent the concentration of irradiated copper which occured in Daphnia calculated from the dose in solution and since the former value is expressed as wt./mg. and the dose as wt./ml. the fraction is multiplied by 1000. Thus for example the concentration factor from a dose solution of 14.9 ng ^{64}Cu/ml. which gave a value of 0.99 ng ^{64}Cu/1mg. Daphnia after 5.5 hours is $\frac{1000 \times 0.99}{1 \quad 14.9} = 66.$ With the exception of the 49.5 hour values which were low from doses of 1.5 and 0.96 ng. ^{64}Cu/ml. the figure shows a reasonably linear response for the remaining treatments. The lower concentrations of ^{64}Cu in the dosing solutions showed a relatively higher degree of accumulation in Daphnia than occured with the higher doses particularly at 5.5 and 16.5 hour exposure intervals.

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6.8. (a) Cont'd

The relative increase in accumulation may be an illustration of the response of an organism to a trace amount of an element essential for growth. Uptake may have been influenced by electro-chemical exchanges at the surface of the cell membrane with an increased ionic density reducing the rate of passage through the membrane.

The regression coefficients have been calculated for the three time intervals and they show for an exposure of 1 hour to 1 ng ^{64}Cu/ml. the average accumulation of ^{64}Cu/mg Daphnia was 0.06 ng ^{64}Cu during the 5.5 hour exposure, 0.23 ng ^{64}Cu during the 16.5 hour exposure and 0.65 ng ^{64}Cu during the 49.5 hour exposure; the difference between the average weights of uptake was statistically significant.

6.5.4. (a) Summary

1) An experiment has been carried out to determine the effect of concentrations of copper in dosing solutions upon the uptake of copper by D. magna.

2) Mature specimens of D. magna were disinfected by immersion in a solution of penicillin and streptomycin and exposed for intervals of 5.5, 16.5 and 49.5 hours to 5 concentrations of copper represented by 14.9, 8.0, 4.0, 1.5 and 0.96 ng ^{64}Cu/ml. as the irradiated portion of the metal which had a specific activity of 225.7 μg ^{64}Cu/mg.

3) Gamma radioactivity was recorded in a sodium azide well linked to a Multi-Channel Analyser for Nuclear Analysis.

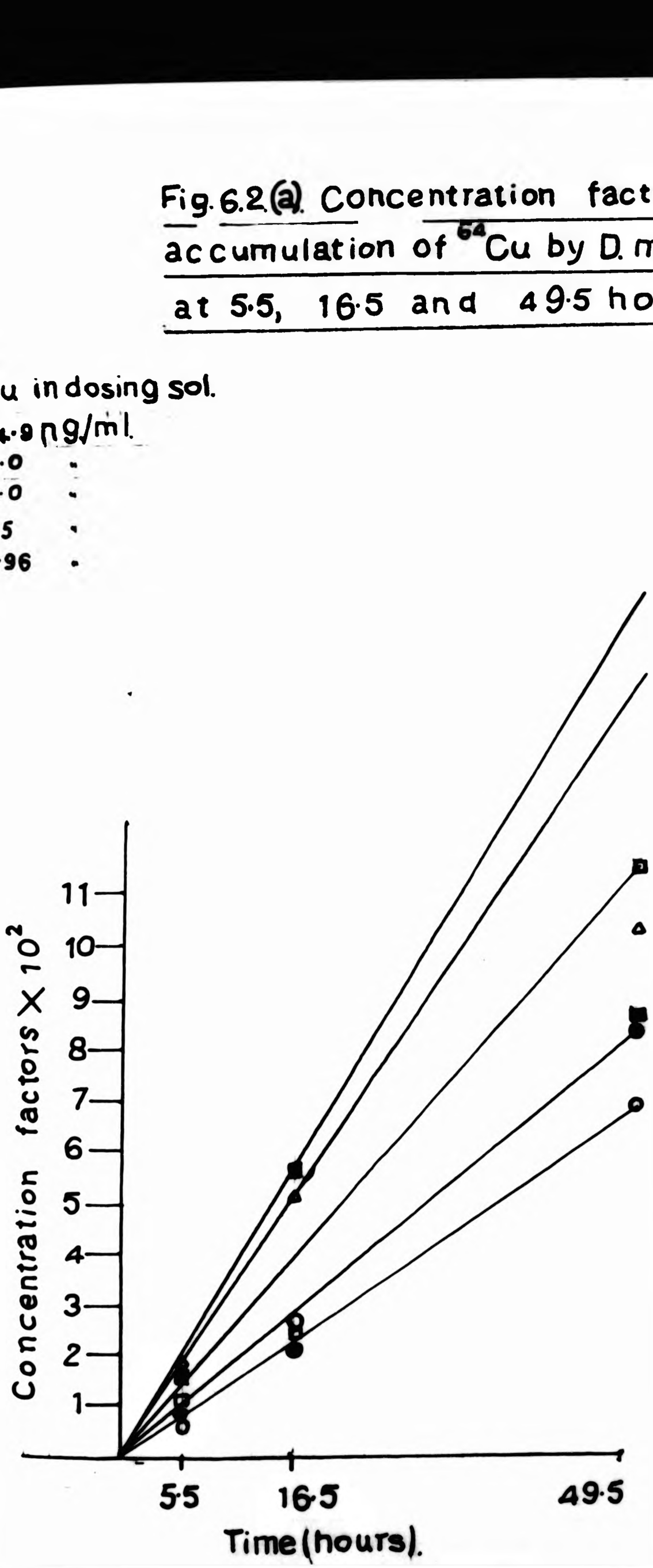
4) The uptake of ^{64}Cu was approximately linear and there was a well defined dose response.

5) The average accumulation of ^{64}Cu/hr/1mg Daphnid was 0.06 ng. ^{64}Cu, 0.23 ng. ^{64}Cu and 0.65 ng. ^{64}Cu during exposures of 5.5, 16.5 and 49.5 hours respectively, and the difference in the rate of uptake was statistically significant.

6) The uptake data can be extrapolated and applied to other experiments in this thesis, for example exposure of 1 mg Daphnia for 1 hour to a concentration of 125 ng. Cu/ml. would result in the accumulation of 7.5 ng. Cu per Daphnid which in that interval would consume 1.5 units of PO_2. Again the uptake values can be related to the sensitivity limits of the atomic absorption spectrophotometer which in this instance could be applied for the analysis of copper in Daphnia when there was a minimum dose of 8.0 ng. Cu/ml. combined with a minimum exposure interval of 16.5 hours.

The linear mode of uptake of copper by Daphnia and the highly significant increases in uptake through time suggest the absence of a secretory mechanism as a means of copper detoxification.

Fig. 6.2(a) Concentration factors in accumulation of ^{64}Cu by D. magna at 5·5, 16·5 and 49·5 hours.



CHAPTER 7

EXPERIMENT TO DETERMINE IF COPPER INITIATES INDUCTION OF THE METAL-BINDING PROTEIN METALLIOTHIONEIN IN *Daphnia hyalina*

7.1 Introduction

The literature search reported in Chapter 1 revealed several references to the apparent association between sulphur or sulphur derivatives and tolerance to copper or reduced toxicity of copper. For example, Brown (1977) identified sulphur in the hepatopancreas of a copper-tolerant strain of Asellus meridianus while none was found in a non-tolerant strain. Holm-Jensen (1948) demonstrated that cysteine reduced the toxicity of copper to daphnia, an effect which was confirmed by Corner and Sparrow (1956) on Artemia salina. The inactivation of several enzyme systems caused by copper or mercury could be partly inhibited by thiol compounds such as cysteine (Stoppani et al., 1953). Peters (1965) found that copper reduced the amount of phosphate split from ATP by membranous ATP ase in pigeon brain, an effect which was partly reversed by the addition of thiols such as deithyldithiocarbamate. Excess copper was deposited as sulphide in the cell periphery in copper-tolerant strains of yeast (Ashida, 1965). A pure strain of sulphur-oxidising bacteria developed a remarkably high resistance to copper after repeated exposures to increasing amounts of $CuSO_4$ in contrast to a pure strain of sulphur-reducing bacteria which remained sensitive to copper (Booth and Mercer, 1963).

This association between copper and sulphur, and the documented induction of metallothioneins by metals in various organisms suggested that the administration of copper to daphnia might stimulate, as a defence system, the synthesis of these specialised binding proteins which are rich in half-cystinyl residues.

7.1.1 The Metallothioneins

According to Rauser and Curvetto (1980) animal tissues normally have low concentrations of metallothionein which increase on administration of metals and this reaction is said to be consistent with their suggested role in detoxification mechanisms. This is interpreted to mean that metallothionein acts by binding the metal in a toxicologically-insensitive site in the animal tissue similar to the sub-cellular compartmentation of copper which occurs in several organisms in the form of vesicles bounded by a membrane. The above authors discovered the presence of copper-thionein in the roots of a clone of Agrostis gigantia treated with copper and they claimed this was the first demonstration of a metallothionein in a vascular plant. George et al. (1979) characterised metallothionein as having an apparent molecular weight of about 20,000 and containing 20% cysteine residues. Carpene and George (1981) demonstrated by Sephadex G - 75 gel chromatography that cytosol extracted from the gills of M. edulis exposed to 0.1 ppm cadmium for three months was ejected in a peak corresponding to metallothionein. As molecular weight markers they used Blue Dextran (M 2,000,000), haemoglobin (65,000), cytochrome C (12,500) and KCl (70). Olafson et al. (1978a) reported that cadmium metallothionein isolated from the hepatopancreas of the crab Scylla servata, which had been exposed to cadmium, had an apparent molecular weight of 9,600 $\pm$ 700. In the same paper the authors comment that Crustacean metallothionein extracted from the Shrimp, Acates sibogae, was similar to vertebrate metallothionein in terms of molecular weight, u.v. absorption spectra, isoelectric points and amino acid composition. In a further paper, Olafson et al. (1978b) found that de novo synthesis of crab hepatopancreas

metallothionein was induced by injection of cadmium or zinc but there was no induction from copper or mercury. No reason is advanced for the failed induction. The absence of response in the case of copper however may be attributed to the fact that this metal is naturally present as the central atom in the respiratory haemocyanin of the crab Scylla servata thus precluding the operation of a defence mechanism against it. In daphnia the respiratory protein is haemoglobin and this reservation would not be applicable.

The techniques employed by Olafson et al. (1978) in the induction and extraction of metallothionein have been adopted in the experiment reported in the following pages.

7.1.2 Objective of the Experiment

The experiment was designed in order to determine if metallothionein was synthesized by daphnia in response to exposure in sub-lethal concentrations of $CuSO_4$. In view of the apparent ubiquitous nature of metallothionein there seemed a possibility that metal-binding by this specialised class of proteins might form a part of the detoxification mechanism in daphnia, particularly since their presence had already been demonstrated in the crustacea. On the other hand the well documented observations on the high susceptibility of daphnia to metals and other toxins suggested the absence of sophisticated defence systems and survival was possibly more likely to be attained by a change from normal parthenogenesis to reproduction by ephippial eggs which would hatch when the poison no longer posed a risk to its survival.

7.1.3 Experimental Methods

(a) Daphnia - The methodology consisted of exposing daphnia to a low level of $CuSO_4$ for two days, followed by a higher

concentration for a further two days in order to initiate and then promote the production of metallothionein which would be identified by the elution pattern of a supernatant extract in a G - 75 Sephadex column. A quantitative measure of the metallo-protein would be sought by the prior introduction of labelled $^{64}Cu^{++}$ into the daphnia sample at the time of homogenisation when it was anticipated that the radioactive copper would bind with the metallothionein.

A considerable quantity of daphnia was required for the experiment and since the laboratory culture was not large enough the requisite amount was acquired by trawling with a plankton net in Loch Lomond, using the facilities supplied by the Field Station of the Universities of Stirling and Glasgow at Rowardennan. Four nets were attached to a catamaran and the water was trawled one foot deep from the surface, earlier trawls at three to four feet having been unsuccessful. At that time of year, the last week in April, the crustacean populations was low and it was necessary to make several runs to obtain a few grammes of specimens. The contents of the nets were transferred to a 1000 gallon tank containing Loch Lomond water and subsequently to a 275 litre tank for treatment with $CuSO_4$. Samples were taken to the laboratory for identification and separation of the species which in terms of numbers and mean length are shown below. I acknowledge the assistance of Dr. A. Henderson, Department of Biology, University of Stirling in the identification of the species.

Sample of Invertebrate Species from Loch Lomond

No. of Daphnia		No. of other species	
300	*D. hyalina*	200	*Bosmina* sp.
2	*D. pulex*	20	*Diaphanosoma* sp.
		10	*Cyclops*
Mean length	0.75 mm		0.35 mm

Separation of the species by placing the entire sample in a 2-litre glass cylinder screened with black polythene, and focussing different wavelengths of light represented by blue, red or green filters was not successful. Daphnia showed some response to blue but the migration was too weak to be of use. The alternative method by sieving through a 0.5 mm sieve was perfectly satisfactory.

After an acclimatization period of three days, 1.9 ug/l $CuSO_4$ was added to the 275 litre tank containing the daphnia and other invertebrates. In order to obtain a uniform distribution of the $CuSO_4$ aliquots of the solution were applied by a rubberized version of a dribble bar, and several passes were made from the surface to the bottom of the tank and across the tank followed by stirring.

Two days after the application of the 1.9 ug/l $CuSO_4$ there was negligible mortality of the daphnia and a second application of 7.8 ug/l $CuSO_4$ was made which was expected to cause some 20% mortality in 24 hours, and at the same time stimulate the possible synthesis of metallothionein in the remaining population. The following day it was found that the entire population of daphnia was dead. This was an unfortunate result since all preparations had been made for starting the experiment including the ordering of the radioisotope and there would have been inadequate time left to repeat the entire experiment before the end of the University term. Normally the experiment would have been abandoned but in the circumstances it was decided to take a sample of the daphnia population on the assumption that if the mortality was due to copper the majority of daphnia must have died a short time before the scheduled sampling and that, therefore, the protein complement could still be determined. The sample was transferred to liquid N_2 in

a thermos flask and on return to the laboratory it was placed in a polythene specimen tube and stored at -70°C.

In the section that follows I acknowledge the assistance of Mr. A. Harris, a post-graduate in the Department of Biochemistry, University of Stirling. He prepared and calibrated the Sephadex column and performed the procedures, as requested, according to Olafson et al. (1978) up to the stage when the sample was added to the column. He also recorded the absorbance readings and prepared that part of the graph covering the absorbance values, and made several comments which are included in the interpretation of the results.

7.1.4 Sephadex Column

According to the manufacturers, Sephadex is a "bead-formed dextran gel"; it is strongly hydrophilic, swelling in water and in electrolyte solutions. It is a chromatographic material capable of separating substances according to molecular size, a process known as gel filtration or gel chromatography. Molecules of the test proteins which are larger than the largest pores of the swollen Sephadex beads cannot penetrate the gel particles which are thus by-passed as the solution proceeds down the column and is ejected first. Smaller molecules penetrate the gel particles, their rate of progress varying with their size and shape, and elution from the column proceeds in order of decreasing molecular size. Graphs can be constructed relating migration rate to the molecular size of the standard molecular weight markers, and to the approximate molecular size of the proteins under test. Several different types of Sephadex are available to cover a range of molecular weights from 700 - 2000,000. In this experiment the object was to identify

a molecular weight of 10,000 - 20,000 and the appropriate type chosen was Sephadex G - 75 Superfine which has a claimed fractionation range of 3,000 to 70,000 molecular weight. The quantity of Sephadex for the column, which was 80 cms high x 2.5 cms diameter, was estimated on the manufacturer's recommendation by dividing the volume of the column by 12 to 15 g dry Sephadex. A slurry was made of 30 g dry Sephadex which was then boiled for three hours and after cooling it was poured into the column.

The buffer for equilibrating the column was 5 m M Tris-HCl adjusted to pH 8.6 and a volume of 2 litres at a controlled flow rate of 18.5 ml/hr was used as a prelude to the elution procedure.

7.1.5 Preparation of Daphnia Sample

The daphnia which had been stored at -70°C was thawed by warming in water at 25°C for 5 minutes and 30 ml Tris-HCl buffer added. The sample was then homogenised in a Sorbex homogeniser for 3 minutes at top speed which reduced the daphnia to a suspension of body parts and fluids. At this stage a volume of 25 ml of 5 mg radioactive copper as $CuSO_4$, which earlier that day had been obtained from The Scottish Universities Research and Reactor Centre, East Kilbride, Glasgow, and which had a specific activity of 262 u Ci/5 mg $^{64}Cu^{++}$, was introduced into the suspension which was then mixed at a slow speed in the Sorbex homogeniser for 10 minutes. The object of the slow mixing was to promote suitable conditions for the binding of the labelled copper to the induced metallothionein. The sample was then centrifuged at 18,000 r.p.m. for 25 minutes and the clear supernatant was collected for subsequent application to the column.

In the meantime the standard marker Dextran Blue in Tris-HCl

the remaining 23 samples for 2 minutes. Duplicate counts were taken and the instrument settings were as follows:

Lower level discriminator 50; upper level discriminator 500, Gain 10%.

Samples of each fraction were also taken for absorbance readings at 280 nm in a Cecil CE 272 Linear Readout Ultraviolet Spectrophotometer.

7.2 Results

The mean radioactivity counts of $^{64}Cu^{++}$ adjusted to zero time for each fraction are shown in Table 7.1.

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TABLE 7.1

Fraction no.	c.p.m. minus background	% Initial Activity Left	c.p.m. adjusted for decay to zero time
1	0	-	0
2	0	-	0
3	0	-	0
4	0	-	0
5	0	-	0
6	0	-	0
7	0	-	0
8	0	-	0
9	0	-	0
10	0	-	0
11	0	-	0
12	0	-	0
13	0	-	0
14	0	-	0
15	0	-	0
16	0	-	0
17	0	-	0
18	0	-	0
19	78	27.0	289
20	0	-	0
21	0	-	0
22	0	-	0
23	0	-	0
24	0	-	0
25	2	25.4	8
26	0	-	0
27	9	24.8	36
28	36	24.5	247
29	92	24.3	379
30	470	24.0	1958
31	3430	23.8	14412
32	14762	23.5	63629
33	28529	23.2	122970
34	39903	22.9	174249
35	42931	22.6	189960
36	51052	22.4	227911
37	79855	22.2	359707
38	111831	22.0	508323
39	157128	21.8	720771
40	137606	21.6	637065
41	107490	21.4	502290
42	77693	21.1	268213
43	38086	20.9	182230
44	18516	20.7	89449
45	9138	20.5	44576
Background	43		

The radioactivity counts showed that the first trace of activity was identified in fraction No. 19. When first observed this was of considerable interest since this would be expected to correspond approximately with the elution of the metallothionein. However, there was no radioactivity recorded in the subsequent five fractions and since the amount of radioactivity recorded in fraction 19 was a minute proportion of the total recorded in the experiment it can be disregarded. Fraction 30 showed an increase in activity compared with the previous three fractions and from that point onwards there was increased activity recorded until fraction 39 which was followed by a regular reduction in activity through to the last sample in fraction 45. Potassium dichromate first occurred in fraction 34, indicating the elution of low to very low molecular weight proteins. By this stage of the experiment it was apparent that there had been no binding of radioactive copper with proteins having molecular weights in the 10,000 - 20,000 N.W. region.

The absorbance readings at 280 nm in Figure 7.1 shows that the bulk of protein eluted at two peaks, one in the high to medium molecular weight range and the other in the very low molecular weight region. The graph demonstrates clearly that there was no binding of radioactive copper to the protein in the high or medium molecular weight region. It is therefore concluded that no metallothionein was detected in this experiment. The techniques used throughout the experiment were validated by earlier workers (Olafson et al., 1978) and the Sephadex C - 75 column functioned normally as observed by the elution pattern of the markers Dextran Blue and Potassium dichromate. It seems unlikely that if metallothionein were present in the sample that this experiment would have failed

to demonstrate its occurrence. The validity of any conclusion however must be qualified by the observation that any metallothionein synthesised during the exposure to $CuSO_4$ may have become proteolized in the interval between death of the organism and sampling. On the other hand the experiment clearly demonstrated the presence of other proteins which supports the comment that mortality probably occurred a short time before sampling and the experimental technique was sufficiently sensitive to identify even very small amounts of metallothionein if present.

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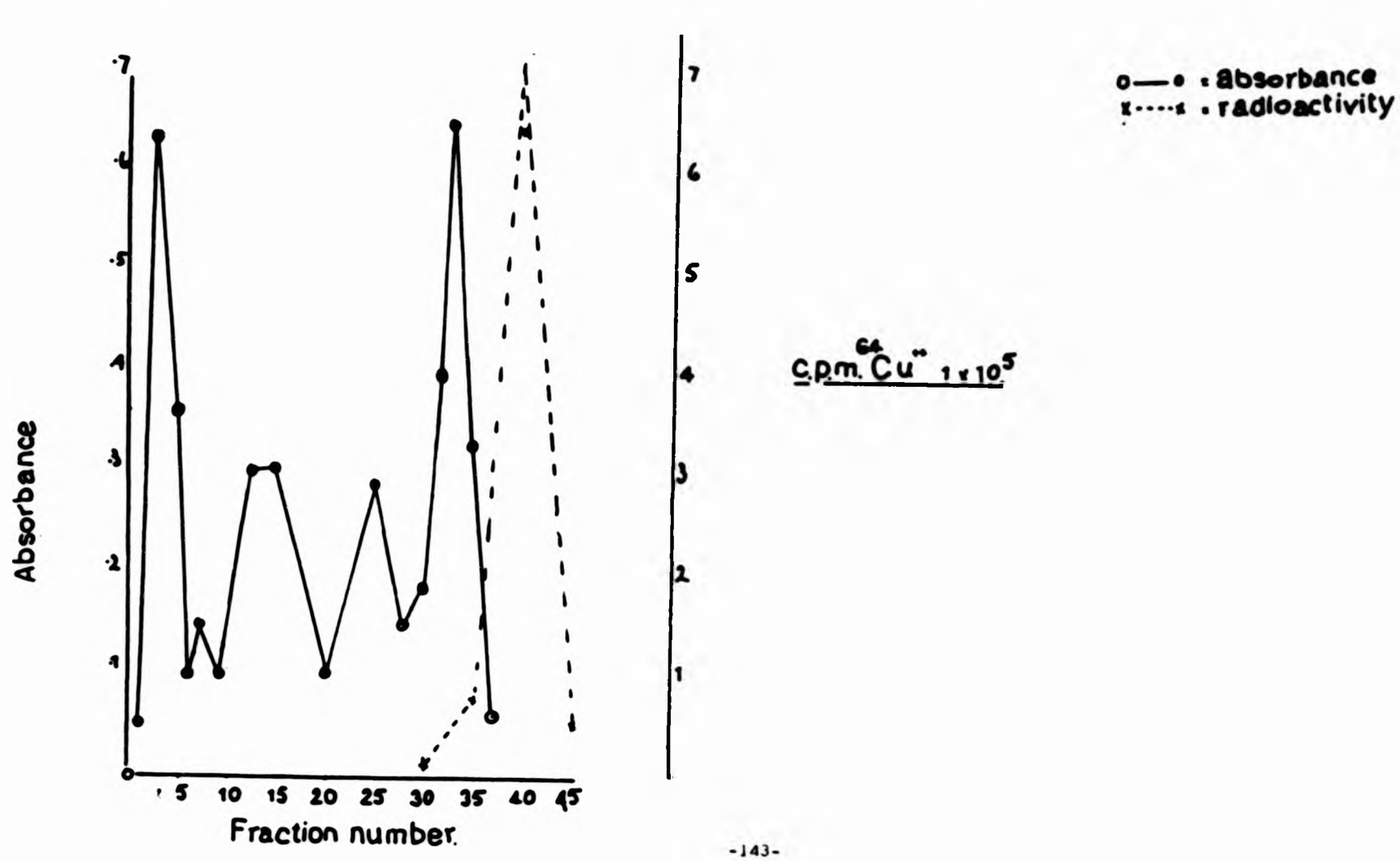
Summary

1. An experiment has been carried out to determine if the specialized binding protein metallothionein could be induced in D. hyalina by exposure to sub-lethal concentrations of $CuSO_4$.
2. In order to promote the synthesis of metallothionein, D. hyalina was exposed to 1.9 ug/l $CuSO_4$ for two days and to an additional 7.8 ug/l $CuSO_4$ for one day.
3. At the time of sampling it was found that all the daphnia were dead but it was decided to continue with the sampling on the assumption that the mortality probably occurred within a recent space of time.
4. Forty-five fractions each of 9 ml were collected and samples were taken for radioactivity determination in a liquid scintillation counter and for absorbance readings at 280 nm in a Cecil Linear Read-out u.v. Spectrophotometer.
5. The daphnia proteins eluted at two peaks, one in the high to medium molecular weight range and the other in the very low molecular weight region. The radioactive copper eluted in the solute volume of the column. There was no binding of copper to the proteins in the high or medium molecular weight fractions.
6. No metallothionein was found in the fractions but no firm conclusion about the presence or absence of this class of proteins in daphnia is advanced since the daphnia were dead at the time of sampling and any induced metallothionein may have proteolized before the samples were taken.

Fig.7.1 Sephadex-G75 elution of protein from D.hyalina.



GENERAL DISCUSSION AND CONCLUSION

The aims and objectives of the project as detailed in 1.3 have not been achieved due to a number of unforeseen difficulties which occurred at critical stages during the programme of studies. Several of the problems are attributed to the high sensitivity of daphnia to copper as illustrated by the toxicity test which demonstrated the 24 hour LC50 to be 15.2 ug Cu^{++}/l. Since the conventional analytical procedures were not sufficiently sensitive to detect this low level, EDTA was added to the culture medium in order to chelate the copper ions and thus reduce their toxicity. In the presence of 0.009 m M EDTA the 24 hour LC50 was 169.1 ug Cu^{++}/l and it was felt that this would facilitate the chemical analysis particularly by the technique of ion concentration which had been examined. However, interpretation of the results from the exposure of daphnia in the continuous flow system indicated that the maximum dosage of copper should be no more than 50 ug Cu^{++}/l and the background level of copper in the system was a minimum of 50 ug Cu^{++}/l. Numerous attempts to reduce background were not successful. It was apparent that a virtually copper-free background would be required to pursue the study adequately and it was concluded that the high costs involved to achieve this would not be justified by the speculative nature of inducing copper tolerance in daphnia in the laboratory. It was therefore necessary to discontinue this part of the study despite the fact that the failure to acquire a tolerant strain of daphnia meant a modification of most of the original programme which was based on the assumption that tolerance would be achievable and subject to elucidation. It also meant there was no application for a considerable

amount of work which had been done in the evaluation of staining techniques for copper in daphnia and in the preparatory procedures for the processing of sections by electron microscopy. However, it is considered that the development of the continuous flow system was a technical innovation which has a wide application in the mass culture of algae used in conjunction with the administration of metals other than copper to daphnia or similar organisms. The use of peristaltic pumps, sophisticated flow meters and a counting mechanism for cells would provide a completely automatic and versatile system capable of exploitation in a wide variety of studies.

In view of the ubiquitous nature of background copper in the continuous flow system it was decided to concentrate on a programme of studies in which the observed levels of background would be most unlikely to occur. These studies included the rate of uptake of radioactive copper in daphnia, the influence of copper on respiratory rate and the binding of copper by metallothionein.

The full exploitation of radioactivity techniques in uptake studies was inhibited by instrumental failure or in one experiment unusually high background radioactivity but it was demonstrated that only a trace of copper, 0.72^{-3} ug Cu^{++}/l per single daphnid per hour was taken up from a radioactive solution in deionized water thus confirming the necessity for reducing background copper to zero or near zero in critical non-labelled studies. Further radioactive studies suggested that daphnia excreted the absorbed copper after intervals of six to nine hours' exposure indicating the action of a direct detoxification system as a defence mechanism rather than isolation of the copper in membrane-bound vesicles which commonly occurs in several other aquatic organisms. The absence of a binding

system was also tentatively concluded from the study designed to seek copper metallothionein in daphnia. It was anticipated that the radioactive studies would be supplemented by autoradiography and an extensive literature search had defined the relevant techniques and all the preparations were made for processing sections cut by freezing microtome. In the event these applications were not made for the reasons already given.

The techniques developed for the removal of superficial copper and in the conversion of daphnia into a form suitable for counting in a liquid scintillation counter represent a positive contribution in radioactive procedures and they are presented as a working model for daphnia studies or as a basis for a model requiring minor modification in other invertebrate species.

The statistical techniques of multiple regression and analysis of covariance applied to the data in experiments designed to determine the influence of copper solutions on the respiratory rate of daphnia revealed that the response is mediated by the concentration of copper. At a concentration of 0.125 mg Cu^{++}/l the respiratory rate did not differ significantly from the untreated control, while concentrations of 0.25, 0.5 and 5.0 mg Cu^{++}/l caused significant increases in the respiratory rate. There was no significant difference between the respiratory rates in 5.0 and 0.25 mg Cu^{++}/l and the maximum stimulus therefore occurred at 0.25 mg Cu^{++}/l and a plateau-type response has been postulated to represent the action of the concentrations examined. It was anticipated that the diminishing PO_2 levels in the respirometer would create stress in daphnia and this would be reflected in a higher toxicity of copper. However, comparisons with identical concentrations of

copper in open specimen tubes clearly demonstrated that there was no interaction of diminishing PO_2 levels and mortality from copper. This apparent independence of daphnia to ambient PO_2 is counter-balanced by a curious change which occurs at about PO_2 10 when haemoglobin is first synthesized and continues with greater intensity until zero PO_2. At PO_2 levels above PO_2 10 in the absence of haemoglobin, oxygen transport is effected by diffusion and convection, while in the presence of haemoglobin transport is assumed to occur by haemoglobin which has been shown in these experiments to give a threefold increase in the rate of respiration. Since it has been demonstrated that daphnia can survive for about one hour in the absence of ambient oxygen, the energy for this survival period may be derived from stored glycogen synthesized in excess by the additional oxygen supply from haemoglobin.

Conclusion

It is postulated from the experiments on respiration that the mode of action of copper occurred by its toxic effect on the neuro-muscular system causing hyperactivity in the appendages supporting the modified gill structures thus promoting an elevated respiratory rate which in turn would deplete the energy reserves leading to death of the organism by starvation.

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